SCREENING HALOARCHAEA AND HALOPHILIC MICROORGANISMS ISOLATED FROM MARINE ORGANISMS FOR BIOACTIVE COMPOUNDS

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

By

SHERYANNE VELHO-PEREIRA

Research Guide

PROF. IRENE J. FURTADO

Goa University,
Taleigao Goa
2013

External Examiner

Research Guide

CERTIFICATE

This is to certify that **MISS SHERYANNE VELHO-PEREIRA** has satisfactorily completed the thesis entitled **"SCREENING HALOARCHAEA AND HALOPHILIC MICROORGANISMS ISOLATED FROM MARINE ORGANISMS FOR BIOACTIVE COMPOUNDS"** submitted to Goa University for the award of the degree of **DOCTOR OF PHILOSOPHY IN MICROBIOLOGY** is a record of original and independent work carried out during the period of January 2007 - January 2013 in the **DEPARTMENT OF MICROBIOLOGY, GOA UNIVERSITY** under my supervision and that it has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University.

I affirm that the thesis submitted by **MISS SHERYANNE VELHO-PEREIRA** is completely independent research work carried by her under my supervision.

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It is hereby declared that this thesis entitled **"SCREENING HALOARCHAEA AND HALOPHILIC MICROORGANISMS ISOLATED FROM MARINE ORGANISMS FOR BIOACTIVE COMPOUNDS"** submitted to the Goa University, for the award of the degree of **DOCTOR OF PHILOSOPHY IN MICROBIOLOGY** is a record of original and independent work carried out by me during January 2007 - January 2013, in the **DEPARTMENT OF MICROBIOLOGY, GOA UNIVERSITY** under the supervision of **Prof. IRENE J. FURTADO**, Professor, Department of Botany, Goa University and that it has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University.

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A reverberating sigh of relief chimes as I come to an end of this journey. At the onset, I am grateful to the Almighty for keeping me in good health and blessing me abundantly in all my endeavors. Without His innumerable blessings I would accomplish very little as He is the one who gave me the strength in times of trials and difficulties.

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Last but definitely not the least I wish to extend my heartfelt thanks to my parents for all their motivation, care,

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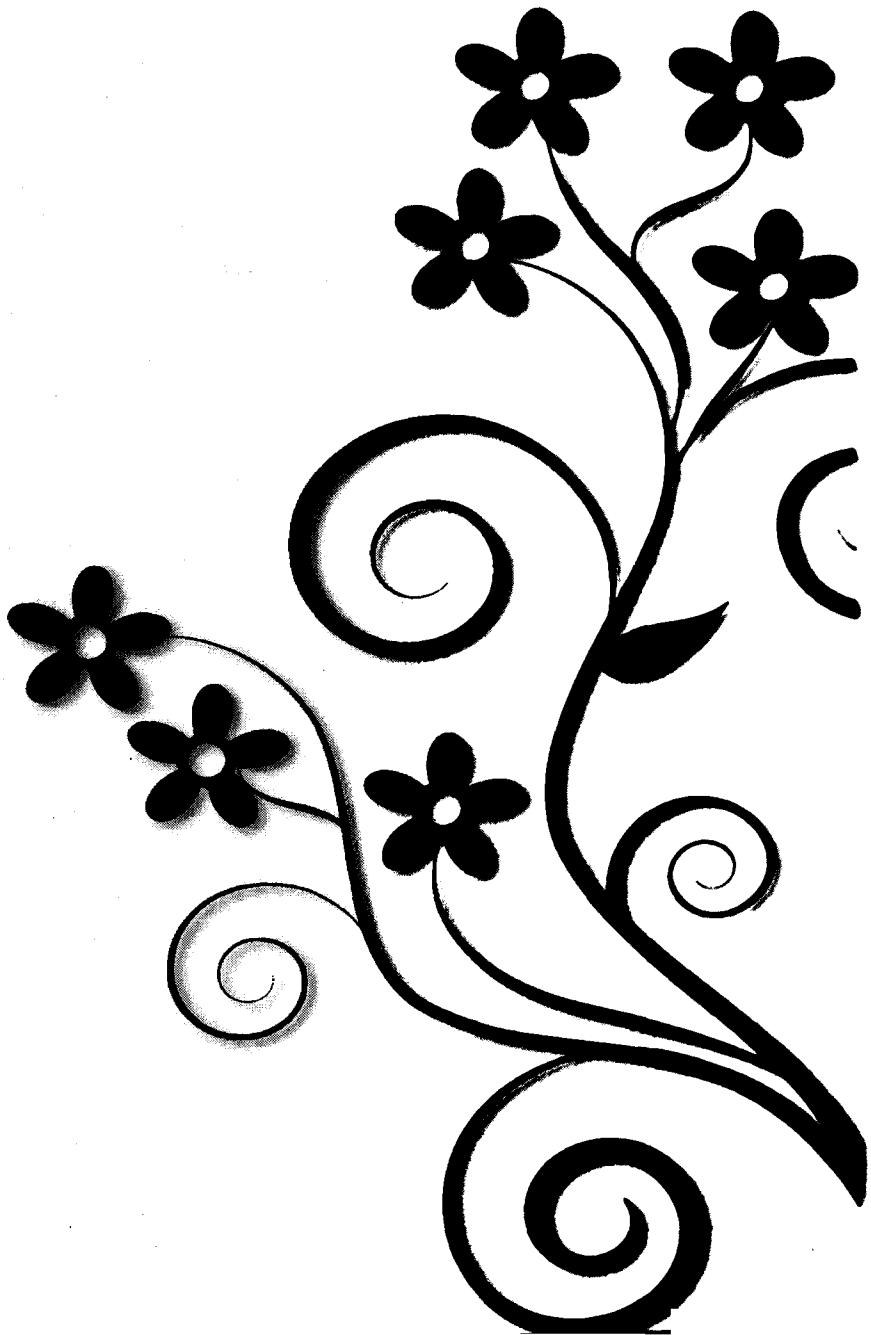
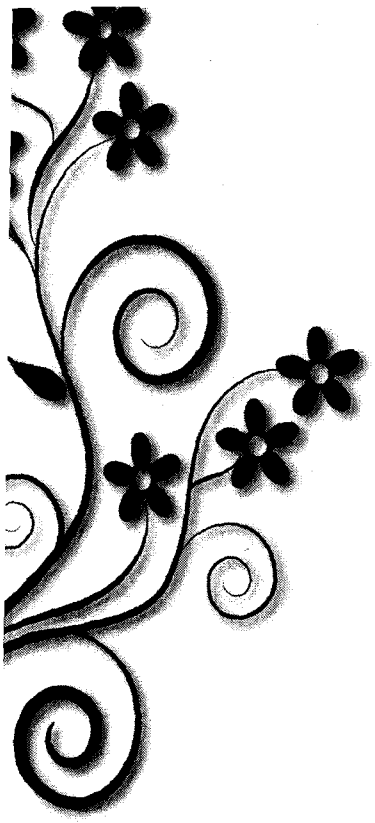
Sheryanne Velho-Pereira





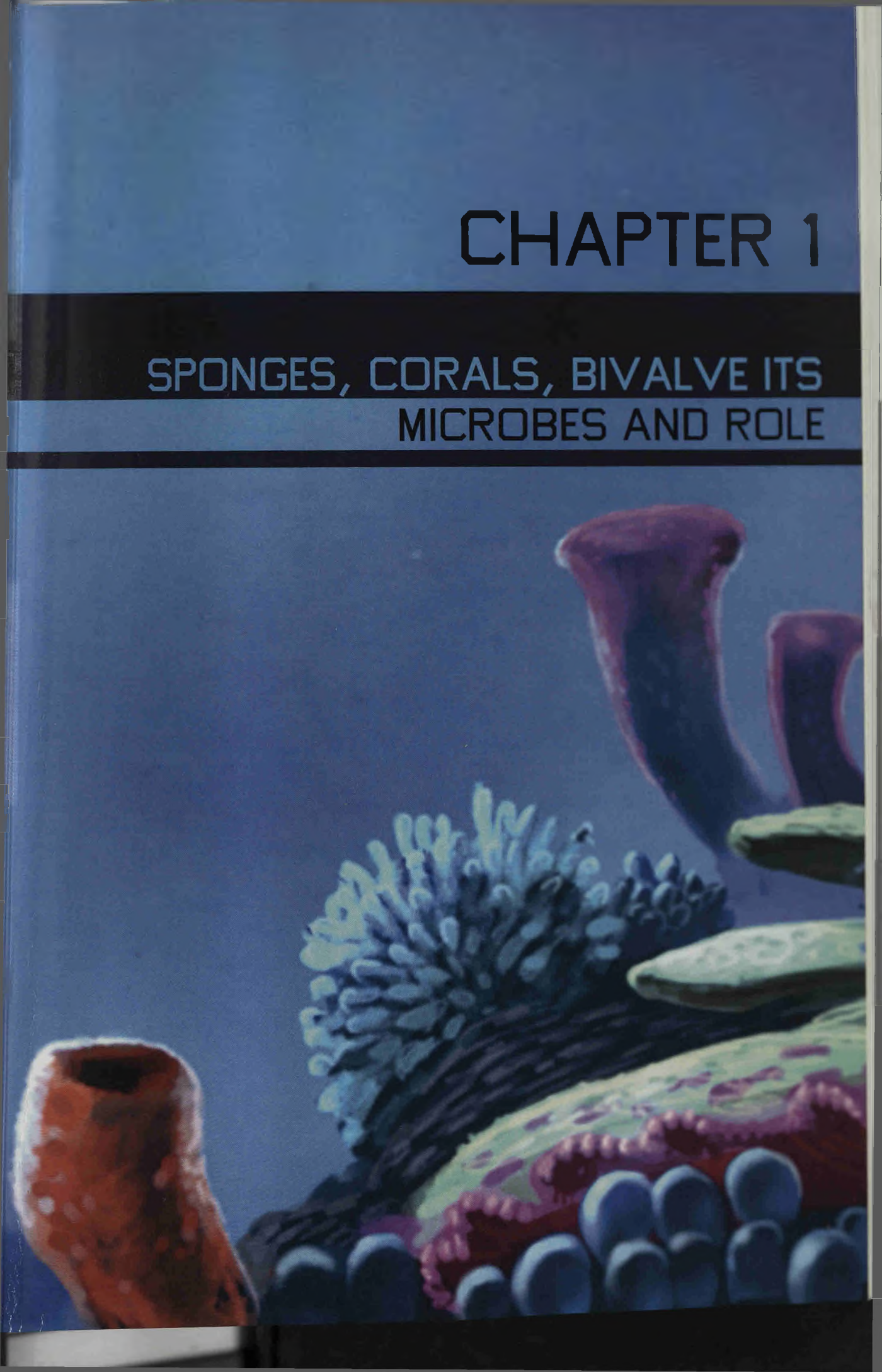
Dedicated to my wonderful parents

Love you both so very much

INDEX

CHAPTER 1

SPONGES, CORALS, BIVALVE ITS MICROBES AND ROLE



1.1 Mapping of the marine econiches harbouring Sponges, Corals and Bivalve (*P.viridis*)

Sponges, Corals and Bivalves are sessile mesozoic, mixotrophic invertebrates of a reef ecosystem. All these invertebartes are benthic, attached and are involved as geographical bafflers, binders, eroders and sediment producers within the reef ecosystem.

Diverse sponges and corals mapped in Figure 1.1 and 1.2 is an effort of exploration for invertebrate diversity and its associated microbial flora. The Caribbean reef, Florida reef, Mediterranean Sea, Davies Reef on the Great Barrier Reef, Australia Tropical reefs of the Atlantic and Pacific (Webster *et al.*, 2001, Silberhorn *et al.*, 2008) are regions widely explored for its invertebrates and its associated microbial diversity. The sponges and corals commonly found in these regions include *Acropora* sp., *Monstastraea* sp., *Agelas* sp., *Petrosia* sp., *Aplysina* sp. (Berna *et al.*, 2011, Hentschel *et al.*, 2001, Rohwer *et al.*, 2002). Research efforts in India span from Tuticorin, Nagapattinum coast, Mandapam, Hare Island in Rameswaram region, Thondi Palk Strait South East coast of India to Ratnagiri Gujarat on the Western coast (Selvin *et al.*, 2009; Kanagasabhabathy *et al.*, 2005). Extensive sponge related microbiological work is being carried out in South East Asia and Europe as indicated in Figure 1.1 while Figure 1.2 shows the scarce reports of coral associated nicroorganisms across the globe. There are no reports on the microbes associated with the single bivalve *P.viridis* used in this study.

Figure 1.1 Global distribution of marine sponges studied for microbial diversity.

1. *Haliclona* sp., 2. *Xestospongia* sp., 3. *Agelas* sp., 4. *Chondrosia* sp., 5. *Petrosia* sp., 6.*Geodia* sp. 7. *Tethya* sp., 8. *Axinella* sp., 9. *Dysidea* sp., 10. *Oscarella* sp., 11. *Mycale* sp., 12. *Aplysina* sp., 13. *Rhopaloeides* sp., 14. *Homophymia* sp., 15. *Fasciospongia* sp., 16. *Cymbastela* sp., 17. *Callyspongia* sp., 18. *Stylinos* sp., 19. *Chondrilla* sp., 20. *Clathrina* sp., 21. *Ircinia* sp., 22. *Suberites* sp., 23. *Acanthella* sp., 24. *Ianthella* sp. ,25.*Terpios* sp., 26. *Aaptos* sp., 27. *Pseudoceratina* sp., 28. *Anoxycalyx* sp.,29. *Sigmadocia* sp., 30.*Stelletta* sp., 31.*Halichondria* sp., 32. *Craniella* sp., 33. *Amphilectus* sp., 34. *Eurypon* sp., 35. *Ceratoporella* sp., 36. *Lissodendoryx* sp.,37. *Crambe* sp., 38. *Tedania* sp., 39. *Gelloides* sp., 40. *Epipolasis* sp.,41. *Psammaplysilla* sp., 42. *Plakortis* sp., 43. *Clathria* sp., 44. *Hymeniacidon* sp.,45. *Characella* sp., 46.*Plakortis* sp., 47. *Amphimedon* sp., 48. *Aiolochroia* sp.,49. *Scopalina* sp., 50. *Erylus* sp., 51. *Echinodictyum* sp., 52. *Pachastrissa* sp., 53. *Coelocarteria* sp., 54. *Halisarca* sp. sp., 55. *Suberites* sp., 56. *Jaspis* sp., 57. *Dendrilla* sp., 58. *Monachora* sp., 59. *Niphates* sp.,60. *Didiscus* sp., 61 *Monanchora* sp., 62. *Acanthostronglyophora* sp., 63. *Kirkpatrickia* sp., 64. *Latrunculia* sp.,65. *Homaxinella* sp., 66. *Sphaerotylus* sp., 67. *Stylissa* sp., 68. *Polymastia* sp., 69. *Hyrtios* sp.

Figure 1.2 Global distribution of marine corals studied for microbial diversity.

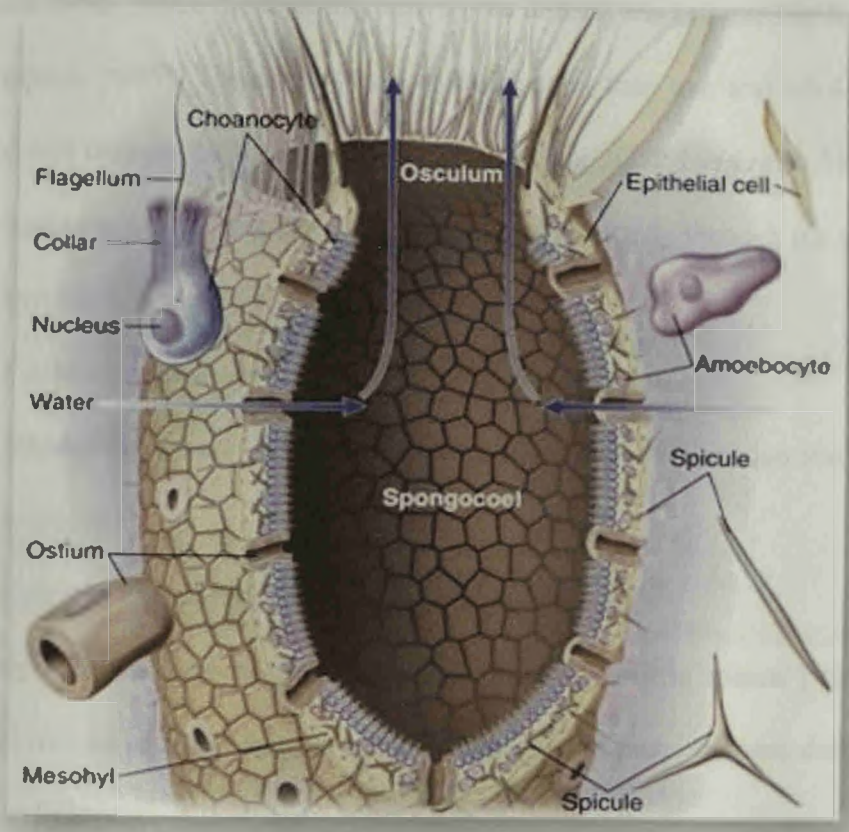
1. *Dendronephthya* sp., *2. Montipora* sp., 3. *Acropora* sp., 4. *Monstastraea* sp., 5. *Diploria* sp.,6. *Porites* sp., 7. *Galaxea* sp., 8. *Pavona* sp., 9. *Lophelia* sp., 10. *Eunicea* sp., 11. *Pollicipora* sp., 12. *Tubastrea* sp.,13. Scleractinian; 14. Octocorallian, *15. Favia sp.;* 16. *Platygyra* sp., 17. *Fungia* sp., 18. *Stylophora* sp., 19. *Acanthastrea sp.;*20. *Xenia* sp., 21. *Subergorgia* sp., 22. *Junceella* sp., 23. *Siderastrea* sp., 24. *Sarcophyton* sp., 25. *Diploria* sp., 26. *Sinularia* sp., 27. *Echinopora* sp., 28. *Alcyonacean;* 29. *Aiptasa sp.*

1.2 Sponges

Sponges are animals of the phylum Porifera (meaning "pore bearer"). They are sessile aquatic animals, heterotrophic, lack cell walls, produce sperm cells, do not have true tissues or organs, and have no body symmetry. Sponges do not have nervous, digestive or circulatory systems. Instead, most rely on maintaining a constant water flow through their bodies to obtain food, oxygen and remove wastes. The shapes of their bodies are adapted for maximal efficiency of water flow through the central cavity, where it deposits nutrients, and leaves through a hole called the **osculum.** Although sponges are freshwater species, the great majority are marine (salt water) species, ranging from tidal zones to depths exceeding 8,800 m (5.5 mi).

While most of the approximately 5,000–10,000 known species feed on bacteria and other food particles in the water, some host photosynthesizing micro-organisms as endosymbionts and these alliances often produce more food and oxygen than they consume. A few species of sponge that live in food-poor environments have become carnivores and prey mainly on small crustaceans (Vacelet and Duport 2004).

1.2.1 Basic structure



1.2.2 Cell types

A sponge's body is hollow and is held in shape by the **mesohyl**, a jelly-like substance made mainly of collagen and reinforced by a dense network of fibers also made of collagen. The inner surface is covered with **choanocytes**, cells with cylindrical or conical **collars** surrounding one **flagellum** per choanocyte. The wave-like motion of the whip-like flagella drives water through the sponge's body. All sponges have **ostia**, channels leading to the interior through the mesohyl, and in most sponges these are controlled by tube-like **porocytes** that form closable inlet valves. The porocytes are tubular cells that make up the pores.

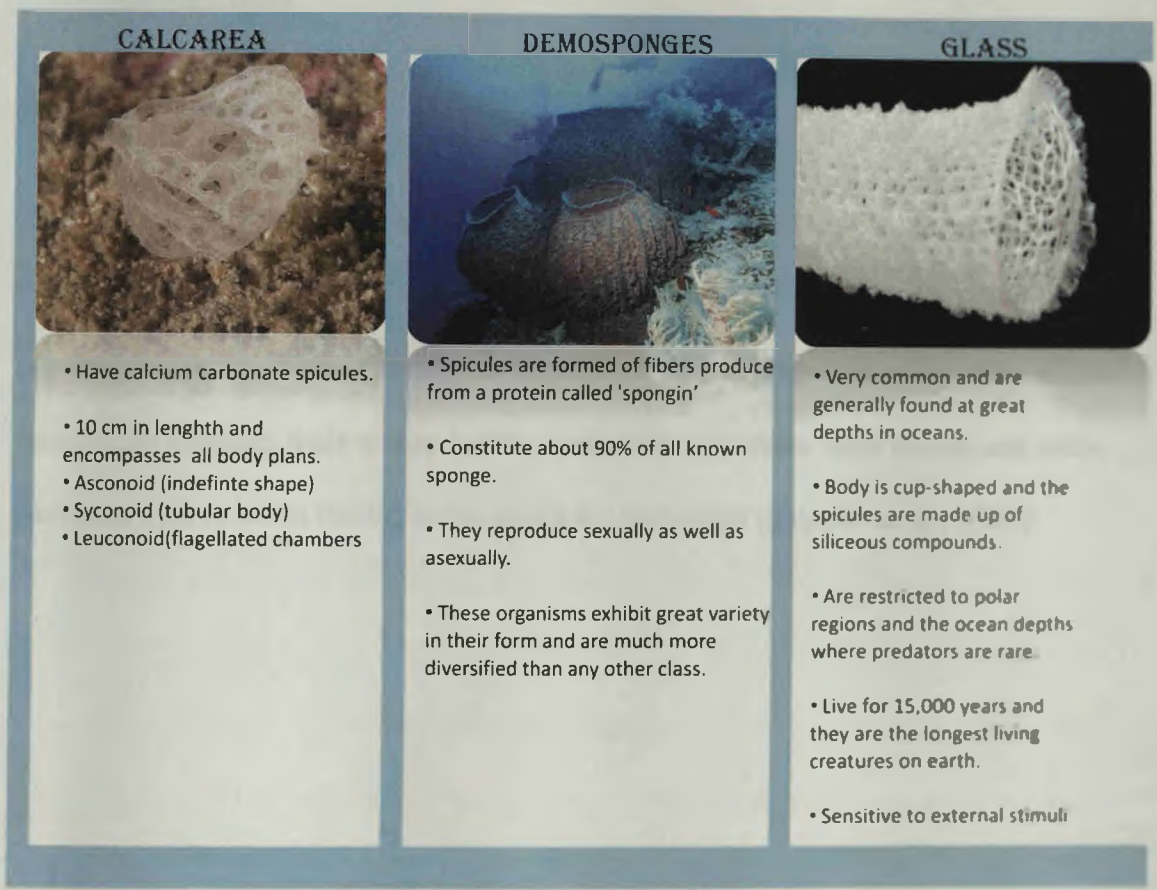
Pinacocytes, are plate-like cells that form a single-layered external skin over all other parts of the mesohyl that are not covered by choanocytes. The pinacocytes also

digest food particles that are too large to enter the ostia, (Ruppert, *et al.*, 2004; Bergquist 1998) while those at the base of the animal are responsible for anchoring it (Bergquist, 1998). There are other types of cells that live and move within the mesohyl and they are enlisted below (Ruppert, *et al.*, 2004; Bergquist, 1998).

- **Lophocytes** are amoeba-like cells that move slowly through the mesohyl and secrete collagen fibres.
- **Collencytes** are another type of collagen-producing cell.
- **Rhabdiferous** are cells that secrete polysaccharides, that also form part of the mesohyl.
- **Oocytes and spermatocytes** are reproductive cells.
- **Sclerocytes** secrete the mineralized **spicules** ("little spines") that form the skeletons of many sponges and in some species provide some defense against predators.
- In addition to or instead of sclerocytes, demosponges have **spongocytes** that secrete a form of collagen that polymerizes into spongin, a thick fibrous material that stiffens the mesohyl.
- **Myocytes** ("muscle cells") conduct signals and cause parts of the animal to contract.
- **Grey cells** act as sponges equivalent of an immune system.
- **Archaeocytes** (or amoebocytes) are amoeba-like cells that are totipotent, in other words each is capable of transformation into any other type of cell. They also have important roles in feeding and in clearing debris that block the ostia.

1.2.3 Classification of Sponges

The sponges are primarily classified on the basis of characteristic structures, for example spicules that are used to reinforce the mesohyl. Different types of sponges or classes formed due to this process of reinforcing are explained below (Bergquist *et al.*, 2001).



1.2.4 Skeleton

.The mesohyl functions as an endoskeleton in most sponges, and is the only skeleton in soft sponges that encrust hard surfaces such as rocks. More commonly the mesohyl is stiffened by mineral spicules, by spongin fibers or both. Spicules may be made of silica or calcium carbonate, and vary in shape from simple rods to three-dimensional "stars" with up to six rays. Spicules are produced by sclerocyte cells, and may be separate, connected by joints, or fused (Hooper *et al.*, 2002). Some sponges also secrete exoskeletons that lie completely outside their organic

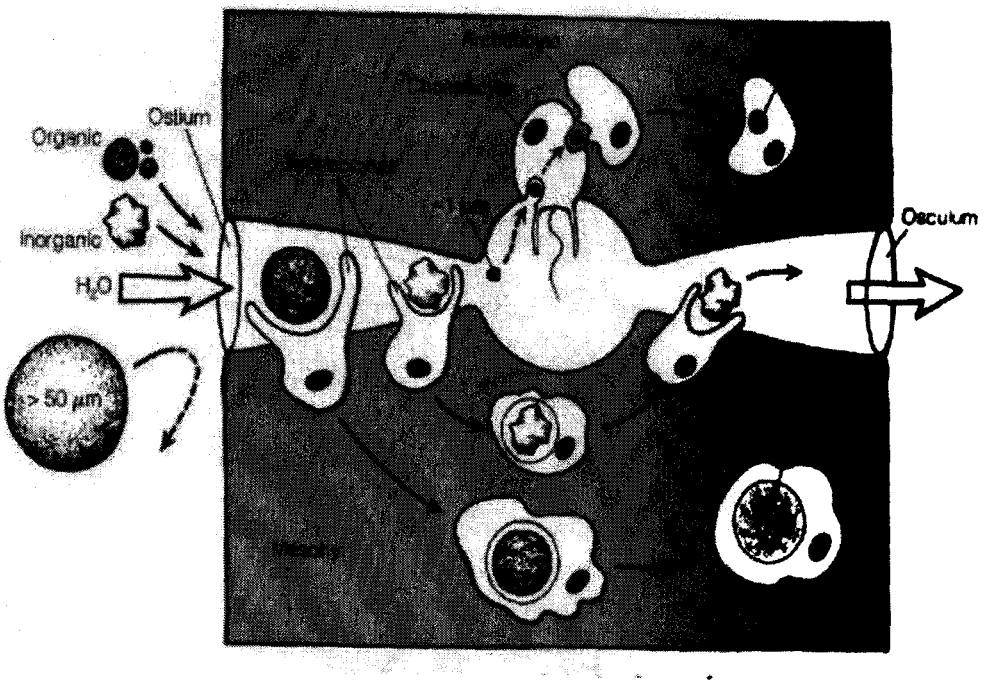
components. For example sclerosponges ("hard sponges") have massive calcium carbonate exoskeletons over which the organic matter forms a thin layer with choanocyte chambers in pits in the mineral. These exoskeletons are secreted by the pinacocytes that form the animals skins (Ruppert *et al.*, 2004).

1.2.5 Vital functions

1.2.5.1 Movement

Although adult sponges are fundamentally sessile animals, some marine and freshwater species can move across the sea bed at speeds of 1–4 mm (0.039–0.16 in) per day, as a result of amoeba-like movements of **pinacocytes** and other cells. A few species can contract their whole bodies, and many can close their oscula and ostia. Juveniles drift or swim freely, while adults are stationary (Ruppert *et al.*, 2004).

1.2.5.2 Respiration, feeding and excretion



Sponges do not have distinct circulatory, respiratory, digestive and excretory systems and instead the water flow system supports all these functions. They filter food particles out of the water flowing through them also called intracellular digestion. Particles larger than 50 micrometers cannot enter the ostia and pinacocytes consume them by phagocytosis (engulfing and internal digestion). Particles from 0.5 µm to 50 µm are trapped in the ostia, which taper from the outer to inner ends. These particles are consumed by pinacocytes or by archaeocytes. Bacteria-sized particles, below 0.5 micrometers, pass through the ostia and are caught and consumed by choanocytes. Since the smallest particles are by far the most common, choanocytes typically capture 80% of a sponge's food supply (Bergquist, 2001). Archaeocytes transport food packaged in vesicles from cells that directly digest food to those that do not. At least one species of sponge has internal fibers that function as tracks for use by nutrient carrying archaeocytes (Ruppert *et al.*, 2004), and these tracks also move inert objects (Bergquist, 1998).

It used to be claimed that glass sponges could live on nutrients dissolved in sea water and were very averse to silt (Krautter, 1998). However a study in 2007 found no evidence of this and concluded that they extract bacteria and other micro-organisms from water very efficiently (about 79%) and the process suspended sediment grains to extract such prey (Yahel *et al.*, 2007). Collar bodies digest food and distribute it wrapped in vesicles that are transported by **dynein** "motor" molecules along bundles of microtubules that run throughout the **syncytium** (Ruppert *et al.*, 2004). Sponge cells absorb oxygen by diffusion from water into cells, as water flows through its body, into which carbon dioxide and other soluble waste products such as ammonia also diffuse. Archeocytes remove mineral particles that threaten to block the ostia, transport them through the mesohyl and generally dump them into the outgoing water current, although some species incorporate them into their skeletons (Ruppert *et al.*, 2004).

1.2.5.3 "Immune" system

Sponges do not have the complex immune systems of most other animals. However they reject grafts from other species but accept them from other members of their own species. In a few marine species, **grey cells** play the leading role in rejection of foreign material. When invaded, they produce a chemical that stops movement of other cells in the affected area, thus preventing the intruder from using the sponge's internal transport systems. If the intrusion persists, the grey cells concentrate in the area and release toxins that kill all cells in the area. The "immune" system can stay in this activated state for up to three weeks (Bergquist, 2001).

1.2.5.4 Reproduction

Asexual

Sponges have three asexual methods of reproduction: **fragmentation, budding**, and by producing **gemmules**. Fragments of sponges may be detached by currents or waves. They use the mobility of their pinacocytes and choanocytes and reshaping of the mesohyl to re-attach themselves to a suitable surface and then rebuild themselves as small but functional sponges over the course of several days (Ruppert *et al.*, 2004). A sponge fragment can only regenerate if it contains both collencytes to produce mesohyl and archeocytes to produce all the other cell types. A very few species reproduce by budding. (Ruppert *et al.*, 2004).

Gemmules are "survival pods" which a few marine sponges and many freshwater species produce by the thousands when dying and which some, mainly freshwater species, regularly produce in autumn. Spongocytes make gemmules by wrapping shells of spongin, often reinforced with spicules, round clusters of archeocytes that are full of nutrients (Ruppert *et al.*, 2004).

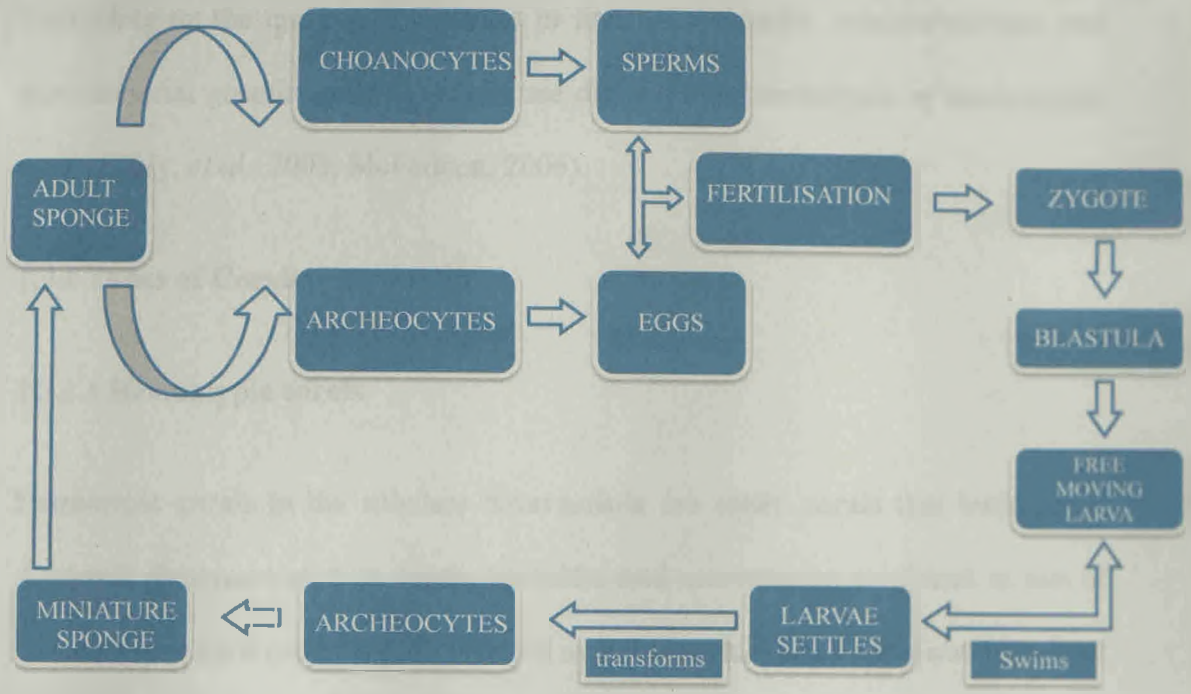
Freshwater gemmules may also include phytosynthesizing symbionts (Smith and Pennak 2001). The gemmules then become dormant, and in this state can survive cold, drying out, lack of oxygen and extreme variations in salinity (Ruppert *et al.*, 2004). Freshwater gemmules often do not revive until the temperature drops, stays cold for a few months and then reaches a near-"normal" level (Smith and Pennak 2001). When a gemmule germinates, the archeocytes round the outside of the cluster transform into pinacocytes, a membrane over a pore in the shell bursts, the cluster of cells slowly emerges, and most of the remaining archeocytes transform

into other cell types needed to make a functioning sponge. Gemmules from the same species but different individuals can join forces to form one sponge (Ruppert *et al.*, 2004). Some gemmules are retained within the parent sponge, and in spring it can be difficult to tell whether an old sponge has revived or been "recolonized" by its own gemmules (Smith and Pennak, 2001).

Sexual

Most sponges are hermaphrodites (function as both sexes simultaneously), although sponges have no gonads (reproductive organs). Sperms are produced by choanocytes or entire choanocyte chambers while eggs are formed by transformation of archeocytes, or of choanocytes in some species. Each egg generally acquires a yolk by consuming "nurse cells". During spawning, sperm burst out of their cysts and are expelled via the **osculum.** If they contact another sponge of the same species, the water flow carries them to choanocytes that engulf them, instead of digesting them, metamorphose to an ameboid form and carry the sperm through the mesohyl to eggs, which in most cases engulf the carrier and its cargo (Ruppert *et al.*, 2004).

A few species release fertilized eggs into the water, but most retain the eggs until they hatch. There are four types of larvae, but all are balls of cells with an outer layer of cells whose flagellae or cilia enable the larvae to move. After swimming for a few days the larvae sink and crawl until they find a place to settle. Most of the cells transform into archeocytes and then into the types appropriate for their locations in a miniature adult sponge (Ruppert *et al.*, 2004).



1.3 Corals

Corals are marine animals in class Anthozoa of phylum. It has **coral head** which represents a group of genetically identical multicellular organisms known as **polyps** and are a few millimeters in diameter. It has layer of outer **epithelium** and inner jellylike tissue known as **mesoglea**. Polyps are radially symmetrical, with a ring of **tentacles** surrounding a **central mouth**, which leads to the stomach or **coelenteron** or **gastrovascular cavity**, through which food is ingested and waste expelled.

The epithelium produces an **exoskeleton** called the **basal plate or calicle**. The calicle is formed by a thickened **calcareous ring** (annular thickening) with six supporting **radial ridges**. When a polyp is physically stressed, its tentacles contract into the calyx so that virtually no part is exposed. This protects the organism from predators (Barnes, 1987; Sumich, 1996).

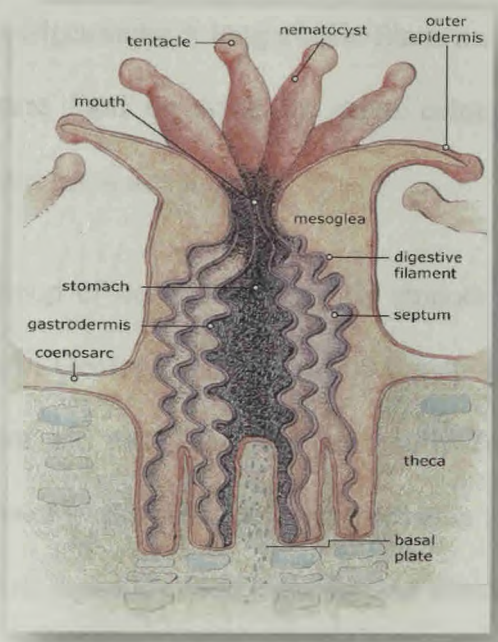
Depending on the number of tentacles or lines of symmetry, nematocyst type and mitochondrial genetic analysis corals are divided into: hermatypic or ahermatypic corals (Daly, *et al.*, 2003; McFadden, 2006).

1.3.1 Types of Corals

1.3.1.1 Hermatypic corals

Hermatypic corals in the subclass **Scleractinia** are **stony corals** that build reefs. Anatomic structures such as **septa, tentacles and mesenteries** are found in sets of six and hence hard corals are also termed as **hexacorals.** They mostly obtain at least part of their energy requirements from zooxanthellae. They secrete calcium carbonate to form a hard skeleton. Those having six or fewer lines of symmetry in their body structure are called **hexacorallia or Zoantharia**. This group includes reef-building corals (scleractinians), sea anemones and zoanthids. Hermatypic genera include *Scleractinia, Millepora, Tubipora* and *Heliopora* (The Greenpeace Book of Coral Reefs). Living coral grow on top of the skeletons of their dead predecessors. Some stony corals are incapable of producing sufficient quantities of calcium carbonate to form reefs. Many of these corals do not rely on the algal metabolites produced by zoozanthellae and live in deeper and or colder waters (Noaa Coral reef information system-What are coral reefs). Some of the deep sea corals may form community structures that resemble the forests but do not build reefs. Other stony deep sea corals *Oculia* and *Lophelia* species for eg. do build reefs (Piper, 2007).

Hard coral anatomy



The figure shows the typical structure of an individual hard coral polyp. **Polyps** are usually a few millimeters in diameter and sit within a **cup or calyx.** The individual polyps are radially symmetrical and are connected via a system of gastrovascular of canals that allow for the sharing of nutrients and symbiotic algae. The tissue that connects individual polyps at the surface of a coral colony is the **coenosarc** while the outer tissue of the polyp is the **epithelium** or **epidermis**. The walls surrounding the calyx are called **theca** and the bottom of the cup is called the **basal plate**. The basal plate is a **calciferous ring with six supporting radial ridges**. The ridges have **tabulae** which are horizontal partitions that allow for upward growth of a polyp. The **stomach** opens in the center of the polyp and is surrounded by **tentacles** and is used to ingest food and to expel waste. The tissue lining the stomach is called the **gastrodermis**. In between the epidermis and gastrodermis is a jelly like substance called the **mesoglea**. **Septa or vertical plates** are also present within the

gastrovascular cavity and this supports the internal folds or the **mesenteries** of the stomach. The mesenteries increase the surface area of the stomach and also contain reproductive cells. Its edges support long mobile filaments that can protrude through the mouth and capture food or assist the coral colony in competing with its neighbouring organisms for space to grow.

Tentacles for this group of corals are typically smooth and occur in sets of **six** (University of Michigan-Geological Sciences 100 Course page). They are mainly for defense and to capture and pass food into the mouth. Tentacles may be retracted when physically stressed to protect them from predators and other elements. Some species also retract their tentacles during the day but extend them at night primarily to feed on plankton. The tentacles contain stinging cells called **nemtocysts or cnidae**. These cells are also found on the epidermis and are used for both incapacitating prey and defense from predators. The nematocysts inject poison into the corals prey immobilizing or killing them. While effective against small prey such as plankton and in discouraging predators, the sting of most corals has no harmful effect on humans. A notable exception would be the fire corals whose stings could be extremely painful for upto two weeks. Nematocysts are also one of the means by which the coral competes for space to grow. (University of Michigan-Geological Sciences 100 Course page).

1.3.1.2 Ahermatypic corals/ Alcyonacea

Ahermatypic corals have no zooxanthellae. They sport eight tentacles and are also called **octocorallia or octocorals** (University of Michigan-Geological Sciences 100 Course page). They include corals in subclass *Alcyonacesdasda*, as well as some species in order *Anthipatharia* (black coral, *Cirripathes, Antipathes*). Ahermatypic

corals, such as sea whips, sea feathers, and sea pens (National Geographic Traveller: The Caribbean) are also known as **soft corals**. Unlike stony corals, they are flexible, undulating in the current, and often are perforated, with a lacy appearance. Their skeletons are proteinaceous, rather than calcareous. Soft corals are somewhat less plentiful (in the Caribbean, twenty species appear) than stony corals. Soft corals are composed of some rigid calcium carbonate but it is blended with protein so it is less rich than hard corals. These corals are "rooted" but because they have no exoskeletons they sway back and forth with the currents appearing to be more like plants blowing in the breeze. Though they don't secrete calcium carbonate as prolifically as hard corals, they do contain spiny skeletal elements called **sclerites** in the cells on the outside of the **colony**. Sclerites are found in the jelly like tissue called **coenenchyme** between polyps. Sclerites are made of protein and calcium carbonate and give coral support to allow them to achieve their vertical structures. The scletrites also give their surfaces a spiky or grainy texture. The inner core consist of **gorgorin** which is aflexible fibrous wood like protein which gives the coral the ability to flex with the ocean waves and currents (Ries *et al.*, 2006).Visually soft coral colonies tend to resemble trees, bushes, fans, whips and grasses.

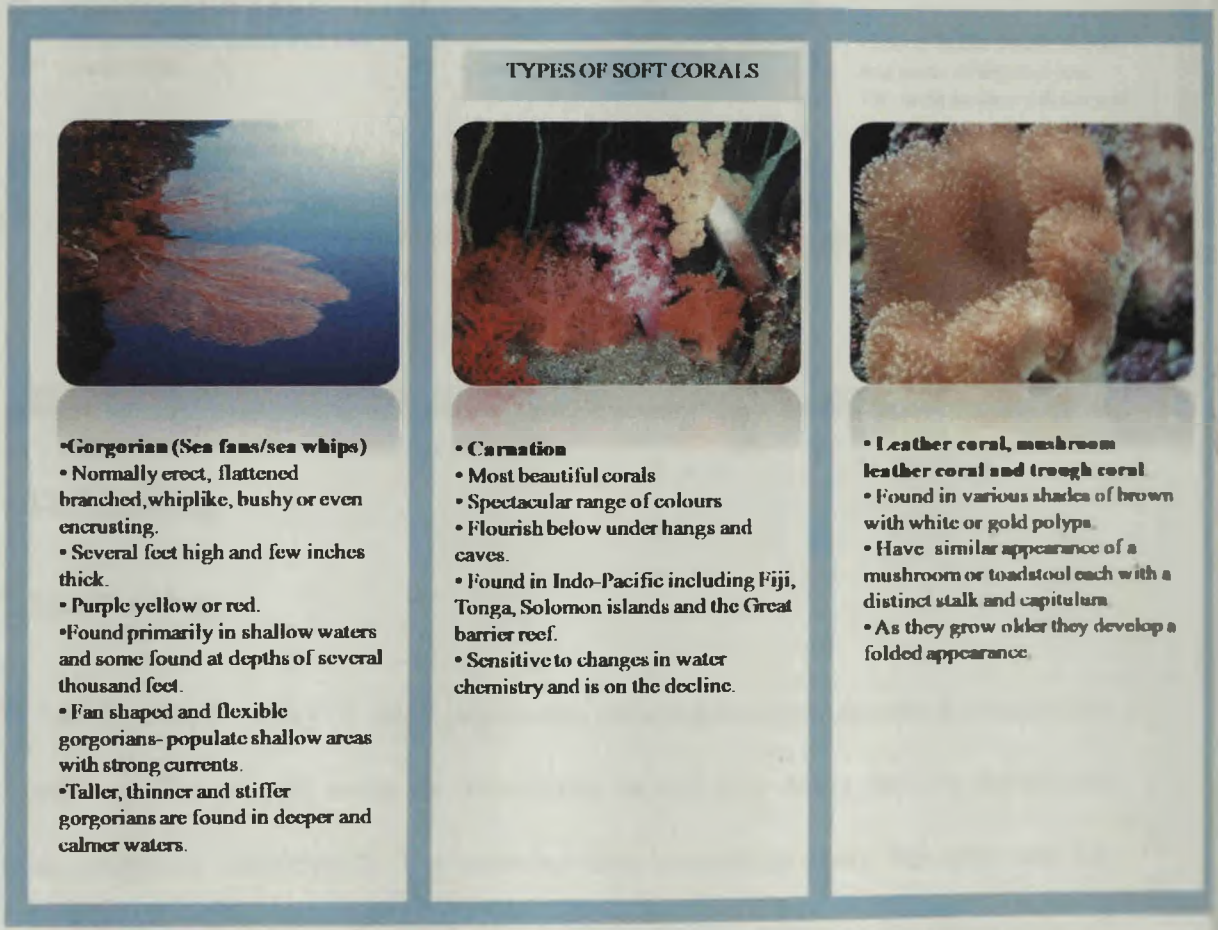
Soft coral anatomy

As these coral species do not secrete calcium carbonate their polyps do not contain calyx, theca, tabulae, septae or a basal plate. **Tentacles and mesenteries** are present in groups of eight rather then six. In addition their tentacles are often fringed and many of them do not contain zoozanthellae (Barnes, 1987; University of Michigan–Geological Sciences 100 Course page). In some soft corals polyps specialise to

perform specific functions for the colony such as formimg supportive core at the center of the main stalk of the **colony** (University of California Museum of Paleontology Website–Introduction to Octocorallia page).

Types of soft coral

The types of corals are sumarised in the figures below



- Tree corals
- Flowery corals are commonly seen in many of our shores.
- Usually attached to hard surfaces including boulders and coral rubble.
- Look like bushes and are generally rubbery and rough to touch.
- A thick main trunk attaches to a hard surface on one end with many small branches on the other hand and hence referred to a tree coral.

- Sea pens
- Reminiscent of antique quill pens.
- Sea pens may rise upto 2 mts (6.6 ft) (*Funiculina quadrangularis*)
- Sometimes brightly colored.
- Rarely found above depths of 10 mts sea pens prefer deeper waters where turbulence is less likely to uproot them

- Bubble coral/Grape coral/Pearl coral.
- have large water filled bubbles (vesicles).
- Found in the Pacific ocean and parts of the Red Sea.
- Occur in various colours and forms.
- Their egg like or grape like appearance during the sunlit hours and then deflate at dark manifesting finger tentacles that feed on plankton etc.

1.3.2 Physiology

1.3.2.1 Feeding

Polyps feed on a variety of small organisms, from microscopic demersal plankton to small fish. The polyp's tentacles immobilize or kill prey using their **nematocysts** (also known as 'cnidocysts'). The tentacles then contract to bring the prey into the stomach. Once the prey is digested, the stomach reopens, allowing the elimination of waste products and the beginning of the next hunting cycle. They can scavenge drifting organic molecules and dissolved organic molecules (Murphy and Richard 2002).

1.3.2.2 Reproduction

Corals can be both **gonochoristic (unisexual) and hermaphroditic**, each of which can reproduce sexually and asexually. Reproduction also allows coral to settle in new areas (Veron, 2000; Barnes *et al.*, 1987; Hatta, *et al.*, 1999; Madl and Yip 2000; Jones and Endean, 1973; Barnes and Hughes 1999). The coral cycle is schematically explained below

CORAL LIFECYCLE

Corals are either Gonochoristic or Hemaphroditic)

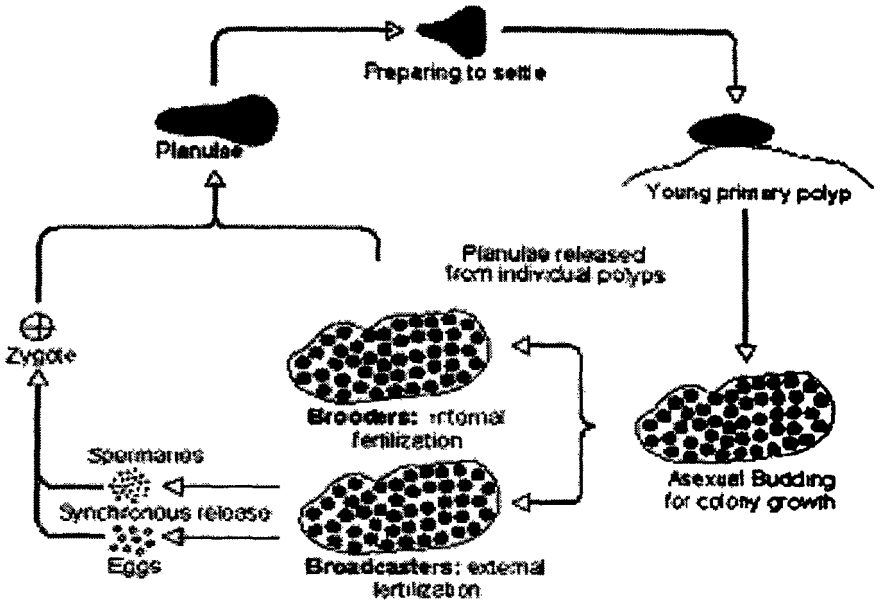
Sexual ⟵ ⟶ **Asexual**

- **BROADCASTERS: broadcast "spawn" by releasing Gametes-eggs and sperms—into the water to spread offspring.**
- **Gametes fuse form planulae (pink and elliptical)**
- **Seen in hermatypic corals (reef building).**
- **Temperature change, lunar cycle, day length, and possibly chemical signalling.**
- **BROODERS: release only sperm, which is negatively buoyant, sinking on to the waiting egg carriers who harbor unfertilized eggs for weeks ⟶ planulae.**
- **Planulae exhibit positive sonotaxis and phototaxis settle on a hard surface ⟶ larvae ⟶ polyp ⟶ coral head by asexual budding**

- **Either via gemmation (budding) or by longitudinal or transversal division**
- **BUDDING: involves splitting a smaller polyp from an adult.**
  - ❖ **Intratentacular—from its oral discs, producing same-sized polyps within the ring of tentacles**
  - ❖ **Extratentacular—from its base, producing a smaller polyp.**

DIVISION: Longitudinal division polyp broadens and then divides its coelenterons. mouth divides and new tentacles form. Transversal division occurs when polyps and the exoskeleton divide transversally into two parts. This means one has the basal disc (bottom) and the other has the oral disc (top).

- **The two "new" polyps then generate their missing body parts and exoskeleton.**



1.4 BIVALVES

Bivalvia, commonly referred to as bivalves, are the class of marine and freshwater molluscs with laterally compressed bodies enclosed by a shell in two hinged parts. They include clams, oysters, mussels, scallops, and numerous other families. The majorities are filter feeders and have no **head or radula**. The gills have evolved into **ctenidia**, specialised organs for feeding and breathing. Most bivalves bury themselves in sediment on the seabed, where they are safe from predation. Others lie on the sea floor or attach themselves to rocks or other hard surfaces. A few bore into wood, clay or stone and live inside these substances. Some bivalves, such as the scallops, can swim.

1.4.1 Classification of Bivalves

Subclass: Pteriomorphia (marine mussels)

Pteriomorphia is a subclass of saltwater clams, marine bivalve mollusks. It contains several major orders and suborders: the Arcoida,Ostreoida, Pectinoida, Limoida, Mytiloida, and Pterioida, and it also contains some extinct and probably basal families, such as the Evyanidae, Colpomyidae, Bakevelliidae, Cassianellidae and Lithiotidae.

This subclass of mollusks has lamellibranch gills, and are epibenthic. Some attach to the substrate using a byssus. The foot is reduced. The mantle margins are not fused. Gills are usually large and assist in feeding. This group includes the well known mussels, scallops,pen shells, and oysters (Barnes, 1982).

Palaeoheterodonta is a subclass of bivalve molluscs Palaeoheterodonta. It contains the extant orders Unionoida (freshwater mussels) and Trigonioida. They are distinguished by having the two halves of the shell be of equal size and shape, but by having the hinge teeth be in a single row, rather than separated into two groups, as they are in the **clams and cockles** (Barnes 1982).

Heterodonta is a taxonomic subclass of saltwater clams, marine bivalve molluscs. This subclass includes the edible clams, the cockles and the Venus clams Heterondonta. These bivalves are distinguished by having the two halves of the shell equally sized, and having a few cardinal teeth separated from a number of long lateral teeth. Their shells lack a nacreouslayer, and the gills are lamellibranch in form. Most species have a siphon (Barnes, 1982).

The mussel's external shell is composed of two hinged halves or "valves". The valves are joined together on the outside by a ligament, and are closed when

necessary by strong internal muscles. Mussel shells carry out a variety of functions, including support for soft tissues, protection from predators and protection against desiccation.

The shell has three layers. In the pearly mussels there is an **inner iridescent** layer of nacre (mother-of-pearl) composed of calcium carbonate, which is continuously secreted by the mantle; **the prismatic layer**, a middle layer of chalky white crystals of calcium carbonate in a protein matrix; and **the periostracum**, an outer pigmented layer resembling a skin. The periostracum is composed of a protein called **conchin**, and its function is to protect the prismatic layer from abrasion and dissolution by acids (especially important in freshwater forms where the decay of leaf materials produces acids).

Like most bivalves, mussels have a large organ called a **foot**. In freshwater mussels, the foot is large, muscular, and generally hatchet-shaped. It is used to pull the animal through the substrate (typically sand, gravel, or silt) in which it lies partially buried. It does this by repeatedly advancing the foot through the substrate, expanding the end so it serves as an anchor, and then pulling the rest of the animal with its shell forward. It also serves as a fleshy anchor when the animal is stationary.

In marine mussels, the foot is smaller, tongue-like in shape, with a groove on the ventral surface which is continuous with the byssus pit. In this pit, a viscous secretion is exuded, entering the groove and hardening gradually upon contact with sea water. This forms extremely tough, strong, elastic, byssus threads that secure the mussel to its substrate. The byssus thread is also sometimes used by mussels as a defensive measure, to tether predatory molluscs, such as dog whelks, that invade mussel beds, immobilising them and thus starving them to death

1.4.2 Physiology

1.4.2.1 Feeding

Both marine and freshwater mussels are filter feeders; they feed on plankton and other microscopic sea creatures which are free-floating in seawater. A mussel draws water in through its **incurrent siphon**. The water is then brought into the **branchial chamber** by the actions of the **cilia** located on the gills for ciliary-mucus feeding. The wastewater exits through the **excurrent siphon**. The **labial palps** finally funnel the food into the mouth, where digestion begins. Marine mussels are usually found clumping together on wave-washed rocks, each attached to the rock by its byssus. The clumping habit helps hold the mussels firm against the force of the waves.

1.4.2.2 Reproduction

Both marine and freshwater mussels are **gonochoristic**, with separate male and female individuals. In marine mussels, fertilization occurs outside the body, with a larval stage that drifts for three weeks to six months, before settling on a hard surface as a young mussel. There, it is capable of moving slowly by means of attaching and detaching **byssal threads** to attain a better life position.

Freshwater mussels reproduce sexually. Sperm is released by the male directly into the water and enters the female via the **incurrent siphon**. After fertilization, the eggs develop into a larval stage called a **glochidium (plural glochidia)**, which temporarily parasitize fish, attaching themselves to the fish's fins or gills. Prior to their release, the glochidia grow in the gills of the female mussel where they are constantly flushed with oxygen-rich water. In some species, release occurs when a fish attempts to attack the mussel's minnow or other mantle flaps shaped like prey;

an example of aggressive mimicry.Glochidia are generally species-specific, and will only live if they find the correct fish host. Once the larval mussels attach to the fish, the fish body reacts to cover them with cells forming a cyst, where the glochidia remain for two to five weeks (depending on temperature). They grow, break free from the host, and drop to the bottom of the water to begin an independent life.

1.5 Microbes associated with Sponges, Corals and Bivalve

They include a diverse range of archaea, intracellular and extracellular heterotrophic bacterial communities, cyanobacteria, green algae, red algae, cryptophytes, viruses, dinoflagellates and diatoms (Duglas, 1994; Larkum *et al.*, 1987; Preston *et al.*, 1996; Santavy *et al.*, 1990; Taylor *et al.*, 2007). The symbiotic microbial community is highly novel and diverse, can be host species specific and displays some level of spatial variability from local to geographic and temporal scales (Rohwer *et al.*, 2002, Webster and Hill 2001). The microbial assemblage differs from those of the overlying water column (Charlott *et al.*, 2010, Rohwer *et al.*, 2002). The bacterial assemblage associated with marine sponges and corals is predominated by γ-Proteobacteria, ß-proteobacteria, δ-proteobacteria, Bacteroidetes, Acidobacteria, with genus *Bacillus* and *Pseudomonas* sp. being the most predominant, followed by Genus *Vibrio* sp., *Pseudoalteromonas* sp. and *Streptomyces* sp. (Penn *et al.*, 2006; Kennedy *et al.*, 2008). The various geographical locations mapped for the microbial diversity associated with sponges and corals are tabulated in Table 1.1 and 1.2 respectively. Table 1.1 indicates that the sponges from the family Spongiidae has been the most extensively studied for its microbial diversity while among the corals, as evident from Table 1.2, the corals from the family Acroporidae is most widely studied for its microbes.

Table 1.1. Sponges and its associated microbial diversity

FAMILY	SPONGES	ECONICHE	GENERA	REFERANCE
Chalinidae	*Haliclona simulans*	Irish waters	*Verrucomicrobia* sp., *Lentisphaerae* sp., *Bdellovibrio* sp.	Kennedy *et al.*, 2008
Petrosiidae	*Xestospongia muta* and *Xestospongia testudinaria*	Tropical reefs of the Atlantic and Pacific Oceans	*Chloroflexi* sp., *Acidobacteria*, *Actinobacteria*	Montalvo and Hill 2011
Agelasidae/ Chondrillidae/ Petrosiidae/ Geodiidae/ Tethyidae/ Axinellidae/ Dysideidae/ Plakinidae	*Agelas oroides*, *Chondrosia reniformis*, *Petrosia ficiformis* , *Geodia sp.*, *Tethya sp.*, *Axinella polypoides*, *Dysidea avara* and *Oscarella lobularis*	Mediterranean Sea	Unidentified bacteria belonging to Cyanobacteria, Actinobacteria, Acidobacteria, Bacteroidetes	Berna *et al.*, 2011
Mycalidae	*Mycale armata*	Hawaii island	Unidentified bacteria belonging to Proteobacteria, Actinobacteria,Bacteroidetes,Cyanobacteria,Acidobacteria,Chloroflexi,Crenarchaeota,Firmicutes, α-proteobacteria	Wang *et al.*, 2009
Aplysinidae	*Aplysina aerophoba* and *Aplysina cavernicola*	Mediterranean Sea	*Bacillus* sp., *Arthobacter* sp., *Micrococcus* sp., *Pseudoalteromonas* sp.	Hentshel *et al.*, 2001
Spongiidae	*Rhopaloeides odorabile*	Great Barrier Reef, Australia	*Cytophaga* sp., *Flavobacterium* sp., unidentified bacteria belonging to *Actinobacteria*, the ß- and γ-subdivisions of the *Proteobacteria* green sulfur bacteria, green nonsulfur bacteria, Planctomycetes	Webster *et al.*, 2001
Neopeltidae	*Homophymia* sp.	off Touho (New Caledonia)	*Pseudomonas* sp.	Bultel-Ponce *et al.*, 1999

Thorectidae	*Fasciospongia cavernosa*	GoM, India	*Streptomyces* sp., *Salinobacter* sp., *Roseobacter* sp., *Pseudomonas* sp., *Vibrio* sp., *Micromonospora* sp., *Saccharomonospora* sp., *Alteromonas* sp.	Selvin *et al.*,2009
Axinellidae/ Asteraceae/ Tethyidae/ Irciniidae/ Petrosiidae/ Suberitidae/ Melastomataceae	*Axinella polypoides*, *Chondrilla nucula*, *Tethya aurantium*, *Clathrina clathris*, *Agelas oroides*, *Ircinia* sp., *Petrosia ficiformis*, *Suberites domuncula*, *Acanthella acuta*	Mediterranean Sea	Unidentified belonging to α - proteobacteria γ-proteobacter, Acidobacteria	Silberhorn *et al.*, 2008
Spongiidae/ Ianthellidae/ Aplysinidae	*Rhopaloeide sodorabile* *Ianthella basta* *Aplysina* sp.	Davies Reef on the Great Barrier Reef, Australia	*Nitrospira* sp. *Deinococcus* sp. and unidentified bacteria beonging to α, γ and Deltaproteobacteria,Acidobacteria, Actinobacteria,Archea,Verrucomicrobia,Bacteroidetes Planctomycetes,Lentisphaerae,Spirochaetes,Fimicutes	Wagner *et al.*,
Suberitidae	*Suberites domuncula*	Northern Adriatic, near Rovinj, Croatia	α-Proteobacterium *Idiomarinaioihiensis*(Alteromonadaceae)	Thakur *et al.*, 2005
Suberitidae	*Aaptos* sp.	Panjang island, Jepara, North Java Sea, Indonesia	*Halomonas aquamarina* *Pseudoalteromonas luteoviolacea* and unidentified bacteria belonging to α-proteobacterium	Radjasa *et al.*, 2007
Pseudoceratinidae	*Pseudoceratina purpurea*	Mandapam GoM, India	*Vibrio* sp., *Bacillus* sp.,*Virgibacillus* sp., *Photobacterium* sp.	Kanagasabhabathy *et al.*, 2005
Dysideidae/ Chalinidae	*Dysidea granulosa*, *Sigmadocia fibulata*	Lakshadweep islands	Unidentified belonging to γ proteobacteria, Firmicutes, Actinobacteria, ß proteobacteria	Feby and Nair ,2010

Ancorinidae/ Halichondriidae/ Tetillidae	*Stelletta tenui, Halichrondria, Dysidea avara, Craniella australiensis*	South China Sea	Unidentified belonging to Proteobacteria, Bacteroidetes Firmicutes, Actinobacteria	Li *et al.*, 2006
Astroscleridae	*Ceratoporella nicholsoni*	Jamaica Bay at Acklins Island, Bahamas	*Vibrio* sp., *Aeromonas* sp.	Santavy and Colwell 1990
Coelosphaeridae / Cruciferae/ Ircinidae/ Tedaniidae	*Lissodendoryx isodyctiyalis, Crambe crambe, Ircinia dendroides, Tedaniaignis* sp.	India	*Shewanella irciniae, Winogradskyella poriferorum, Gillisia myxillae, Fabibacter halotolarans, Microbulbifer mediterranus, Stenothermonacter spongiae*	Dash, 2009
Niphatidae	*Gelliodes carnosa*	Hainan Island Coastal Waters of the South China Sea	*Bacillus* sp., *Halobacillus* sp., *Pontibacillus* sp., *Brevibacillus* sp., *Oceanobacillus* sp., *Staphylococcus* sp., *Rhodococcus* sp., *Micromonospora* sp., *Verrucosispora* sp., *Gordonia* sp., *Pseudonocardia* sp., *Sphaerisporangium* sp., *Streptomyces* sp., *Saccharomonospora* sp., *Vibrio* sp., *Photobacterium* sp., *Shigella* sp., *Pseudoalteromonas* sp., *Alteromonas* sp., *Marinobacter* sp., *Marinomonas* sp., *Kangiell* sp., *Alcanivorax* sp., *Phaeobacter* sp., *Pseudomonas* sp.	Li *et al.*, 2011
	Antarctic sponge	Antartica	*Psychrobacter* sp., *Pseudoalteromonas* sp., *Arthrobacter* sp.	Papaleo *et al.*, 2012
Dictyonellidae/ Chalinidae/ Mycalidae/ Axinellidae/ Callyspongiidae/	*Acanthella ramosa, Sigmadocia fibulatus, Mycale mannarensis, Axinella carteri, Callyspongia*	Hare Island and Rameswaran region, South East coast of India	*Bacillus* sp., *Alteromonas* sp., *Flavobacterium* sp., *Micrococcus* sp., *Vibrio* sp., *Pseudomonas* sp., *Plesiomonas* sp., *Corynebacterium* sp., *Photobacterium* sp., *Staphylococcus* sp.	Saravanakumar *et al.*, 2011

Petrosiidae/ Aplysinellidae/ Plakinidae/ Thorectidae/ Ancorinidae/ Dysideidea/ Tedaniidae/ Dysideidae	*fibrosa, Epipolasis topsenti, Petrosia testudinaria, Psammaplysilla purpurea, Plakortis simplex, Fasciospongia cavemosa, Stelletta tenui, Dysidea fragilis, Carniella australiensis, Dysidea avara*			
Microcionidae/ Chalinidae/ Dysideidae	*Clathria procera, Sigmadocia fibulata, Dysidea granulosa*	Lakshadweep archipelago	*Acinetobacter calcoaceticus*	Gopi *et al.*, 2012
Pseudoceratinidae	*Pseudoceratina clavata*	Australia	*Actinobacteria, cyanobacteria*	Kim *et al.*, 2006
Hymeniacidonidae	*Hymeniacidon perleve*	Nanjiisland,China	*Pseudoalteromonas piscicida*	Zheng *et al.*, 2005
Irciniidae	*Ircinia muscarum*	bay of Naples (Italy)	*Pseudomonas* sp.	Mitova *et al.*, 2003
Pachastrellidae	*Characella* sp.	Sumisu Caldera, Ogasawara Island chain, Japan	Thioautotrophic bacteria belonging to the γ-proteobacteria	Nishijima *et al.*, 2010
Axinellidae	*Axinella corrugata*	Caribbean reef Florida reef	Unidentified belonging to γ-proteobacteria, Flavobacteria, α - proteobacteria, Cyanobacteria, Acidobacter, Nitrospira	White *et al.*, 2004
Chalinidae	*Haliclona* sp.	Waigeo Island, Indonesia	*Bacillus* sp.	Tokasaya, 2010
Aplysinidae/	*Aplysina fistularis,*	Bahamas	*Microbacterium* sp., *Rhodococcus* sp., *Streptomyces* sp.,	Tabares *et al.*, 2011

Plakinidae/ Niphatidae/ Aplysinellidae/ Aplysinidae/ Agelasidae/ Agelasidae/ Irciniidae/ Dictyonellidae/ Geodiidae/ Chondrillade/ Aplysinidae	*Plakortis sp., Amphimedon compressa, Aiolochroia crassa, Agelas clathrodes, Agelas cerebrum, Ircinia felix, Scopalina ruetzleri, Erylus formosus, Chondrilla nucula, Aplysina archeri*		*Mycobacteriu* sp., *Micromonospora* sp., *Knoellia* sp., *Gordonia* sp., *Curtobacterium* sp., *Arthrobacter* sp., *Salinispora* sp., *Saccharopolyspora* sp., *Nocardioides* sp., *Citromicrobium* sp., *Sanguibacter* sp., *Lapillicoccus* sp., *Kocuria* sp., *Dietzia* sp. ,*Cellulosimicrobium* sp., *Cellulomonas* sp., *Agrococcus* sp.	
Irciniidae	*Ircinia ramosa*	Ratnagiri, located along the central west coast of India	*Bacillus* sp., *Xanthomonas* sp., *Pseudomonas* sp.	Thakur and Anil 2000
Mycalidae/ Raspmliiiiae/ Microcionidae/ Raspmliiiiae/ Microcionidae/Pseudoceratinidae/Niphatidae/ Calthropellidae/ Tethyidae	*Mycale* (Zygomycale) *parishii, Echinodictyum spp., Clathria (Microciona) sp., Echinodictyum spp., Clathria (Thalysias) reinwardti, Pseudoceratina sp., Gelliodes petrosioides, Pachastrissa nux, Coelocarteria singaporensis, and Tethya seychellensis*	Chaolao Beach of Chanthaburi Province, Mak Island of Trat Province, and Kood Island of Trat Province located in the Gulf of Thailand.	*Sphingomonas* sp.	Thawornwiriyanun *et al.*, 2012
Raspmliiiiae/Chalinidae/ Mycalidae	*Echinodictyum sp., Spongia sp., Sigmadocia*	Tuticorin coast, Gulf of	*Bacillus* sp., *Vibrio* sp., *Idiomarina* sp., *Dietzia* sp., *Staphylococcus* sp., *Marinobacter* sp.,	Elyakov *et al.*, 1991

	fibulatus, Mycale mannarensis	Mannarregion,India	*Pseudomonas* sp., *Ruergia* sp., *Salinicoccus* sp.	
Chalinidae	*Sigmadocia medussa*	Nagapattinum coast, Tamil Nadu,India	Unidentified bacteria	Kalaivani and Krishnapriya 2012
Steilettidae	*Stelletta* sp.	off the coast of Geoje Island, Korea.	*Psychrobacter* sp.	Li *et al.*, 2009
Agelasidae	*Agelas robusta*	coral reef in the South China Sea	*Chloroflexis* sp., *Synechococcus* sp.	Sun *et al.*, 2010
Jaspidae	*Jaspis* sp.	Waigeo Island, Raja Ampat West Papua Province, Indonesia	*Providencia* sp., *Paracoccus* sp., *Bacillus* sp.	Wahyudi *et al.*, 2010
Aplysinidae/ Dysideidae/ Tethyidae/ Chondrillidae	*Aplysina aerophoba, Dysidea avara, Tethya* sp., *Chondrosia reniformis*	offshore Banyuls-sur-Mer (France) and from Rovinj (Croatia)	*Nitrosospira* cluster 1 of the ß proteobacteria *Nitrosomonas*	Bayer *et al.*, 2007
Haliclonidae	*Haliclona grant*	Thondi in the Palk Strait region of Tamil Nadu,India	RJAUTHB 14 *Unidentified*	Inbaneson and Ravikumar, 2012
Axinellidae/ Mycalidae/ Crambeidae/ Niphatidae/ Crambeidae/ Petrosiidae	*Axinella corrugata, Mycale laxissima, Monanchoraunguifera,* and *Niphates digitalis Didiscus oxeata* and *Monanchora unguifera Acanthostronglyophora* sp.	Key Largo, Florida; from Discovery Bay, Jamaica; from the Cheasapeake Bay in Maryland	*Pseudovibrio denitrificans*	Enticknap *et al.*, 2006
Axinellidae/	*Cymbastela concentric*	Sydney, Australia;	*Pseudovibrio* sp.	Penesyan *et al.*, 2010

Spongiidae	*Rhopaloeides odorabile*	Great Barrier Reef		
Mycalidae	*Mycale Adhaerens Bowerbank*	at three sites in the eastern Hong Kong waters, namely, Three Fathoms Cove Long Harbour and Clear Water Bay	*Pseudoalteromonas* sp., *Bacillus* sp., *Alteromonas* sp. *Shewanella* sp., *Vibrio* sp., *Tenacibaculum* sp., *Microbulbifer* sp., *Micrococcus* sp., *α-proteobacterium*,Uncultured *Ruegeria*	Lee *et al.*, 2006
Niphatidae	*Amphimedon ochracea*	Red Sea (El-Gona station)	*Bacillus* sp.	Aboul-Ela *et al.*, 2012
Polymastiidae	*Polymastia cf. corticata*	at the Kahouanne Basin (Lesser Antilles, Caribbean Sea)	Unidentified bacteria belonging to Crenarchaeota, α - proteobacteria, ß-proteobacteria, γ-proteobacteria, Deltaproteobacteria, Acidobacteria, Actinobacteria, Nitrospira	Meyer and Kuever, 2008
Darwinellidae/ Halichondriidae/ Dysideidae/ Tetillidae	*Stelletta tenuis, Halichondria rugosa, Dysidea avara, Craniella australiensis*	South China Sea	*Alcaligenes* sp., *Staphylococcus* sp., *Bacillus* sp.	Zhang *et al.*, 2009
Aplysinidae	*Aplysina aerophoba*	Banyulssur Mer, France	Unidentified belonging to *Poribacteria, Planctomycetes, Verrucomicrobia, Chlamydia*	Fieseler *et al.*, 2004
Chondrillidae	*Chondrilla nucula*	Limskikanal,Croatia	*Chloroflexi* sp. and unidentified bacteria belonging to γ - and δ proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Gemmatimonadales.	Thiel *et al.*, 2007
Thorectidae/ Niphatidae	*Hyrtios erectus, Amphimedon* sp.	Ras Mohamed, Sinai, Egypt Red Sea	Unidentified bacteria belonging to γ- proteobacteria, ß-proteobacteria, Bacteroidetes, Actinobacteria, α-proteobacteria	Radwan *et al.*, 2010

Aplysinidae/ Theonellidae/ Spongiidae/ Halichondriidae/ Axinellidae	*Aplysina aerophoba* and *Theonella swinhoei* *Rhopaloeide sodorabile* *Halichondri apanicea* *Axinella mexicana*	BanyulssurMer, France Western Caroline Islands, Palau Eilat, Israel Hachijo-jima Island, Japan Davies Reef, Australia Santa Barbara, California	*Nitrospira* sp. and unidentified bacteria belonging Actinobacteria, Cyanobacteria, Acidobacteria, δ-proteobacteria Bacteroidetes	Hentschel *et al.*, 2002
Spongiidae	*Spongia officinalis*	Aegean Sea, Mediterranean Sea	*Escherichia* sp., *Morganella* sp., *Proteus* sp. *Pasteurella* sp., *Aeromonas* sp., *Pseudomonas* sp. *Acinetobacter* sp.	Kefalas *et al.*, 2003
Thorectidae/ Ancorinidae	*Dysidea avara* *Stelletta tenui*	China Sea	*Alcaligenes* sp., *Acinetobacter* sp., *Staphylococcus* sp., *Providencia* sp.	Li *et al.*, 2007
Suberitidae	*Suberites domuncula*	near Rovinj (Croatia)	*Proteobacterium* sp.	Muller *et al.*, 2004
Petrosiidae	*Petrosia ficiformis*	Paraggi (Portofino Promontory, Ligurian Sea)	*Pseudoalteromonas* sp., *Flavobacter* sp., *Micrococcus* sp., *Bacillus* sp., *Corynebacteria* sp., *Streptomyces* sp., *Vibrio* sp., *Aeromonas* sp.	Chelosi *et al.*, 2004
Chalinidae	*Haliclona simulans*	Irish coastal waters	Unidentified bacteria belonging to γ -Proteobacteria	Dobson *et al.*, 2007
Tethyidae	*Tethya californiana*	Monastery Beach USA	Unidentified bacteria belonging to α -Proteobacteria, c-Proteobacteria ß-Proteobacteria, δ-Proteobacteria, e-Proteobacteria, Bacteroidetes, Firmicutes,Verrucomicrobia. Actinobacteria	Sipkema and Blanch 2010
Esperiopsidae/ Raspailiidae	*Amphilectus fucorum* and *Eurypon major*	Ireland	*Proteobacteria* sp., *Pseudovibrio* sp.	Margassery *et al.*, 2012

Halisarcidae	*Halisarca ectofibrosa*	Thailand	*Pseudoalteromonas* sp.	Rungprom *et al.*, 2008
Suberitidae	*Suberites domuncula*	Naples Italy	*Ruegeria* strain	Mitova *et al.*, 2004
Myxillidae/Latrunculiidae/Suberitidae/Mycalidae/Polymastiidae	*Kirkpatrickia varialosa, Latrunculia apicalis, Homaxinella balfourensis, Mycale acerata, Sphaerotylus antarcticus*	Antarctica	*Cytophaga/Flavobacterium* of Bacteroidetes group and unidentified bacteria belonging to γ and α- proteobacteria	Webster *et al.*, 2004
Suberitidae	*Terpios hoshinota*	Taiwan	Unidentified bacteria belonging to α- proteobacteria γ-proteobacteria, Cyanobacteria	Tang, 2011
Rosselidae/Coelosphaeridae	*Anoxycalyx joubini, Lissodendoryx nobilis*	Antarctica	Unidentified bacteria belonging to α- and γ- Proteobacteria respectively), the CFB group of Bacteroidetes,Actinobacteria	Mangano *et al.*, 2009
Axinellidae	*Cymbastela concentrica*	Australia	*Bacillus* sp.,*Kinecoccus* sp.,*Mycobacterium* sp. *Escherichia* sp.*Burkholderia sp.*,*Moraxella* sp. *Acanthamoeba* sp. *Schizosaccharomyces* sp.	Yung *et al.*, 2011

Table 1.2 Corals and its associated microbial diversity.

FAMILY	ECONICHE	CORALS	BACTERIA	REFERANCE
Nephtheidae	Port Shelter, Hong Kong	*Dendronephthya* sp.	*Vibrio* sp., *Photobacterium* sp., *Pseudoalteromonas* sp.	Harder *et al.*, 2003
Acroporidae	Davies Reef, Great Barrier Reef, Australia	*Montipora aequituberculata, Acropora millepora*	*Roseobacter sp., Spongiobacter sp. Vibrio sp., Alteromonas* sp.	Raina *et al.*, 2009
	Gulf of Mannar-India	*Acropora latistella* sp.	*Bacillus* sp., *Enterobacter* sp.	Babu *et al.*, 2004
	Teluk awur north Java sea Indonesia	*Acropora* sp.	*Pseudoalteromonas* sp., *Luteoviolacea* sp., *Vibrio harveyi*	Radjasa *et al.*, 2005
	Gulf of Mannar-India	*Acropora digitifera*	*C. violaceum*	Thenmozhi *et al.*, 2009
	Heron reef, Great barrier reef, Australia	*Acropora millepora*	*Vibrio* sp., *Pseudoalteromonas* sp., *Bacillus* sp.	Kvennefors *et al.*, 2010
	Heron island Australia	*Acropora muricata*	*Pseudidiomarina* sp., *Klebsiella* sp., *Roseobacter* sp.	Sweet *et al.*, 2011
	Gulf of Mannar-India	*Acropora digitifera*	Unidentified bacteria belonging to α- proteobacteria, γ-proteobacteria, Actinobacteria, Firmicutes	Paramasi *et al.*, 2011
	Great barrier reef-Australia	*Acropora millepora*	*Vibrio* sp.	Bourne *et al.*, 2008

	Bidong island, Terengganu Malaysia	*Acropora cervicornis*	*Pantoea dispersa, Pseudomonas* sp., *Enterobacter agglomerans, Cadacea darisae Serratia plymuthica, Citrobacter youngae, Erwinia herbicola* *Vibrio* sp., *Klebsiella pneumonia, Alteromonas* sp. *Moraxella* sp., *Photobacterium* sp., *Yersinia bercovieri* *Brucella* sp., *Acinetobacter* sp., *Micrococcus* sp., *Flavobacterium* sp.	Kalimutho *et al.*, 2007
	China	*Acropora solitaryensis*	*Psychrobacter* sp., *Rhizobium* sp., *Ochrobactrum* sp.	Liu *et al.*, 2012
	Caribbean	*Acropora palmata*	*S. marcescens, Photobacterium leiognathi, Halomonas meridian, Photobacterium mandapamensis*	Krediet *et al.*, 2009
Acroporidae/Pocci lloporidae	Great Britan reef	*Acropora millepora, A.muricata, Pollicipora damicormis*	Unidentified bacteria belonging to α- proteobacteria, δ-proteobacteria,Cyanobacteria	Lema *et al.*,2012
Acroporidae/Pocci lloporidae/ *Dendrophylliidae*	Ningaloo Reef in Western Australia	*Acropora tenuis, Pocillopora damicornis, Tubastrea faulkneri*	*Acidobacteria, Flavobacteria, Sphingobacteria, Bacilli, Clostridia*	Ceh *et al.*, 2012
Pocilloporidae	Great barrier reef Australia	*Pocillopora damicornis*	*Vibrio* sp., *Enterobacteria* sp., *Alteromonas* sp.and Unidentified bacteria belonging to α- protobacteria sp.,ß protobacteria sp.Cyanobacteria sp.Firmicutes sp.,Atinobacteria sp.	Bourne and Munn 2005

Acroporidae/Faviidae	Tuticorin, India	*Acropora formosa, Favia palida*	Unidentified	Chellaram *et al.*, 2011
Faviidae/Faviidae/Poritidae	Whale Bone Bay, Bermuda, Hog Breaker Reef, Bermuda and Bocas del Toro, Panama	*Monstastraea franksi, Diploria stigosa, Porites astreoides*	*Bacillus* sp., *Clostridium* sp., *Cytophaga* sp. *Flavobacter* sp., *Flexibacter* sp., *Bacterioides* Cyanobacteria	Rohwer *et al.*, 2002
Faviidae/Poritidae	St Thomas U.S. Virgin Island. Summerland key, Florida	*Montastraea faveolata* *Porites asteroides*	Cyanobacteria sp., Protobacteria sp. Actinobacteria, Firmicuties, Bacteroidetes	Morrow *et al.*, 2012
Faviidae	Caribbean and Panama	*Montastraea faveolata*	Unidentified bacteria belongiong to Protobacteria sp. Firmicutes and *Clostridia* sp.	Sunagawa *et al.*, 2009
Poritidae	Hawaii	*Porites lobata*	Unidentified bacteria	Garren and Azam 2010
Caryophylliidae	Northeastern gulf of Mexico	*Lophelia pertusa*	*Mycoplasma* sp.	Kelogg *et al.*, 2009
Plexauridae	Hillsboro Ledge, Deerfield Beach, Florida, USA.	*Eunicea fusca*	Proteobacteria Acidobacteria, Actinobacteria, Cyanobacteria, Planctomycetes, δ-Proteobacteria, Lentisphaerae and Nitrospirae.	Duque-Alarcón *et al.*, 2012
Acroporidae/Poritidae/Poritidae	Oahu Hawaii	*Montipora capitata, Porites compressa, Porites lobata*	*Micrococcus* sp., *Photobacterium* sp., *Psuedoalteromonas* sp., *Psychrobacter* sp., *Vibro* sp., *Alteromonas* sp.	Ritchie, 2005

	Red Sea	Stony Scleractinea and soft Octocorallia corals	*Chloroflexi* sp., *Chlamydiae* sp.	Lee *et al.*, 2012
Faviidae/Faviidae/ Fungiidae/Fungiidae/Pocilloporidae/ Musiidae/ Xeniidae	Gulf of Eilat Israel	*Platygyra sp.* *Porites sp.* *Fungia granulose* *Favia sp.* *Stylophora sp.* *Acanthastrea sp.* *Pocillopora sp.* *Xenia sp.*	Unidentified bacteria belonging to α-proteobacteria, γ-proteobacteria	Golberg *et al.*, 2011
Fungiidae	Gulf of eilat, red sea.	*Fungia granulosa*	Unidentified bacteria belonging to α, ß, γ, proteobacterium sp., Cynobacteria sp., Acidobacteria sp. Firmicutes sp., Planctomycetes sp.	Kooperman *et al.*, 2007
	Red sea	*Fungia scutaria*	Unidentified bacteria belonging to γ protobacteria α protobacteria andActinobacteria	Lampert *et al.*, 2006
	Gulf of Alaska seamounts	*Octocorals*	Protobacteria sp., Firmicutes, Bacteroidetes, Acidobacteria	Penn *et al.*, 2006
Subergorgiidae/Ellisellidae	Tuticorin India	*Subergorgia suberosa, Junceella juncea*	*Micrococcus* sp., *A. formicans, Proteus mirabilis*	Gnanambal *et al.*, 2005
Siderastreidae	Horseshoe reef, Bahamas	*Siderastrea siderea*	*Aurantimonas coralicida, Serratia marcescenes, Vibrio shiloi*	Gantar *et al.*, 2011

Alcyoniidae	Peucang island ,Indonesia	*Sarcophyton sp.*	*Pelagiobacter variabilis, Arthrobacter nicotianae, Pseudomonas synxantha, Bacillus aquamaris, Pseudovibrio denitrificans*	Sabdono and Radjasa 2006
Faviidae	Curacao, Netherlands Antilles	*Diploria strigosa*	*Shewanella oneidensis, Methanosarcina mazei, Vibrio parahaemolyticus*	Friaz-Lopez *et al.*, 2004
	Indian Ocean	*Echinopora lamellosa*	Protobacteria sp., Actinobacteria *Flavobacteria* and Bacilli	Piskorska *et al.*, 2007
	Hong Kong	*Latygyra carnosus*	Unidentified bacteria belonging to α- proteobacteria	Chiu *et al.*, 2012
Caryophylliidae	cold-water coral reefs along the continental margins	*Lophelia pertusa*	Unidentified bacteria belonging to α- and γ proteobacteria, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, *Firmicutes*, *Planctomycetes.*	Neulinger *et al.*, 2008
Alcyoniidae	Indonesia	*Sinularia polydactyla*	*Pseudomonas sp.*	Salasia *et al.*, 2008
	Red Sea	Scleractinian corals and alcyonacean soft corals	*Arthrobacter* sp. *Vibrio* sp.	Kelman *et al.*, 2006
Aiptasiidae	South Carolina ,western atlantic to the Caribbean	*Aiptasa pallida*	*Vibrio* sp. and other unidentified bacteria belonging to α- and γ protobacteria Firmicutes, Cyanobacteria	Plovie, 2010

1.5.1 Techniques adopted for detection and isolation of associated microbial flora

The use of molecular approaches for describing microbial diversity has greatly enhanced the knowledge of population structure in natural microbial communities. It is widely accepted that culture-based techniques are inadequate for studying bacterial diversity from environmental samples, as many bacteria cannot be cultured using current and traditional techniques (Hugenholtz *et al.*, 1998). The culturing strategy has been improvised greatly by use of selective media. Amending media with sponge extracts has also led to the isolation of bacteria not previously recovered. However the advantage of working on culturable bacteria is that they hold a promise for isolation of bioactive metabolites of therapeutic value.

Molecular techniques employed to document the microbial diversity associated with the marine invertebrates include Fluorescence in situ hybridization (FISH) (Webster *et al.*, 2001) Random

Amplified Polymorphic DNA (RAPD) (Chelosi *et al.*, 2004), 454-based tag sequencing strategy (Wagner *et al.*, 2002).The phylogenetic affiliation of the associated bacteria is assessed by 16S rRNA sequencing of cloned DNA fragments. The analysis of 16S rDNA genes through clone libraries and fingerprinting approaches such as denaturing gradient gel electrophoresis (DGGE) has also greatly extended our knowledge about the Phylogenetic richness of invertebrate associated bacteria (Thiel *et al.*, 2007; Mohamed *et al.*, 2008).

1.5.2 Location of micro-oranisms in inveterbrates

Microbes are located both intra and extra cellularly and each symbiotic microorganism seems to have a specific habitat in the host. Extracellular symbionts are present on the outer layers as exosymbionts (like the coral mucus layer known to harbor a large diversity of coral bacterial communities (Ritchie 2006) or in the inner tisue as endosymbionts (Althoff *et al.*, 1998, Flowers, 1998, Hinde, 1994). Some intracellular or intranuclear symbionts permanently reside in host cells or nuclei. In the case of the sponge *Theonella swinhoei*, all populations of symbiotic bacteria are located extracellularly (Bewley *et al.*, 1996). Bacteria, e.g., *Pseudomonas* sp. and *Aeromonas* sp. inhabit as free-living cells in the mesohyl and/or as intracellular symbionts in sponge *Verongia* (Vacelet, 1975, Wilkinson, 1978b). The amount of symbiotic microorganisms varies between from host to host. While bacteria constitute up to 60% of the biomass (Wilkinson 1978 a,b,c), others contain only a small number of bacteria inside their tissue (Vacelet and Danadey 1977).

1.5.3 Existence of species-specificity in host-microorganism relationship

Some symbionts are species specificity, but others don't. Wilkinson *et al.*, 1981 found that a symbiotic microorganism was specific to a single species. A species of *δ-proteobacteria* and the sponge *Theonella swinhoei* also show a specific association (Schmidt *et al.*, 1998). Vibrionaceae and Altermonadaceae (Rohwer *et al.*, 2001; Bourne and Munn, 2005) are bacterial isolates showing species specificity in corals. Some invertebrates have a dominant symbiotic microorganism. A species of *α-proteobacteria* dominates in sponge *Rhopaloeides odorabile* over various habitats but is not detected from seawater, which strongly suggests that the symbiont is species specific (Webster and Hill 2001). On the other hand, one symbiont occurs

commonly in various invertebrates from different regions, so seems to possess a wide host range (Wilkinson *et al.*, 1981). For example, cyanobacteria *Aphanocapsa* sp., *Phormidium* sp., or *Oscillatoria spongeliae* are found in numerous sponges (Wilkinson, 1978c.), while bacteria from the genus *Vibrio* sp., *Pseudomonas* sp.,*Bacillus* sp. are associated with a wide range of host such as *Dendronephthya* sp., *Porites* sp., *Galaxea* sp., *Acropora* sp. (Harder *et al.*, 2003, Kalimutho *et al.*, 2007).

1.5.4 Advantages to the host and associated symbiont

The surfaces or internal spaces of the hosts are more nutrient-rich than seawater and sediments; therefore they offer nourishment and a safe habitat to their symbionts (Bultel-Poncé, 1999). On the other hand, symbiotic microorganisms help in the nutritional process, either by intracellular digestion or by translocation of metabolites including nitrogen fixation, nitrification and photosynthesis (Wilkinson and Fay 1979). Microorganisms also stabilize the host skeleton (Wilkinson, 1987) and participate in the host's chemical defense system against predators and biofouling by occupying entry niches and space or by the production of antimicrobial compounds (Unson, 1994; Kelman, 2006).

1.6 Bioactive compounds produced by invertebrates

1.6.1 Antimicrobials vis á vis humans pathogens

Large numbers of invertebrates, in particular marine corals, sponges and bivalves have been screened against various human test pathogens, including multidrug resistant strains. Efforts to screen sponges against harmful microbial pathogens are enlisted in Table 1.3 while the efforts to screen corals extracts for antimicrobial

compounds are enlisted in Table 1.4. The antibacterial activity of bivalve *P.viridis* remains restricted to the reports of Annamalai *et al.*, 2007, Zulpikar *et al.*, 2010) and a single report on antiviral activity by Debasis and Chatterji, 2004. The compounds identified were peptidic in narure.

Table 1.3. Summary of diverse antimicrobial activity associated with sponges

SPONGE	BIOACTIVE PROFILEOF COMPONDS EXTRACTED FROM SPONGES				REFERENCES
	ANTIMICROBIAL				
	ANTIBACTERIAL	ANTIPARASITIC	ANTIVIRAL	ANTIFUNGAL	
Discodermia sp.	-	-	-	Discobahamins	Zhang *et al.*, 2009
Theonella swinhoei	-	-	-	Swinholide Theopederins	Brigmann *et al.*, 2003, Laatsch *et al.*, 2006
Theonella mirabilis, T. swinhoei	-	-	Papuamides C and D (HIV-1)	-	Ford *et al.*, 1999
Discodermia kiiensis	Discodermins B, C, and D	-	-	-	Matsunaga *et al.*, 1985
Pachastrissa sp.	-	-	-	Bengamides Bengazoles	Hill *et al.*, 2006
Jaspis sp.	-	Bengazoles*	-	Cyclodepsipeptide Psammaplin	Haygood *et al.*,1999, Wicke *et al.*, 2000, Miki *et al.*, 1994*
Agelas nakamurai	Ageliferine Debromosceptrin Nakamuric acid	-	-	-	Varoglu *et al.*, 2000
Agelas dispar	Aminozooanemonin Pyridinebetaine A	-	-	-	Osinga *et al.*, 2001
Agelas sp.	Agelasine I Agelasine F	-	-	-	Radjasa *et al.*, 2007 Braekman *et al.*, 2004
Agelas mauritiana	Sceptrin	-	-	-	Fieseler *et al.*, 2004

Cortichum sp.	-	-	-	Meridine	Varoglu *et al.*, 2000
Hanacantha sp.	-	-	-	Hamacanthin	Hentschel *et al.*, 2001
Erylus lendenfeldi	-	-	-	Eryloside	Taylor *et al.*, 2007
Leucetta cf. chagosensis	-	-	-	Isonaamidine D	Zang *et al.*, 2005
Psammaplysilla purpurea	Bastadin	-	-	-	Wang *et al.*, 2006
Poecillastra wondoensis	Wondosterols	-	-	-	Kelecom, 2002
Acanthella sp.	Kalihinol-A	-	-	-	Gunasekera *et al.*, 2004
Ianthella basta	Bastadin	-	-	-	Jadulco *et al.*, 2004
Axinella sp.	Axinellamines B-D	-	-	-	Urban *et al.*, 1999
Hyrtios erecta	-	-	-	Spongistatin	Pettit *et al.*, 1998
Fascaplysinopsis reticulata	-	b-carbolium salt	-	-	Arillo *et al.*,1993
Hyrtios erecta		Heteronemin	-	-	Oclarit *et al.*, 1994
Fascaplysinopsis reticulata	-	b-carbolium salt	-	-	Arillo *et al.*,1993
Dysidea sp.	-	Furodysinin	-	-	Bugni *et al.*, 2004
Dysidea avara	-	-	Avarol, UAG suppressor glutamine tRNA inhibitor	-	Muller *et al.*, 1987, Muller *et al.*, 1991
Notodoris citrina-leucetta chagosensis	-	Naamidines and naamines	-	-	Taylor *et al.*, 2007
Verongia	Aeroplysinin	-	-	-	Teeyapany *et al.*, 1993

aerophoba	Aeroplysinin and dienone				
Arnosclera brasiliensis	Alkaloid toxins	-	-	-	Torres *et al.*, 2002
Callyspongia truncata	-	-	Callyspongynic acid	-	Nakao *et al.*, 2002
Arenosclera brasiliensis	Arenosclerins A, B, and C	-	-	-	Torres *et al.*, 2002
Petromica citrina	Halistanol trisulphate	-	-	-	Marinho *et al.*, 2012
Xetospongia sepra	-	-	-	Xestiquinone	Hendrik *et al.*, 2008
Xestospongia sp.	-	-	Haplosamates A and B, Antiviral (HIV-1 integrase inhibitor)	-	Qureshi and Faulkner, 1999
Petrosia weinbergi	-	-	Weinbersterols A and B, Antiviral (feline leukemia, mouse influenza, mouse corona)	-	Sun *et al.*, 1991
Halicortex sp.	-	-	Dragmacidin F	-	Cutignano *et al.*, 2000
	-	-	Mololipids (HIV-1)	-	Ross *et al.*, 2000
Hamigera tarangaensis	-	-	Hamigeran B Antiviral (herpes and polio)	-	Wellington *et al.*, 2000
Kirkpatrickia varialosa	-	-	Variolin B, Antiviral	-	Perry *et al.*, 1994

Many sponges	-	-	2-5A, Interferon mediator	-	Kelve *et al.*, 2003
Polyfibrospongia sp.	-	-	Hennoxazole A, Antiviral	-	Ichiba *et al.*, 1991
Acanthella klethra	-	Axisonitrile-3,antimalarial	-		Angerhofer *et al.*,1992
Acanthella sp.	-	Kalihinol A	-	-	Miyaoka *et al.*, 1998
Haliclona sp., Cymbastela hooperi, Diacarnus levii	-	Manzamine A	-	-	Ang *et al.*, 2001 Konig *et al.*, 1996
Topsentia sp.	Topsentiasterol sulfates A–E	-	-	Topsentiasterol sulfates D and E	Fusetani *et al.*, 1994
Acanthodendrilla sp.	-	-	-	Acanthosterol I and J	Tsukamoto *et al.*, 1998
Oceanapia phillipensis	-	-	-	Oceanapiside	Nicolas *et al.*, 1999
Leucascandra caveolata	-	-	-	Leucascandrolide A	D'Ambrosio *et al.*, 1996

Table 1.4: Summary of the diverse bioactivity reported from different corals

CORAL	BIOACTIVE PROFILE			REFERENCES
	CYTOTOXIC	ANTIINFLAMMATO-RY	ANTIMICROBIAL	
Sarcophyton crassocaule	Three new cembranoids, sarcocrassocolides M–O (**1–3**)	Three new cembranoids, sarcocrassocolides M–O (**1–3**)	-	Lin *et al.*, 2011
Sarcophyton crassocaule	Five new cembranoids, sarcocrassocolides A–E (1–5), along with three known cembranoids 6–8	Five new cembranoids, sarcocrassocolides A–E (1–5), along with three known cembranoids 6–8	-	Lin *et al.*, 2011
Sarcophyton stolidotum	14 membered carbocyclic cembranes,sarcostolides (A-G) Sarcostolide E	-	-	Cheng *et al.*, 2008
Sarcophyton, Lobophytum	cembrane diterpenoid, (+)-sarcophytol-A (1), along with a new lobane diterpenoid, carbomethoxyfuscol (2), from *Sarcophyton* sp., and a new cembranoid, crassumolide E (3), f	cembranoid 3	-	Bonnard *et al.*, 2010
Menella sp., *Lobophytum crassum*	-	A new germacrane-type sesquiterpenoid, menelloide E (**1**), and a new cembrane-type diterpenoid, lobocrassin F (**2**)	-	Lee *et al.*, 2012
Sarcophyton crassocaule	-	Five new cembranoids, sarcocrassocolides A–E (1–5), along with three known cembranoids 6–8	-	Lin *et al.*, 2011

Paraminabea acronocephala	Six new withanolides, paraminabeolides A_F (1_6), along with five known compounds, minabeolides-1, -2, -4, -5, and -8 (7_11)	Six new withanolides, paraminabeolides A_F (1_6), along with five known compounds, minabeolides-1, -2, -4, -5, and -8 (7_11)	-	Chao *et al.*, 2011
Cladiella krempfi.	four new eunicellin-based diterpenoids, krempfielins A–D (**1–4**), along with two known compounds (**5** and **6**)	four new eunicellin-based diterpenoids, krempfielins A–D (**1–4**), along with two known compounds (**5** and **6**)	-	Tai *et al.*, 2011
Cladiella krempfi	Four new eunicellin-based diterpenoids, krempfielins A–D (**1–4**), along with two known compounds (**5** and **6**)	Four new eunicellin-based diterpenoids, krempfielins A–D (**1–4**), along with two known compounds (**5** and **6**)	-	Su, 2011
Klyxum molle	-	Eight new eunicellin-based diterpenoids, klymollins A-H (1-8),	-	Hsu, 2011
Lobophytum crassum	Three new cembranoids, culobophylins A–C (**1–3**), along with two known compounds (**4** and **5**)/ two new α-tocopherols, designated as crassumtocopherols A (1) and B (2)	Three new cembranoids, culobophylins A–C (**1–3**), along with two known compounds (**4** and **5**)	-	Lee, 2012, Su, 2011, Chen *et al.*, 2011
Lobophytum durum	-	seven new cembranolides, durumolides F–L (1–7), as well as one previously characterized cembranolides, sinularolide D (8). Anti-inflammatory	-	Cheng *et al.*, 2011
Lobophytum laevigatum	four new cembranoids, namely laevigatol A–D (1–4), and six known metabolites (5–10), (+)-Sarcophine (5), 7b,8b-Epoxy-4a-	Four new cembranoids, namely laevigatol A–D (1–4), and six known metabolites (5–10)	-	Quang *et al.*, 2011

	hydroxycembra-1(15), 2,11-trien-16,2-olide (6),Emblide (7),. Ximaolide F (8),Methyl tortuoate B (9), Nyalolide (10)			
Lobophytum laevigatum	-	four new cembranoids, namely laevigatol A–D (1–4), and six known metabolites (5–10), (+)-Sarcophine (5), 7b,8b-Epoxy-4a-hydroxycembra-1(15), 2,11-trien-16,2-olide (6),Emblide (7),. Ximaolide F (8),Methyl tortuoate B (9), Nyalolide (10) from the Vietnamese soft coral *Lobophytum laevigatum*	-	Quang *et al.*, 2011
Lobophytum crassum	four new cembranoids, crassumols A–C (**1–3**) and 13-acetoxysarcophytoxide (**4**)	-	-	Lin *et al.*, 2011
Lobophytum michaelae	Six new cembranolides, michaolides L–Q (1–6), and a known cembranolide, lobomichaolide (7)	-	-	Wang *et al.*, 2012
Sinularia crassa	crassarosterol A (**1**), and four new steroidal glycosides, crassarosterosides A–D (**2–5**)	one new sterol, crassarosterol A (**1**), and four new steroidal glycosides, crassarosterosides A–D (**2–5**)	-	Chao *et al.*, 2012
	new cembranoids, crassarines A–H (1–8)	-	-	Chao *et al.*, 2011
	Two new cembrane diterpenes, sicrassarines A and B (compounds 1 and 2)	-	-	Lin *et al.*, 2012

Sinularia gibberosa	three new polyoxygenated sterols, gibberoketosterols B (1) and C (2) and gibberoepoxysterol (3), along with two known steroids, gibberoketosterol (4) and 24-methylenecholest-5-en-3â-ol (5)	three new polyoxygenated sterols, gibberoketosterols B (1) and C (2) and gibberoepoxysterol (3), along with two known steroids, gibberoketosterol (4) and 24-methylenecholest-5-en-3â-ol (5)	-	Ahmed *et al.*, 2006
Sinularia capillosa	Capilloquinol (**1**)	one new tetraprenylbenzoquinone, capilloquinone (1), two new furano benzo sesquiterpenoids, capillobenzopyranol (2) and capillobenzofuranol (3), one new furano sesquiterpenoid, capillofuranocarboxylate (4), and five previously characterized metabolites, comprising (*E*)-5-(2,6-dimethylocta-5,7-dienyl)furan-3-carboxylic acid (5), 2-[(2*E*,6*E*)-3,7-dimethyl-8-(4-methylfuran-2-yl)octa-2,6-dienyl]-5-methylcyclohexa-2,5-diene-1,4-dione (6), 2-[(2*E*,6*E*)-3,7-dimethyl-8-(4-methylfuran-2-yl)octa-2,6-dienyl]-5-methylbenzene-1,4-diol (7), (-)-loliolide (8), and 3,4,11-trimethyl-7-methylenebicyclo[6.3.0]undec-2-en-11R-ol (9)	-	Cheng *et al.*, 2009
Sinularia crassa	-	Eight new cembranoids, crassarines A–H (1–8)	-	Chao *et al.*, 2011
Sinularia sp.	ethyl 5-[(20S,50E)-20,60-dimethylocta-50,70-dienyl]furan-3-carboxylate (1)4.Two new furanobenzosesquiterpenoids,capillobenzopyranol (2)	Four new polyhydroxylated steroids (1–4)	-	Su, 2011

	andcapillobenzofuranol(3), onenewfuranosesquiterpenoid, capillofuranocarboxylate(4),			
Sinularia granosa	new cembranoids, querciformolide B (2) and granosolides A (5) and B (6) Two new cembranoids, namely granosolides C (1) and D (2), along with one known cembranoid 4	Two new cembranoids, namely granosolides C (1) and D (2), along with one known cembranoid 4,	-	Lu *et al.*, 2008
Sinularia querciformis	-	one new cembranoid, querciformolide E (3), along with four known -cembranoids 4—7.	-	Lu *et al.*, 2008
Sinularia discrepans	A new cembranoid, discrepanolide A (**1**), along with four known cembranoids **2–5**	A new cembranoid, discrepanolide A (**1**), along with four known cembranoids **2–5**	-	Lu *et al.*, 2008
Sinularia crassa.	A new cubitane diterpenoid, crassalone A (1)/ Eight new cembranoids, crassarines A–H (**1–8**)	crassarosterol A (**1**), and four new steroidal glycosides, crassarosterosides A–D (**2–5**)	-	Cheng *et al.*, 2012
Sinularia sp.	-	resultedintheisolationofethyl 5-[(20S,50E)-20,60-dimethylocta-50,70-dienyl]furan-3-carboxylate (1)4.Two new furanobenzosesquiterpenoids,capillobenzopyranol (2) andcapillobenzofuranol(3), onenewfuranosesquiterpenoid, capillofuranocarboxylate(4),	-	Su, 2011
Sinularia gyrosa	-	Three new diterpenoids, designated as gyrosanols A-C (1-3).	-	Cheng ,2008

Sinularia triangular	five cembrane-based diterpenoids 1–5, including two new metabolites, triangulenes A (1) and B (2).	five cembrane-based diterpenoids 1–5, including two new metabolites, triangulenes A (1) and B (2).	-	Sui and Wen 2011
Sinularia querciformis.	four new cembranoids, querciformolides A-D (1-4), along with two known cembranoids, 7 and 8,	four new cembranoids, querciformolides A-D (1-4), along with two known cembranoids, 7 and 8,	-	Lu *et al.*, 2008
Sinularia crassa and *Lobophytum*	-	new sphingosine derivative (1) and cembrenoid diterpene (lobohedleolide) (2)	-	Radhika *et al.*, 2005
Sinularia capillosa and *Nephthea chabroli*	Capillosanol and Chabranol	-	-	Cheng *et al.*, 2009
Clavularia viridis	-	Four new steroids, stoloniferones R–T (1–3), and (25*S*)-24-methylenecholestane-3_,5_,6_-triol-26-acetate (4)	-	Chang, 2008
Clavularia viridis	marine prostanoid clavulones	Four new steroids, stoloniferones R–T (1–3), and (25*S*)-24-methylenecholestane-3_,5_,6_-triol-26-acetate (4)	-	Huang *et al.*, 2005
Pseudopterogorgia elisabethae	-	-	Pseudopterosins and *seco*-Pseudopterosins	Cheng *et al.*, 2008
Erythropodium caribaeorum	Eleutherobin	-	-	Fenical and Pawlik 1991
Galaxea fascicularis	novel antitumour compound	-	-	Faith and Ding 1998
Nephthea chabroli	-	Eight new 4-methylated steroids,	-	Huang, 2005

		nebrosteroids A–H (1–8)		
Nephthea chabrolii	19-oxygenated steroids, Nebrosteroids N–P (1–3)	-	-	Wang *et al.*, 2012
Paralemnalia thyrsoides	Paralemnolide A (1)	-	-	Wang *et al.*, 2012
Octocoral *Pseudopterogorgia elisabethae*	Pseudopterosins and *seco*-Pseudopterosins	-	-	Cheng *et al.*, 2008
Gorgorian octocoral	The compounds were 1) dinosterol, 2) plakortide O, 3) pseudoplexauric acid methyl ester, 4) 3-epi-14-deoxycrassin, 5) kallolide A, 6) kallolide A acetate, 7) asperdiol 8) asperdiol acetate 9) 2-deoxyasperdiol acetate 10) isoasperdiol, 11) asperdicin, 12) Eupalmerin acetate (EPA) 13) isoeupalmerin acetate and 3'-o-acetyl-pseudopterosin-N-methylpyridinium-3-sulphonate	-	-	Iwamaru *et al.*, 2007

The antimicrobial activity of coral extracts remains restricted to the work done on some gorgorian corals, octocorals, scleractinian corals and alcyonacean softcorals. However it may be noted that there are sparse reports (indicated in Table 1.4) which have worked on the aspect of identification of the bioactive compound. However among the ones that have been identified they belong to the class of sphingolipids, steroids, pseudopterosins and terpenes. Our literature survey clearly showed that the work done on sponges is much more than that reported from corals. The sponge *Agelas* sp. has been widely studied for the presence of antimicrobial compounds. The compounds reported from this sponge were were Ageliferine, Nakamuric acid, Aminozooanemonin, Pyridinebetaine A, Agelasine I and F, Debromosceptrin, Sceptrin etc.

1.6.2 Cytotoxicity

Since, cancer is recognized as one of the most dreaded diseases, a major cause to many deaths, there is felt need for developing drugs to combat the same. The most frequent cancers in man are of the lung, lip, oral cavity and in women these include those of the cervix uterus, breast and ovary. Anticancer drug research in India is based on lead molecules from invertebrates like sponges and corals and its associated micro-organisms. The cytotoxic compounds and its source coral are highlighted in Table 1.4, while the cytotoxic effects of sponges and the identified compound showing cytotoxic activity is tabulated in Table 1.5. It was interesting to note that there were no reports on the cytotoxic effects of the bivalve *P.viridis.* The research efforts on the cytotoxic effects of coral tissue extracts has been extensive with several researchers having successfully isolated the compounds showing cytoyoxic activity against several cell lines and also identified the same. It was interesting to note that the cembranoid group of compounds was found in many of the corals studied for its

cytotoxic activity namely *Sinularia querciformis, Lobophytum laevigatum, Sinularia crassa, Sarcophyton crassocaule, Sinularia discrepans, Lobophytum crassum* and the alike. Sponges have been proven to be a prolific resource of cytotoxic compounds and are known to harbor a rich chemical diversity ranging from dieones, agelasines, avarol, jaspisamides etc. from sponges such as *Agelas* sp., *Jaspis* sp., *Verogia* sp. etc.

Table 1.5: Bioactivity associated with sponge extracts

SPONGE	BIOACTIVE PROFILE				REFERENCES
	CYTOTOXIC	ANTI-INFLAMMATORY	HEMOLYTIC	OTHERS	
Amphimedon sp.	Pyrinodemin	-	-	-	Suzumura *et al.*, 2003
Amphimedon sp.	Pyrinodemins B—D (1—3)	-	-	-	Hirano *et al.*, 2000
Amphimedon sp. (SS-1059)	Nakinadines B (1) and C(2)	-	-	-	Nishi *et al.*, 2008
Aplysina aerophoba	Aeroplysinin I	-	-	-	Amagata *et al.*, 2003
Aplysina caissara	Unidentified	-	-	-	Azevedo *et al.*, 2008
Verongia aerophoba	Dienone	-	-	-	Nagai *et al.*, 2002
Verongia spengelii	Aplysinopsin	-	-	-	Selvin *et al.*, 2009
Verongia aerophoba	Aeroplysinin-1 and dienone	-	-	-	Teeyapany *et al.*, 1993
Suberea sp.	-	Subersic acid, Lipoxygenase inhibitor	-	-	Carroll *et al.*, 2001
Chondropsis sp.	-	-	Chondropsin A and B, v-ATPase inhibitor	-	Cantrell *et al.*, 2000, Bowman *et al.*, 2003
Batzella sp.	Discorhabdin	-	-	-	Li, 2009
Batzella sp.	Secobatzelline	-	-	Secobatzelline/ Phosphatase inhibitor	Zheng *et al.*, 2000

Crella sp.	Crellastatins	-	-	-	Laport *et al.*, 2009
Disidea avara	Avarol	-	-	-	Abrell *et al.*,1996
Dysidea sp	-	-	-	S1319/, antiasthmaticuterine relaxation	Suzuki *et al.*, 1999
Dysidea avara	Avarol	Avarol	-		Muller *et al.*, 1987, Muller *et al.*, 1991
Dysidea herbacea	-	-	-	Dysiherbaine/Glutamate receptor antagonist	Sakai *et al.*, 1997
Dysidea sp	-	-	-	Polyoxygenated Sterols/, IL-8 inhibitor	De Leone *et al.*, 2000
Dysidea incrustans	-	-	Incrustasterols A and B	-	Casapullo *et al.*, 1995
Dysidea arenaria	-	-	Arenastatin A, Tubulin polymerisation inhibitor	-	Koiso *et al.*, 1996
Spongionella gracilis	Gracilin B	-	-	-	Govek and Overman 2001
Spongionella sp.	-	-	-	Okinonellin B, muscle relaxant	Kato *et al.*, 1986
Dysidea sp.	-	Dysidotronic acid, Phospholipase A2 inhibitor	-	-	Giannini *et al.*, 2000
Druinella purpurea	Psammaplysin C	-	-	-	Ridley *et al.*, 2005

Haliclona tulearensis	Halitulin	-	-	-	Beasada *et al.*, 2005
Haliclona sp.	Manzamine A	-	-	-	Blunt *et al.*, 2009
Haliclona sp	-		Salicylihalamide A, v-ATPase inhibitor	-	Erickson *et al.*, 1997
Haliclona sp.	-	-	Unidentified;specific inhibitors of cell growth	-	Blackburn *et al.*, 1999.
	-	-	halichondrin B; potent microtubule-interfering compounds	-	Bai *et al.*,1991
	-	-	Spongistatin; potent microtubule-interfering compounds	-	Bai *et al.*, 1993
	-	-	Discodermolide; potent microtubule-interfering compounds	-	Ter Haar *et al.*, 1996
	-	-	Peloruside A; potent microtubule-interfering compounds	-	Hood *et al.*, 2002
	-	-	dictyostatin potent microtubule-interfering compounds	-	Isbrucker *et al.*, 2003

Haliclona osiris	Osirisynes	-	-	Osirisynes Na+/K+-ATPase inhibitor	Amagata *et al.*, 1999.
Haliclona nigra	-	-	Haligramides A and B	-	Rashid *et al.*, 2000
Haliclona sp.	-	-	Adociasulfates,Kinesin motor protein inhibitors	-	Blackburn *et al.*, 1999
Jaspis johnstoni	Jasplakinolide	-	-	-	Jensen and Fenical 1994
Jaspis sp.	Bengamides	-	-	-	Nagai *et al.*, 2003
Jaspis sp.	Jaspisamides	-	-	-	Bringmann *et al.*, 2003
Jaspis splendens	-	Jaspaquinol, Lipoxygenase inhibitor	-	-	Carroll *et al.*, 2001
Neosiphonia superstes	Sphinxolides	-	-	-	Proksch *et al.*, 2002
Reidispongia coerulea	Reidispongiolide	-	-	-	Proksch *et al.*, 2002
Pandaros acanthifolium	Acanthifolicin	-	-	-	Althoff *et al*, 1998
Echinoclathria sp.	-	-	-	Echinoclathrines ; Immunosuppressive	Muller *et al.*, 2004

Petrosia contignata	-	-	-	Contignasterol; Histamine release inhibitor	Takei *et al.*, 1994, Bramley *et al.*, 1995
Petrosia sp.	Petrotetrayndiols	-	-	-	Scheuermayer *et al.*, 2006
Petrosia sp.	C46 (1-3) and three C30 (4-6) polyacetylenic alcohols	-	-	-	Kim, 1999a
Xestospongia sp.	-	-	-	Xestospongin C, IP3-inhibitor	De Smet *et al.*,1999
Xestospongia berquistia	-	-	-	Xestobergsterols A and B; Histamine - release inhibitor	Shoji *et al.*, 1992
Stronglyophora hartmani-	Puupehenone	-	-	-	Fredenhagen *et al.*, 1987
Psammaplysilla purpurea	Purealidin A	-	-	-	Selvin, 2004
Psammaplysilla	Psammaplin A	-	-	-	Jiang *et al.*, 2004
Tedania ignis	Tedanolide	-	-	-	Perovic *et al.*, 1998
Tedania ignis	-	-	Tedanolide	-	Schmitz *et al.*, 1984
Tedania digitata	-	-	-	1-Methylisoguanos	Quinn *et al.*, 1980

				ine, muscle relaxant, antiallergic	
Theonella sp.	Koshikamide	-	-	-	Newman and Hill 2006
Theonella sp.	-	-	-	Cyclotheonamide A, Serine protease inhibitor	Maryanoff *et al.*, 1993
Theonella swinhoei	-	-	Swinholide A, Actin-depolymerization	-	Bubb *et al.*, 1995
Discodermia dissoluta	-	-	Discodermolide Stabilization of microtubules	-	Ter Haar *et al.*, 1996
Mycdle hentschett	-	-	Peloruside A, Stabilization of microtubules	-	Hood *et al.*, 2002
Zyzzya fuliginosa	Veiutamine	-	-	-	Konig *et al.*, 2005
Agelas mauritianus	-	-	Agelasphin(KRN7000), NKT cell activator	-	Shimosaka, 2002
Agelas novaecaledoniae	Ageliferine/ Sceptrin	-	-	Xetospongine B Somatostatin/VIP inhibitor	Holler *et al.*,1999
Agelas oroides	-	-	-	Taurodispacamide A, IL-2 inhibitor	Fattorusso and Taglialatela-Scafati, 2000
Agelas sp.	-	-	-	Keramadine, Serotonergic receptor	Nakamura *et al.*, 1984

				antagonist	
Erylus lendenfeldi	Eryloside A	-	-	-	Taylor *et al.*, 2007
Halichondria okadai	Halichondrin B	-	-	-	Blunt *et al.*, 2009
Halichondria okadai	-	-	Halichondrin B, Tubulin polymerisation inhibitor	-	Hirata and Uemura, 1986; Bai *et al.*, 1991
Hadromerida	-	Also has antiinflammatory activity	Protein kinase C inhibitory	-	Fedoreev *et al.*, 1989
Aaptos aaptos	3-(phenethylamino)demethyl(oxy)aaptamine and 3-(isopentylamino)demethyl(oxy) aaptamine	-	-	-	Sharii *et al.*, 2009
Aaptos sp.	aaptamine alkaloids	-	-	-	Bihud *et al.*, 2005
Marine sponges	Sesquiterpenequinones and polybrominated diphenyl esters	-	-	-	Popov *et al.*, 1999
Ianthella quadrangulata.	bastadin	-	-	-	Greve *et al.*, 2008
Ianthella sp	34-sulfatobastadin 13	-	-	Endothelin A receptor inhibitor	Unson *et al.*, 1994
Petromica citrina	halistanol trisulphate	-	-	-	Marinho *et al.*, 2012

Mycale sp.	-	-	-	Pateamine A, IL-2 inhibitor	Northcote *et al.*, 1991
Mycale sp.	1,5-Diazacyclohenicosane	-	-	-	Coello *et al.*, 2009
Mycale sp.	-	-	Mycalolide B, Actin-depolymerization	-	Fusetani *et al.*, 1989 Saito *et al.*, 1994
Marine sponge	latrunculin A, batzelline A, chondrillin, aureol, epihippuristanol, theonellamine B, discorhabdins A and C, kabiramide C, dercitin, meridine, manzamines A, B, and C, 8,15-diisocyano-11(20)-amphilectene and the corresponding C-15 formamide, a 20-carbon acetylenic alcohol, 4,5-dihydro-6"-deoxybromotopsentin, epispongiadiol, isospongiadiol, puupehenone, reiswigin	-	-	-	Longley *et al.*, 1993

	A, and-demethyl- and demethyloxyaaptamine. Latrunculin A, batzelline A, chondrillin, and aureol				
Callyspongia truncata	-	-	Callystatin A	-	Kobayashi *et al.*, 1997
Arenosclera brasiliensis.	alkaloid toxins	-	-	-	Torres *et al.*, 2002
Callyspongia truncata	-	-	-	Callyspongynic Acid, a-glucosidase inhibitor	Nakao *et al.*, 2002
Aplysinopsis digitata	aplysinoplides A-C (1-3)	-	-	-	Ueoka *et al.*, 2008
Fasciospongia cavernosa	-	Cacospongionolide B, Phospholipase A2 inhibitor	-		Garcia Pastor *et al.*, 1999
Aplysinopsis digitata	sesterterpenoids, aplysinoplides A-C (1-3)	-	-	-	Ueoka *et al.*, 2008
Hyrtios sp.	P-carboline, puupehenone, 12-epi-heteronemin heteronemin	-	-	-	Bourguet-Kondracki *et al.*,1996
Cacospongia mycofljiensis	-	-	Laulimalide, Stabilization of	-	Mooberry *et al.*, 1999

			microtubules		
	-	Manoalide, Phospholipase A2 inhibitor	-	-	Bennet *et al.*, 1987
Petrosaspongia nigra	-	M-R Cheilantane, Phospholipase A2 inhibitor	-	-	Randazzo *et al.*, 1998a
Rhabdastrella globostellata	isomalabaricane-type sesterterpenes	-	-	-	Bourguet-Kondracki *et al.*, 2000
Stelletta sp.	alkaloids	-	-	-	Kondracki *et al.*,2000
Penares sp.	-	-	-	Penaresidin A, Actomyosin ATPase inhibitor	Kobayashi *et al.*, 1991
Stylinos sp.	Mycalamides	-	-	-	Amagata *et al.*, 2006
Auletta sp.	-	-	Hemiasterlin, Stabilization of microtubules	-	Anderson *et al.*, 1997
Aplysilla glacialis	-	-	Glaciasterols A and B	-	Pika *et al.*, 1992
Axinella carter	-	-	Axinellins A and B	Also has immunosuppressive property	Randazzo *et al.*, 1998b
Ircinia sp.	Haterumalides	-	-	-	Amagata *et al.*,1999.
Sarcotragus sp.	-	-	Unidentified arthritis or for combating tumor growth	-	Wakimoto *et al.*, 1999
	.-	-	A1, 3-	-	Wakimoto *et al.*, 1999

			fucosyltransferase inhibitor		
Ircinia oros	-	Ircinin-1 and -2, Phospholipase A2 inhibitor	-	-	Ciminoe *et al.*, 1972
Latrunculia magnifica	-	-	latrunculin A disrupt the polymerization of actin	-	Coue *et al.*, 1987
Leucetta cf. chagosensis	imidazole alkaloid Na amine	-	-	prevent events in the early phases of tumorigenesis	Dunbar *et al.*, 2000
Hippospongia metachromia	Metachromin C	-	-	-	Kobayashi *et al.*, 1989
Spongia sp.	-	-	Agosterol A, Reverses drug resistancy of dancer cells	-	Aoki *et al.*, 1998
Spongia sp.	-	Spongidines A-D, Phospholipase A2 inhibitor	-	-	De Marino *et al.*, 2000
	-	-	Spongistatin 1, Tubulin polymerisation inhibitor	-	Bai *et al.*, 1993
Topsentia genitrix	-	Topsentin, Phospholipase A2 inhibitor	-	-	Jacobs *et al.*, 1994
Hymeniacidonaldis	-	-	Debromohymenialdisine, Protein kinase C inhibitor	-	Kitagawa *et al.*, 1983

Corallistidae sp.	-	-	Dictyostatin, Stabilization of microtubules	-	Isbrucker *et al.*, 2003
Latrunculia magnified	-	-	Latrunculin A, Actin-depolymerisation	-	Kashman *et al.*, 1980
Plakinastrella sp.	-	-	Elenic acid, Topoisomerase II inhibitor	-	Juagdan *et al.*, 1995
Hemiastrella minor, Xestospongia cf carbonaria	-	-	Jaspamide Neoamphimedine, Topoisomerase II inhibitor	-	De Guzman *et al.*, 1999
Leucetta cf. chagosensis	-	-	Naamine D, Nitric oxide synthetase inhibitor	Immunosuppressive activity	Dunbar *et al.*, 2000
Cinachyrella sp.	-	-	6-Hydroximino-4-en-3-one Steroids, Aromatase inhibitor	-	Holland *et al.*, 1992
Crambe crambe	-	-	Crambescidins 1-4, Ca2+/channel blocker	-	Jares-Erijman *et al.*, 1991, Berlinck *et al.*, 1993
Latrunculia brevis, Prianos sp.	-	-	Discorhabdin D	-	Perry *et al.*, 1988
Plakortis simplex	-	-	-	Simplexides, Inhibitor of T-cell	Costantino *et al.*, 1999

				proliferation	
Eryltus formosus	-	-	-	Eryloside F, Thrombin receptor antagonist	Stead *et al.*, 2000
Halichondria okadai	-	-	-	Halichlorine, VCAM-1 inhibitor	Arimoto *et al.*, 1998
Spongosorites sp.	-	-	-	Bromotopsentin, a1-Adrenergic receptor antagonist	Phife *et al.*, 1996

1.6.3 Antiinflammatory effect:

The research efforts all around the globe on the antiinfalmmatory effects of invertebrates namely corals, sponges and bivalves are restricted to the corals and it is tabulated in Table 1.4. It was also of interest that the actual producer of the bioactive compound having antiinflammtory properties is the host coral itself and not the associate bacterial symbiont. There was a single report on the antinflammatory properties of the host sponge and no reports from bivalve. The single report from sponge is by *Axinella sp* attributable to the bromo compounds, cyclopeptides, polyethers, sterols, and terpenes present in the extracts (Yalcin, 2007).

1.6.4 Other Bioactive Compounds

The other bioactivities studied and reported from sponges; include hemolytic activities, phosphatase inhibitors, histamine release inhibitor etc. (Table 1.5). It was interesting to note the antioxidant activity was restricted to the bivalve. The reports on *P.viridis* include Jenna *et al.*, 2010; Amutha *et al.*, 2008; Chen *et al.*, 2011). However the key antioxidant factor remined unidentified.

1.7 Bioactive compounds produced by invertebrate associated microorganisms

Marine microorganisms are good candidates for new pharmaceuticals and bioactive natural products (Kobayashi and Ishibashi, 1993). There is accumulating evidence that demonstrates the involvement of symbiotic microorganisms in the natural products originally attributed to the host (Kobayashi and Ishibashi, 1993, Shigemori *et al.*, 1992). For example, symbiotic bacterium *Micrococcus* sp.

produces diketopiperazines previously ascribed to the host sponge *Tedania ignis* (Stierle *et al.*, 1988).

1.7.1 Antimicrobial compounds from sponge associated bacteria

Among the sponges studied for the antimicrobial compounds produced by the associate bacterial bionts, the sponge *Suberites domuncula, Haliclona* sp. and *Aplysina aerophoba* need a special mention on account of the extensive work reported from these sponges. The genus *Bacillus* sp., *Pseudomonas* sp. and *Vibrio* sp. serve as microbial mines from which compounds of potential antimicrobial activity can be extracted and marketed. The antimicrobial activity of sponge associated bacteria is involving the identification of the antimicrobial compound remains restricted to the few reports tabulated in Table 1.6

Table 1.6: Antimicrobial activity of sponge associated bacteria

SPONGE	BACTERIA	COMPOUND	ACTIVITY	REFERENCES
Dysidea avara	Unidentified bacterium	2-methylthio-1,4-naphthoquinone	Antimicrobial	Thakur and Muller 2005; Muller *et al.*, 2004
Hyatella sp.	*Vibrio* sp. M22-1 (γ- Proteobacteria)	Andrimid	Antibiotic	Oclarit *et al.*, 1994
Hyrtios altum	*Vibrio* sp. (γ-Proteobacteria	Trisindoline	Antibiotic	Kobayashi *et al.*, 1994
Haliclona simulans	*Pseudo-alteromonas* sp. PA2 (γ-Proteobacteria) *Pseudo-alteromonas* sp. PA4 (γ-Proteobacteria) *Pseudo-alteromonas* sp. PA5 (γ-Proteobacteria) *Pseudo-alteromonas* sp. PA5 (γ-Proteobacteria) *Halomonas* sp. HM4 (γ-Proteobacteria) *Psychrobacter* sp. PB1 (γ-Proteobacteria), *Marinobacter* sp. MB1 (γ-Proteobacteria), *Pseudovibrio* sp. PV1 (α-Proteobacteria), *Pseudovibrio* sp. PV2 (α-Proteobacteria),*Pseudovibrio* sp. PV4 (α-Proteobacteria), *Bacillus* sp. BC1 (Firmicutes) Unidentified *Bacillus* sp. BC2 (Firmicutes) Unidentified	Unidentified	Antimicrobial	Wang *et al.*, 1998
Homophymia sp.	*Pseudomonas* sp. 1537-E7 (γ-	2-undecyl-4-quinolone	Antimalarial	Bultel-Ponce *et al.*, 1999

	Proteobacteria)		Anti HIV	
		2-nonyl-4-hydroxyquinoline *N*-oxide	Antibacterial,	Bultel-Ponce *et al.*, 1999
Sponge	[*Candidatus Entotheonella palauensis* (δ-Proteobacteria)	Theopalauamide	Antifungal	Schmidt *et al.*, 1998, Bewley and Faulkner 1998
Theonella swinhoei	*Entotheonella palauenis* (δ-Proteobacteria)	Theonegramide	Antifungal	Bewley *et al.*, 1996, Bewley and Faulkner 1998
Isodictya setifera	*Pseudomonas aeruginosa* (γ-Proteobacteria)	Cyclo-(L-proline-Lmethionine)	Antibacterial	Jayatilake *et al.*, 1996
Aplysina aerophoba	*Bacillus subtilis* A184 (Firmicutes)	Surfactin, iturin and fengycin	Antifungal, antibacterial	Pabel *et al.*, 2003
	Bacillus subtilis A190 (Firmicutes)	Surfactin	Antifungal	Pabel *et al.*, 2003
	Bacillus subtilis A202 (Firmicutes)	Iturin	Antifungal	Pabel *et al.*, 2003
Sponge	*Bacillus pumilus* A586 (Firmicutes)	Pumilacidin containing β-hydroxyl fatty- acid	Antibacterial	Pabel *et al.*, 2003
Halichondria sp.	*Bacillus* sp. SAB1	indole (**1**), 3-phenylpropionic acid (**2**) and a dimer 4,4¢-oxybis[3-phenylpropionic acid] (**3**)	Antimicrobial	Prabha *et al.*, 2010
Petrosia ficiformis	*Rhodococcus* sp. *and Pseudomonas* sp.	Unidentified	Antimicrobial	Chelossi *et al.*, 2004
Dysidea sp.	*Vibrio* sp. (γ-Proteobacteria)	Tetrabromo-diphenylethers	Antibacterial	Elyakov *et al.*, 1991

1.7.2 Sponge associated bionts with cytotoxic activity

The reports on the sponge associated bionts showing cytotoxic activity remains restricted to a few reports. A few of these compounds could be identified while the rest remained unidentified. The genera showing cytotoxic activity include *Vibrio* sp., *Bacillus* sp., *Alteromonas* sp., *Idiomarina* sp., and *Pseudoalteromonas* sp. Table 1.7 summarises the compounds isolated from sponge associated bacteria.

Table 1.7: Sponge associated bacteria showing cytotoxic effects

SPONGE	BACTERIA	BIOACTIVE COMPOUND	ACTIVITY	REFERENCE
Dysidea sp.	*Vibrio* sp. (γ- Proteobacteria)	Tetrabromo-diphenyl ethers	Cytotoxic	Elyakov *et al.*, 1991
Fascaplysinopsis reticulate	*Pseudoalteromonas maricaloris* KMM 636T (γ-Proteobacteria)	Bromo-alterochromide A	Cytotoxic	Speitling *et al.*, 2007
Theonella swinhoei	Uncultured bacterium	Onnamide A	Antitumor	Grozdanov *et al.*, 2007
Theonella swinhoei	Unidentified bacterium	Swinholide A	Cytotoxic	Bewley *et al.*, 1996; 1998
Homophymia sp.	*Pseudomonas* sp. 1537-E7 (γ-Proteobacteria)	[2-undecen-1' –yl-4-quinolone	Cytotoxic	Bultel and Ponce 1999
		2-nonyl-4-hydroxyquinoline *N*-oxide	Cytotoxic	Bultel and Ponce 1999
Acanthella acuta	*Bacillus pumilus* AAS3 (Firmicutes)	GG11	Antitumor	Ramm *et al.*, 2004
Halichondria okadai	*Alteromonas* sp. (γ-Proteobacteria)	Alteramide A	Anticancer, cytotoxic	Shigemori *et al.*, 1992

1.7.3 Bioactivity of coral associated bacteria

As it is well known that the activity which was once ascribed to the corals are now known to be produced by the resident micrroflora. The coral most widely studied for its antimicrobial activity is *Acropora* sp. (Harder *et al.*, 2003; Radjasa *et al.*, 2005). The associated bionts most widely implicated for the coral associated antimicrobial activity belong to the genus *Vibrio* sp. and *Pseudoalteromonas* sp. (Gowrishankar *et al.*, 2012; Harder *et al.*, 2003; Chellaram *et al.*, 2011). As yet there are no reports on the identification of antimicrobial compounds produced by the resident microflora of sponges.

The reports on coral associated bacteria showing cytotoxic activity are limited or scarce. Interestingly the single report of coral associated bacteria showing cytotoxic activity is by bacteria *Marinobacterium* strains, *Pseudoalteromonas* sp., *Vibrio* sp., *Enterovibrio* sp., *Tateyamaria* sp., *Labrenzia* sp.and *Pseudovibrio* sp. from corals *Briareum* sp., *Sinularia* sp., *Sarcophyton* sp., *Nephtheidae* sp., and *Lobophytum* sp. (Ritchie 2005).

The incredible chemical diversity associated with invertebrates has prompted marine biologists to harness the innumerable bioactive compounds, having pronounced and incredible pharmacological effects ranging from anticancer to antimicrobial, antioxidant, hemolytic anti-inflammatory, and the alike. Reports recorded in the foregoing literature treatise points out that the bacterial bionts of sponges, corals and bivalve are responsible for the activity of its associated host. Hence such organisms are worth examining for associated bacteria

AIMS AND OBJECTIVES

Globally, marine econiches particularly those within the tidal reaches are inhabited by barnacles, bryozoans, barnacles, ascidians, holothurians, bivalves sponges, corals etc. These marine invertebrates such as sponges, corals and bivalve are colonized by micro-organisms inhabiting the habitat waters. The colonizers termed as "BIONTS" (epibionts and endobionts) contribute to 60% of the body mass and indirectly help the host in its defense against predators and biofoulers. Additionally these microbial bionts evolve physical and chemical defense strategies to prevent the growth and attachment of co-occurring bacterial species for the same niche on the surface of the host by producing secondary metabolites that exhibit antibacterial, antifungal, antiprotozoal and antialgal activities. Alkaloids, polyketides, sterols, terpenes, and sesterterpenes (Mayer *et al.*, 2011, Thakur and Muller, 2004) having pharmacological and toxicological properties are reported from sponges, corals and bivalve. Studies indicate that the bioactive compounds obtained from the tissues of the marine organisms are actually produced by the resident microbial bionts (Kobayashi *et al.*, 1993). Not many studies are devoted to microbes and their bioactive potentials from sponges, corals and bivalves of a particular region in India, although the GoM in India is reported as a hotspot for invertebrate diversity (Ramadhas *et al.*, 1999). Hence the aim of the research work recorded in this thesis to be submitted to Goa University as thesis for evaluation and award of Ph.D. in Microbiology was to screen haloarchaea and halophillic micro-organisms isolated from marine organisms for bioactive compounds.

This study devoted to sponges, corals and bivalve of Mandapam in GoM-India records the research efforts carried out in the laboratory of Microbiology,Goa University to: Enumerate; Isolate growing cultures; Determine their phylogenetic

and taxonomic position of key isolates as halophillic eubacteria and haloarchaea; Determine the bacterial community of *Acropora formosa* from Lakshadweep-India using DGGE, Evaluate the potential for producing antibacterial, antioxidant, hemolytic compounds; Elucidate the chemical structure of key antibacterial compound.

The various experimental set up, the results obtained, inferences and conclusions drawn forming the content of the thesis is organized into five Chapters, followed by the summary, conclusions, bibliography, outcome of Research study/research publications and Appendix

CHAPTER 2

PHYLOGENETIC IDENTIFICATION OF BACTERIA FROM SPONGES, CORALS, AND BIVALVE FROM MANDAPAM - INDIA

Marine environments are complex and dynamic largely due to hydrography, changing physico-chemical conditions and biota. Globally, marine sponges, bivalves and corals are reported to be widely distributed in the intertidal regions of estuarine reaches. These regions undergo fluctuations in salinity, temperature and light that are reported to adversely affect the bacterial flora. Among marine invertebrates, sponges of phylum Porifera and bivalves during their benthic lifestyle and filter feeding habits encounter microorganisms inherent or transient in the waters they live. Microorganisms that get access into the interior of the invertebrate during the efflux and influx of water establish therein as endobionts or exobionts (Muller *et al.*, 1981). Bacteria are reported to contribute up to 40% of the biomass (equal to about 10^8 to 10^9 bacteria/g of tissue).

Mandapam in India with shallow waters is intertidal and is rich in marine resource (Nanditha *et al.,* 2007). In recent times, the region has been reported as a water body for disposal of sewage, industrial effluents and terrestrial runoff (Wild and Maier, 2010; Ramadas and Rajeshwari, 2001). Sponges, Bivalves and Corals inhabiting such waters due to their feeding habits are expected to sieve in bacteria singly or along with dissolved, particulate organic and inorganic matter, through their pores into its system of internal channels. The process thus offers an opportunity for the bacteria to establish as bionts in the host tissue. Bacteriological studies related to unveiling the possible role of retrieved bionts; state that they are expected to exhibit activities that are antagonistic/ protagonistic/ remediative. Studies on retrieval of bacteria from sponges and corals inhabiting the GoM-India have been carried out using culture media having 3.5% NaCl incapable of supporting growth of halophiles requiring higher concentrations of NaCl (Prem Anand *et al.*, 2006; Vasanthabharathi and Jayalakshmi, 2012; Saravankumar *et al.*,

2011) although moderately halophilic/halotolerant heterotrophic bacteria,requiring 15-20% (w/v) NaCl, have been reported from various non hypersaline marine habitats (Mimura and Nagata, 2000; Raghavan and Furtado 2004). Standard isolation and cultivation methods enable only some of the identification of the associated bacterial bionts (Burja *et al.*, 1999, Lopez *et al.*, 1999). Molecular techniques employing PCR amplification followed by DGGE analysis have provided additional information on associated microbial flora.

In the light of the above, in the current study, which forms the content of this chapter we selected nine different sponges, namely *Petrosia testudinaria, Cinachyra cavernosa, Haliclona sp., Callyspongia fibrosa, Heteronema erecta, Fasciospongia cavernosa, Callyspongia reticutis var solomonensis*, two unidentified sponges, four different corals namely *Telesto sp., Echinogorgia complexa, Echinogorgia reticulata, Echinomuricea indica* and one commonly occurring bivalve *Perna viridis* sampled from the shallow waters of Mandapam. We have documented the predominant bacterial community structure and diversity, retrieved bacterial bionts in culture using strategies of manipulation of NaCl concentration in the nutrient rich growth media, characterized them upto generic level using keys of Bergey's Systematic Biology and SYSTAT v.12.01 and evaluated their predominance and commonality with respect to the hosts through PRIMER v.5.

2.1 Methodology

2.1.1 Sampling Site and Collection of Sponges, Corals and Bivalve samples

Sponges and Corals were collected from Mandapam in Tamil Nadu (Lat. 09°19' 37.3" N and Long. 79° 10' 20.5" E), by scuba diving at depths of 8-10 m, 2 nautical miles off the South Eastern Coast in the Gulf of Mannar (GoM) (Figure 2.1),and deposited under the accession code MAM 1, MAM 2, MAM 4, MAM 5, MAM 6, MAM 10, NIO 1, NIO 2; NIO 3, MAM 3, MAM 7, MAM 8, MAM 9 respectively (Figure 2.2 a and b) in the Biological Chemistry laboratory at the National Institute of Oceanography, Dona Paula, Goa-India. A single bivalve was also collected locally in the sub-tidal regions, of Kurusadai island. The sample (Figure 2.2 c) was deposited in the Haloarchaea laboratory of the Department of Microbiology, Goa University, Goa-India. Working sample of the sponge/coral/bivalve for microbiological analysis were processed and analyzed at the Haloarchaea laboratory of the Department of Microbiology, Goa University.

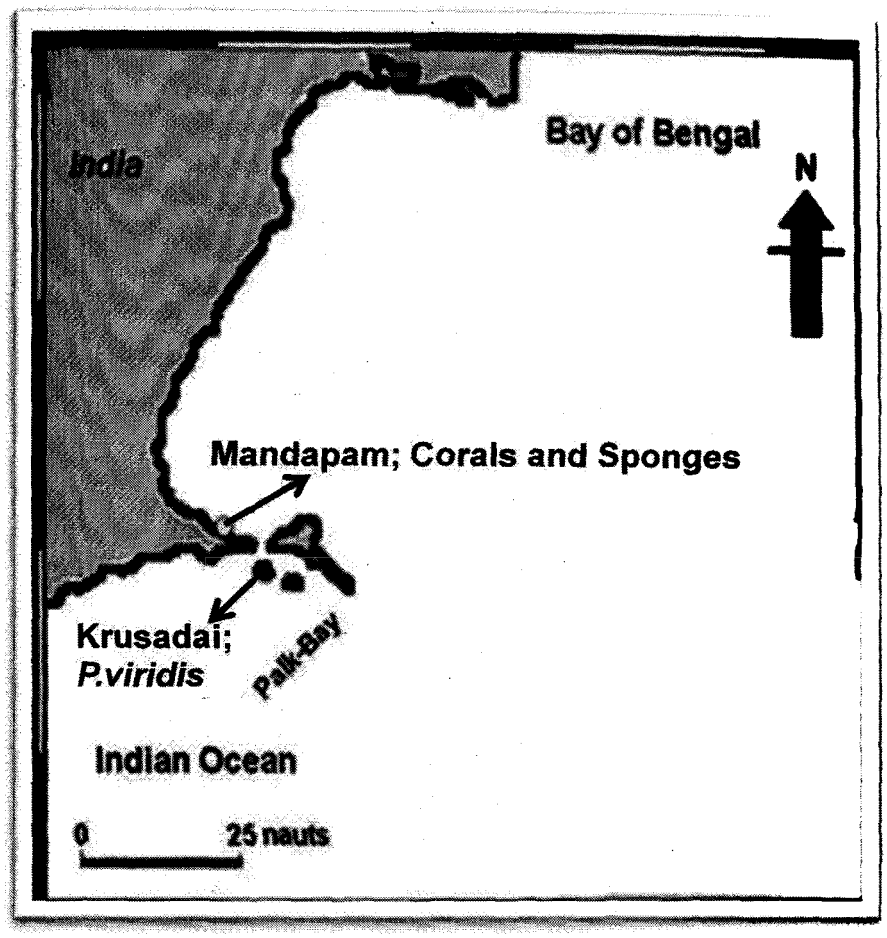


Figure 2.1 Mandapam in Tamil Nadu on the Eastern coast of the Indian peninsula situated at 9°17'0" North and 7°7'0" on East Coast in the Gulf of Mannar (GoM).

(MAM 1)

Class: Demospongiae
Order: Haplosclerida
Family: Petrosiidae
Genus: *Petrosia*
Species: *testudinaria*

(MAM 2)

Class: Denospongiae
Order: Tetillidae
Family:Spirophorida
Genus: *Cinachyra*
Species: *cavernosa*

(MAM 4)

Class: Demospongiae
Order: Haplosclerida
Family: *Chalinidae*
Genus: *Haliclona*

Unidentified (MAM 5)

(MAM 6)

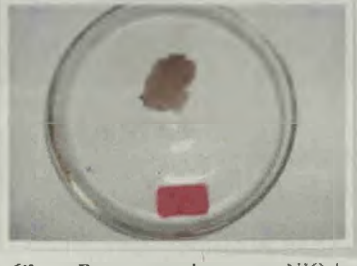
Class: Demospongiae
Order: Haplosclerida
Family: Petrosiidae
Genus: *Callyspongia*
Species: *fibrosa*

(MAM 10)

Class: Demospongiae
Order: Dictyoceratida
Family: Thorectidae
Genus: *Heteronema*
Species: *erecta*

(NIO 2)

Class: Demospongiae
Order: Dictyoceratida
Family: Thorectinae
Genus: Fasciospongia
Species: cavernosa



NIO 1

Class: Demospongiae
Order: Haplosclerida
Family: Petrosiidae
Genus: *Callyspongia*
Species: *reticutis var solomonensis*

Unidentified sponge (NIO3)

Figure 2.2 a Sponge samples under study

(MAM 3)

Class: Anthozoa
Order: Alcyonacea
Family: Clavulariidae
Genus: *Telesto*

(MAM 7)

Class: Anthozoa
Order: Alcyonacea
Family: Clavulariidae
Genus: *Echinogorgia*
Species: *reticulata*

(MAM 9)

Class: Anthozoa
Order: Alcyonacea
Family: Plexauridae
Genus: *Echinogorgia*
Species: *complexa*

(MAM 8)

Class: Anthozoa
Order: Alcyonacea
Family: Plexauridae
Genus :*Echinomuricea*
Species: *indica*

Figure 2.2 b Coral samples under study

Figure 2.2c Bivalve sample under study

Class: Bivalvia; Family: Mytilidae; Order: Mytiloida; Genus: *Perna*; Species: *viridis*

2.1.2 Enumeration and retrieval of bacteria from sponges, corals and bivalve tissue

2.1.2.1 Preparation of sponge macerate

One gram of sponge tissue was excised from the middle of the whole sponge using a sterile scissor, weighed and washed several times in 3% (w/v) NaCl. Following discard of these washings, the sponge samples were homogenized in 3% (w/v) NaCl using a mortar and pestle under aseptic conditions.

2.1.2.2 Preparation of coral macerate

The working sample was washed twice with 0.2 µm of filter sterilized and autoclaved sea water to remove any loosely associated microbes. An aseptically excised core of the coral was rewashed in 3% NaCl (w/v) solution and then crushed using a sterile mortar and pestle in 5 ml of 3% sterile NaCl (w/v) solution.

2.1.2.3 Preparation of bivalve macerate

The external surfaces of the shells were washed by scrubbing under running water. Then the shells were carefully opened by cutting the abductor muscle with a sterile knife. The soft tissues were externally flushed with sterile 3% (w/v) NaCl solution to ensure removal of any non adherent bacteria from the surface. The soft tissues were then homogenized in 3% (w/v) NaCl solution.

2.1.2.4 Enumeration of bacteria by Acridine Orange Direct Count (AODC) from sponge tissue using epifluorescent microscopy.

The sponge macerate was filtered to remove any solid debris and used to determine the total bacterial counts, a defined volume of the tissue homogenate was fixed with 2.5% (w/v) glutaraldehyde(Scanning electron microscope (SEM) grade) to a final concentration of 4% (w/v) and stored at 4°C until further use. It was filtered through a 25 mm diameter polycarbonate filter (GE Osmonics, Minneapolis, MN) and stained with 200 µl of Acridine Orange (10 mg/ml) solution for 5 minutes as described previously (Turley, 1993). The filters were then air dried and mounted with immersion oil onto a microscopic slide. Bacterial numbers were determined using an epifluorescence microscope (Axioplan microscope; Zeiss, Germany). 20-40 quadrats with a minimum of 600 cells were counted, to give a precision of

±10%. The bacterial cell numbers were calculated using the Generalization: cells/ml = (SC-BC) x CF x F/V wherein, SC is the mean of sample counts/quadrat; BC is the mean of background counts/quadrat; CF is the effective filter area/quadrat area; F is the volume of preservative/volume sample preserved +1; V is the volume of sample filtered.

2.1.2.5 Retrieval and isolation of pure bacterial cultures from Sponges/ Corals Bivalve

The macerate was serially diluted, and 10 µl of the filterate was plated separately onto i) Tryptone Yeast Extract medium (TYE) (Steensland and Larsen, 1969) consisting of (g/l) $MgSO_4$-20; $CaCl_2$-0.2; Tryptone-5; Yeast Extract-3, pH was adjusted to 7, using 1N NaOH, ii) TYE supplemented with 3% NaCl (3% TYE) and iii) TYE with 25% of NaCl (NTYE) (Braganza and Furtado, 2009) iv) NTYE supplemented with 700U of penicillin. Additionally the coral and bivalve macerate were plated onto Zobells marine agar. All the chemicals used were from Himedia-India. Plates were incubated at R.T. until visible colonies were seen on the plates. The bacterial colonies were enumerated to obtain cfu/g of sponge/coral/bivalve tissue. Morphology of the colonies growing on TYE medium, 3% TYE, and NTYE were recorded.

Distinct colonies were purified by repeated streaking on corresponding agar medium, designated and maintained as axenic cultures on agar slopes of the different isolation media at 4°C for a month. Bacterial isolates from sponges were numbered with a prefix GUVFPM; GUVFCCM; GUVFHM; GUVFUM; GUVFCFM; GUVFHEM; GUVFCM; GUVFFM; GUVFSM denoting GU (Goa University),V (Velho), F (Furtado), P (*Petrosia*), C (*Callyspongia*), F

(*Fasciospongia*), S (Unidentified Sponge), CC (*Cinachyra cavernosa*), H (*Haliclona*), U (Unidentified Sponge),CF (*Callyspongia fibrosa*), HE (*Heteronema erecta*) and M (Mandapam). Bacterial isolates from corals were numbered with a prefix GUVFTM; GUVFECM; GUVFERM; GUVFEIM denoting GU (Goa University), V (Velho), F (Furtado), T (*Telesto sp.*), EC (*Echinogorgia complexa*), ER (*Echinogorgia reticulata*), EI (*Echinomuricea indica*) and M (Mandapam). The isolates from the single bivalve were designated as GUVFPM denoting GU (Goa University), V (Velho), F (Furtado), P (*Perna*).

2.1.3 Characterization of bacterial bionts isolated from Sponges, Corals, and Bivalve

Various characteristics of individual isolates were examined using Bergeys keys of Systematic Bacteriology (Smibert and Krieg, 1994 and Holt *et al.*, 1994).

A. Morphological

2.1.3.1 A. Determination of Gram character:

A clean grease free slide was wiped with alcohol. Using a nichrome loop, a thin smear of culture suspension was made, air dried and heat fixed. The slide was flooded with crystal violet (1 minute) and washed with water. Grams iodine was added on this smear and kept for 1 minute. The slide was again washed with water. Alcohol was flooded on the smear and washed after 30 seconds. Safranine was added to the smear and washed after 1 minute. Excess water was drained off, and the slide was dried. It was then observed under oil immersion lens of the

microscope. Gram positive cultures stained purple while Gram negative cultures stained pink.

2.1.3.2 A. Modified Gram reaction

Cellular morphology and Gram character of the bacterial isolates growing at 20% NaCl were examined by performing modified Gram staining method for halophiles (Dassault, 1955). An even smear made on a clean grease free slide was air dried and fixed in a 2 % (w/v) acetic acid solution for 1 minute. The fixed smear was air dried and flooded for 30 seconds with 2% (w/v) crystal violet solution Excess of the stain was washed with a gentle stream of water, and it was subsequently replaced with Gram's iodine for the next 30 seconds. Thereafter the smear was decolorized with70% (w/v) absolute alcohol and exposed for 1 minute to 0.5% (w/v) safranine, washed, air dried and observed under an oil immersion lens of a bifocal light microscope (Olympus BX40).

2.1.3.3 A. Determination of cellular characteristics by scanning electron microscopy

Cellular dimensions of the bacterial isolates growing at 20% (w/v) NaCl were recorded using scanning electron microscopy (JEOL-5800LVSEM). A thin smear of a culture suspension ($A_{600=}$ 0.8) was made on square cover slips;air dried, fixed in 2% (v/v) of glutaraldehyde, overnight at room temperature. Smear was subjected to 10 minutes each of an increasing gradient of acetone: water solution (30%, 50%, 70%, 90% and 100% (v/v) respectively followed by air drying. The coverslips were mounted onto aluminium stubs which were in turn fixed on a sputter coater

(SPI module specimen holder) for coating the cells with a 10-15 nm gold film before visualization.

2.1.3.4 A. Determination of presence of spores

A smear of the bacterial cultures were prepared on a clean grease free slide which was heat fixed. The slide was flooded with malachite green and steamed over a Bunsen burner for 5 minutes ensuring that the slide does not dry. The slide was washed with tap water and counter stained with safranine for 30 seconds tol minute. It was washed under tap water air dried and examined under the oil immersion lens to check for the presence of endospores which stained green inside a pink bacterial cell.

2.1.3.5 A. Stability of whole cells in water

Aliquots of 2 ml resting cells of isolates growing at 20% (w/v) NaCl (A_{600} ~ 1) were taken in an eppendorf tube and centrifuged in a microfuge (Eppendorf 140 R) at 14000 rpm at 4^0C for 10 minutes to obtain a tight pellet. The pellet was then resuspended in deionized water, mixed on a cyclomixer (Remi-India), transferred to a spectrophotometric cuvette rapidly within 10 seconds and the absorbance was monitored at 600 nms in a Spectrophotometer (Shimatzu-Japan) at intervals of 20 seconds.

B. Biochemical Characterisation

2.1.3.1 B. Oxidase test

The filter paper (Whatman no. 40) was placed into a petridish and wetted with 0.5 ml of 1% dimethyl-p-phenylenediamine dihydrochloride. Then, the bacterial cells were streaked onto the reagent zone of the filter paper. The development of a deep blue color at the inoculation site within 10 seconds indicated a positive result.

2.1.3.2 B. Catalase test

1 ml of H_2O_2 solution was poured over 24 hr old culture on a slide. The formation of effervescence indicated a positive test.

2.1.3.3 B. Sugar fermentation

The sugar fermentative medium was inoculated with 24 hr old culture and incubated for 24-48 hrs at 37^0C for the production of acid and gas. Acid production is indicated by a colour change from pink to yellow and an air bubble in Durham tubes indicated gas production.

2.1.3.4 B. Indole Production

Tryptone water was inoculated with culture and incubated for 48-96 hrs at 37^0C for accumulation of indole. Indole was concentrated from the medium in the organic solvent like xylene. To each test tube 0.5 ml Kovac's reagent was added to the organic layer and shaken gently. A red colour in the alcohol layer indicated a positive reaction.

2.1.3.5 B. Methyl red test

Glucose phosphate peptone water medium was inoculated from a young agar slope culture and incubated at 37^0C for 48 hrs. Five drops of methyl red reagent were added to each tube; the solution was mixed and colour change was observed immediately. Positive tests were indicated by a red colour while negative with a yellow colour.

2.1.3.6 B. Vogues Proskauer Test

Glucose phosphate peptone water was inoculated with a 24 hr old culture suspension and the tubes were incubated. 0.5ml of O'Mearas reagent was added to the tubes that were then incubated at 37^0C for 30 minutes. Development of pink colour ring indicated a positive test.

2.1.3.7 B. Citrate utilization

Simmon's Citrate agar slants were streaked with the culture suspension and incubated at 37^0C for 48 hrs. The development of blue colour indicated a positive test.

2.1.3.8 B. Motility test agar

24 hr old culture suspensions were stab inoculated into the motility agar (the top surface is not inoculated) and incubated at 37^0C for 48 hrs. The presence of growth away from the line of streaking indicated a positive test.

2.1.3.9 B. Nitrate reduction test

0.1 ml was inoculated into nitrate reduction broth and incubated at 37°C for 48 hrs until bacterial growth was observed. Then 1.0 ml of solution A (α- naphthylamine) was added to each culture tube, followed by 1.0 ml of solution B(sulphanilic acid) and mixed thoroughly. The development of a red color indicated a positive test. The result of a negative test was confirmed by adding small amounts of zinc powder. The true negative test was indicated by the development of the red colour.

2.1.3 C.Chemotaxonomic Characterization of isolates growing on 20% NaCl

2.1.3.1 C. *Analysis of pigment of isolates.*

10 ml of resting cells (A_{600} ~ 2) were centrifuged at 12000 rpm and the pellet thus obtained was dispersed into 20% NaCl and transferred into a glass beaker along with 5ml of acetone. The beaker was placed in an ice bath and the cells were sonicated at 100 mA (8 pulses of 15 seconds duration with 15 seconds cooling time) using a sonicator (B Braun Biotech International, U.S.A.).

2.1.3.2 C. Detection of Diaminopimelic acid (DAP)

a. Hydrolysis

100 ml of resting cells (A_{600} ~ 1) were centrifuged at 8000 rpm for 20 minutes to obtain a pellet. The pellet was suspended in 3 ml of 6 N HCl in a glass pyrex tube with stopper and was hydrolysed at 100^0C in a boiling water bath for a period of 16 hrs (Rhuland *et al.*, 1955). Resting cells of *E. coli* and *S. aureus* of (A_{600} ~ 2) obtained by centrifuging well grown nutrient broth were treated in the same way to serve as eubacterial controls.

b. Ascending paper chromatographic analysis of hydrolysates

Presence of DAP was sought specifically by analyzing the hydrolysates by paper chromatography using the solvent system of Rhuland *et al.*, 1955. Each of the hydrolysate was spotted separately at a distance of 2 cms from one end of the long end of a rectangular chromatographic paper 160 x 100 x 3 mm thickness using glass capillaries of 1 mm bore size. The paper was suspended in methanol: water: 10N HCl: pyridine (80:17.5:2.5:10 v/v/v/v) contained in a rectangular glass chamber 200x 100x 150 mm and allowed to run for approximately 40 minutes. Care was taken to ensure presaturation of the chamber and that the hydrolysed spots were not immersed in the solvent system. The air dried chromatogram was then sprayed with 0.1% ninhydrin in acetone, heated in an oven at 110^0C to develop the spots if any.

2.1.3.3 C. Detection of GDEMs

Whole cell methanolysis

200 mg of freeze dried cells were mixed with 3 ml of methanol, 3 ml of toluene and 0.1 ml of concentrated sulphuric acid. The sample was hydrolyzed at 55^0C for 18 hours in teflon coated screw capped glass tubes. 1.5 ml of hexane was then added to each tube and contents were transferred to a separating funnel, mixed vigorously by shaking and thereafter the hexane layer present at the top was collected by phase separation and concentrated separately under a stream of nitrogen. The concentrate was spotted onto Silica gel plate F_{256} (Merck) and developed in a chamber containing a solvent system (pet ether (60:80) and diethylether in the ratio of 85:15 (v/v). The chromatogram was air-dried and the

lipids were revealed by spraying 10% (w/v) dodecaphosphoric acid in absolute ethanol, heated for 15 minutes at 150^{0}C. The lipids appeared dark blue on a yellow background. Rf values of 0.2 were attributed to the glycerol diether moieties (GDEM's) while Rf values greater than 0.6 were attributed to methyl esters of non hydroxylated fatty acids (FAME's). Absence of ester containing lipids was seen as a negative reagent with ferric chloride while FAME stain purple against a yellow background (Ross *et al.*, 1981).

2.1.4.1 C. Demonstration of polar lipids

Cell pellets were suspended in 2 ml of 4 M NaCl and extracted with 3.75 ml of chloroform: methanol (1:2) (v/v) for 12 hrs. The extract was collected by centrifuging at 10000 rpm and pellet obtained was reextracted with 4.75 ml methanol: chloroform: water (2:1:0.8 v/v/v) (Oren, 1993).The chloroform layer was collected using a separating funnel and concentrated under a stream of nitrogen. Individual concentrates were applied ~~individually~~ onto Silica gel G plates and developed in the solvent system chloroform: methanol: acetic acid: water (85:22.5:10:4v/v/v/v). Lipid spots were visualized by exposure to iodine vapours. Glycolipids were visualized by spraying the chromatogram with 0.5% (v/v) α-naphthol in 50% (v/v) methanol-water followed by spraying with 5% (v/v) sulphuric acid in ethanol and heating the plates at 100^{0}C (Siakatos and Rouser, 1965). Phospholipids were visualized using ammonium molybdate-sulphuric acid spray (Dittmer and Lester, 1964).

Mass data was recorded on an Electrospray Ionization Tandem Mass Spectrometer (ESI/MS-MSa QTOF-XL MS/MS Applied Biosystem instrument equipped with MDS Sciex Analyst software (Concord, Ontario, Canada). The sample dissolved in

1:1 Methanol: 0.1% (v/v) TFA and was directly infused at a constant flow rate of 10μL/min into the ion spray source using an integrated syringe pump. The instrument was operated in positive ionization mode with the following settings: Nebulizer gas (N2) 28 (arbitrary units); curtain gas (N2) 18 (arbitrary units); Ion spray voltage-5700 V; Declustering potential (DP)-120V; Focusing potential (FP)-365V; Declustering potential (DP2)-14V and Collision gas (CAD) 3 (arbitrary units). Full-scan data acquisition was performed, scanning from m/z 100 to m/z 2000 in profile mode and using a cycle time of 1s (Lattanzio, 2009)

2.1.3.5 C. Antibiotic sensitivity of isolates

The susceptibility of bacterial isolates to antimicrobial agents was tested in NTYE. The antibiotics tested were penicillin, chloramphenicol, tetracycline, vancomycin, and kanamycin. The antibiotics were amended in NTYE media, and the cultures were streaked onto the surface of the agar medium. The incubated plate was left to dry for a few minutes at room temperature with the lid closed. Then it was incubated at 42^0C until bacterial growth was observed. At the end of the incubation period, the plate was checked for the presence of growth of the isolates. Growth was indicant of resistance to the antibiotics while the absence of growth indicated susceptibility.

2.1.3.6 C. Fluorescence transmission infra red spectroscopy of cultures growing at 20% NaCl

Bacterial cultures GUVFCM, GUVFFM-1, GUVFFM-2, and GUVFCCM-2 were streaked onto NTYE agar plates amended with 700 U of benzyl penicillin. Following incubation a loopful of culture was dried in an aluminium foil at 42^0C. The bacterial cultures were then subjected to diffused reflectance spectral analysis between 400-4000 nm using an FTIR spectrophotometer (Shimatzu-Japan).

2.1.3 D. Molecular characterization

2.1.3.1 D. Extraction of genomic DNA of individual isolates from sponges, corals and bivalve

Single colony growing on TYE agar medium with 3% NaCl/NTYE was inoculated in TYE liquid medium with 3% NaCl/NTYE. After 2/7days, the cell mass was extracted for DNA by suitably modifying the CTAB-NaCl method of Asubel *et al.*, 1995, wherein cell pellet was incubated with 270 µl of 1% CTAB-NaCl solution, at 225 rpm, 37^0C for 30 minutes followed by the addition 150 µl 10% SDS and incubation at 65^0C for 10-15 minutes or till the solution becomes viscous. The supernatant obtained on spinning at 8000 rpm, 15^0C, 20 minutes were extracted in 800 µl chloroform: isoamylalcohol (24:1 v/v). This was followed by two subsequent extractions, each using 800 µl of tris equilibrated phenol: chloroform: isoamylalcohol (25:24:1 v/v). Finally, DNA was precipitated from the supernatant with chilled isopropanol. The DNA pellet was washed in chilled ethanol (90%), blotted dry and suspended in 0.1X SSC buffer, pH 7 and checked for purity by assessing A_{280}/A_{260} and A_{230}/A_{260} ratios (Johnson, 1994).

2.1.3.2 D. Determination of G+C content of bacterial isolates

Aliquots of extracted DNA were dispensed in quartz cuvettes, placed in the sample chamber of the UV–VIS spectrophotometer having provision for circulating water preheated using (Julabo-F_{25}) water bath. The instrument was programmed for temperature ramp of (28^0C- 30^0C) to 90^0C with a temperature increase of 1^0C min^{-1}. Changes in absorbance at 260nm were recorded spectrophotometrically. A thermal denaturation curve of A_{260} against melting temperature was plotted by the method of Marmur and Doty, 1962 and the midpoint value (Tm) was determined. The G+C content was calculated using the equation of Craveri *et al.*, 1965.

2.1.3.3 D. PCR amplification of 16S rDNA gene of bacterial isolates

PCR was performed in a 100 µl microfuge tube with 1 µl genomic DNA, 5 µl of 10 X Taq buffer, 0.5 µl of 50 nM each of forward and reverse universal primers 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1522R (5'AAG GAG GTG ATC CAG CCG CA 3')(Genei-India) (Hicks *et al.*, 1992), 5 µl of 25 mM $MgCl_2$, 0.5 µl of 25 mM of dNTP, 0.5 µl (5 U/µl) of Taq polymerase (Genei-India) and 38 µl of sterilised MilliQ water and was placed in a thermocycler (Takara-Japan). The programme consisted of an initial delay at 94°C for 2 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 90 seconds and a final elongation at 72°C for 5 minutes. 10µl of the PCR amplified products were examined by electrophoresis using a 1% agarose gel containing ethidium bromide (0.5 µg/mL) at 8 volts/cm and visualised by a gel doc system, (Model GGS 12/D-E Gene Genius Classic Syngene UK) and the PCR product was purified using the DNA purification kit (Invitrogen-India). 16S rDNA coding gene amplicons corresponding to 1.5 kb were *bidirectionally* sequenced

using the Bigdye terminator sequencer, (Applied Biosystems, Foster City USA) and analysed through automated sequencer (ABI-3730 DNA analyser) and electropherograms were imported in Chromas-pro, ver 1.5.

2.1.3.4 D. PCR amplification of 16S rDNA gene of isolates growing on 20%NaCl

About 10 ng of extracted DNA was used as the template in a PCR in which prokaryotic 16S ribosomal DNA (rDNA)-specific primers and archaeal specific primer sets were used. The PCR mixtures (20µl) contained each deoxynucleoside triphosphate at a concentration of 200 mM, 50 mM $MgCl_2$, each primer at a concentration of 0.3 mM, 2.5 U/ml of Taq DNA polymerase (Invitrogen), and the PCR buffer supplied with the enzyme. The archaeal primer set used was Arch165F 5' TCCGGTTGATCCTGCCAG3' and RPP2 5' CCAAGCTTCTAGACGGTACC TTGTTACGACTT 3'. The PCR program included an initial denaturation at 94°C for 5 minutes and 10 touchdown cycles of denaturation at 94°C for 1 minute, annealing at 56°C (with the temperature decreasing 1°C each cycle) for 1 minute, and extension at 72°C for 1 minute, followed by 30 cycles of 94°C for 1 minute, and 72°C for 20 minutes. PCR was performed in Gene amp PCR System 9700 Applied Biosystems. The PCR product was checked for amplification by loading 10µl of DNA onto 0.8% agarose (SD fine Chems) gel having 0.5µg/ml of ethidium bromide (SD fine Chems), and quantified by using a standard (Low DNAMass Ladder; Gibco BRL). The gel was electrophoresed in TAE buffer pH 8 at 1-5 v/cm in an electrophoretic system (Hoeffer Scientific Instruments). The electrophorogram was visualised using a gel doc system (Model GGS 12/D-E Gene Genius Classic Syngene UK). The

amplified DNA was excised from the gel and purified using the DNA purification kit (Invitrogen-India). 16S rRNA amplicons corresponding to 1.5 kb were bidirectionally sequenced using the Bigdye terminator sequencer, Applied Biosystems, Foster City (USA). The reactions were analysed through automated sequencer (ABI-3730 DNA analyser). Sequence data was imported automatically into the BioEdit version 5.0.9 sequence editor (Hall, 1999). Contiguous sequence was obtained by base calling. The 16S rRNA sequence thus obtained was aligned with sequences available in the ribosomal database project RDP (Maidak *et al.*, 2001) and Gene Bank databases (Benson *et al.*, 1999) using ClustalW available at European bioinformatics site (www.ebi.eic.uk/clustalW) and then subjected to a (BLAST)n at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) in order to identify the closest homologues for the 16S rRNA of GUVFCCM-2. A dendrogram was constructed using the aligned sequences by the neighbour-joining method (Felsteinstein, 1981). The 16S rRNA sequences thus obtained for GUVCCM-2 was deposited in the GenBank database.

2.1.3.5 D. 16S rDNA gene Sequence Analysis of individual isolates from sponges/corals and bivalve

Partial sequences (300 - 1000 bp) were obtained for all the isolates. All sequence electropherograms were manually curated to remove few extra nucleotides flanking the sequences, checked for their query coverage (100%) using BLASTn and then uploaded in the naïve Baleysian classifier algorithm Ribosomal Database Project (Cole *et al.*, 2009) and EZ taxon e (Kim *et al.*, 2012) for alignment using 'infernal' secondary structure alignment of RDP.10. and EX taxon e. Sequence data was imported automatically into the BioEdit version 5.0.9 sequence editor (Hall, 1999) The contiguous 16S rRNA gene sequence was obtained by base calling and alignment with sequences available in the ribosomal database project RDP (Maidak *et al.*, 2001) and Gene Bank databases , using the CLUSTAL_X program followed by (BLAST)n at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The resulting alignments were subjected to the PhyML program (Guindon *et al.*, 2005) and the maximum-likelihood analysis (Felsenstein, 1981) in order to derive the evolutionary tree whose topology was evaluated using boot strap analysis based on 100 re-samplings. The 16S rRNA gene sequences thus obtained was deposited in the GenBank database.

2.1.3.6 D. Phylogenetic identification of bacterial isolates from sponges, and corals and bivalve

To reach the best possible generic/species level taxonomic assignments and select the closest phylotype/s to the sequences, we followed a common frame-work based upon: (a) Isolates referred reliably to 'species' level in cases of > 97 % 16S rDNA

sequence homology to a 'single nearest described Type strain' in both RDP-II and EZtaxon-e (Kim *et al.*, 2012) database with supporting phenotypic characteristics. (b) At >97% homology, isolates referred to 'genus' level when species level distinction based on 16SrDNA and phenotypic characters were unreliable (c) At >97% homology, isolates referred to 'groups' of genus when generic level distinction based on 16SrDNA and phenotypic characters were unreliable. (d) Sequences showing <97% match with nearest Type strains referred to as unclassified family/genus level for further characterization.

A neighbor-joining phylogenetic tree was constructed after multiple-alignment of near-complete 16SrDNA sequences of closest relatives using Clustal-X and MEGA-5. Pairwise evolutionary distances were calculated using the Kimura two-parameter model Phylogenetic trees were constructed by three different tree-making algorithms (UPGMA, Maximum likelihood and neighborjoining) of the MEGA-5. The stability among the clades of a phylogenetic tree was assessed by taking boot-strap values expressed as percentages of 1000 replications.

2.1.4 Determination of affiliation of eubacterial isolates to phenon using statistical analysis

For every cultured eubacterial isolate (obtained from sponge/coral), the absence and presence of specific phenotypic character, was assessed and rated as 0 (for negative) or 1 (for positive) respectively. Bacterial cultures were sorted out by measuring the similarity and diversity between two bacteria namely A and B, by dividing the size of the intersection by the size of their union as in Jaccard coefficient. Hierarchical rearrangement of clusters attained through group-average linking method based on Jaccard distance, which measures dissimilarity between

sample sets and is obtained by subtracting the Jaccard coefficient from 1, by dividing the difference of the sizes of the union and the intersection of two sets by the size of the union. Computations were carried out using the software SYSTAT v.12.01 (Ghozlan *et al.*, 2006) to generate dendrograms, which reflect the phenotypic relationships between members and expressed as a *phenogram.*

2.1.5 Statistical measures of diversity

Margalef's species richness (*d*), Pielou's evenness (*J'*) and Shannon-Wiener's diversity index (*H'*) were analyzed using PRIMER v.5, inorder to arrive at univariate measures of the retrieved bacterial community in sponges and corals using the strategy of 0% (w/v), 3% (w/v) and 25% (w/v) NaCl in media for culturing bacteria.

Margalef's species richness (*d*), is given by d = (S-1) / ln N, where S is the number of genera and N is the number of individuals. The evenness of a community is represented by Pielou's evenness index: $J'=H'/H'$maxwhere H' is the number derived from the Shannon diversity index, and H'_{max} is the maximum value of H' given by the equation:

$$H_{\max} = -\sum_{i=1}^{S} \frac{1}{S} \ln \frac{1}{S} = \ln S.$$

The diversity of the community is represented by the Shannon diversity index (*H'*), H'= $-\Sigma$ (*Pi* ln (*Pi*) where *Pi=ni/N* (ni, the relative abundance (RA) of the genera calculated as the proportion of individuals of a given genera to the total number of individuals in a community, *N* (Shannon and Weaver, 1949).

2.2 Results:

The observation of enumeration of bacteria; record of the bacterial isolates; individual morphological; biochemical; chemotaxonomic; molecular characterization; delineation of the identity of individual isolates, taxonomic placements inferences and discussions are described separately as under Section A: Sponges, Section B: Corals and Section C: Bivalve.

Section A: Bacteriology of Sponges

2.2.1 A. Enumeration of bionts associated with sponges by Acridine Orange Direct Count

The Acridine orange direct counts for each of the nine sponges macerates ranged from 2.3 - 8.7 x 10^9 cells/g. The total bacterial counts averaged at 5.2 ± 2.2 x 10^9 cells/g. The highest count of 8.7 x 10^9 cells/g was observed in unidentified sponge MAM 5 and the lowest count of 2.30 x 10^9 cells/g in *C.cavernosa* and *H.erecta* (Figure 2.3) Bacterial morphotypes resolved were cocci, coccobacilli, curved rods, long rods, and short rods (Figure 2.4).

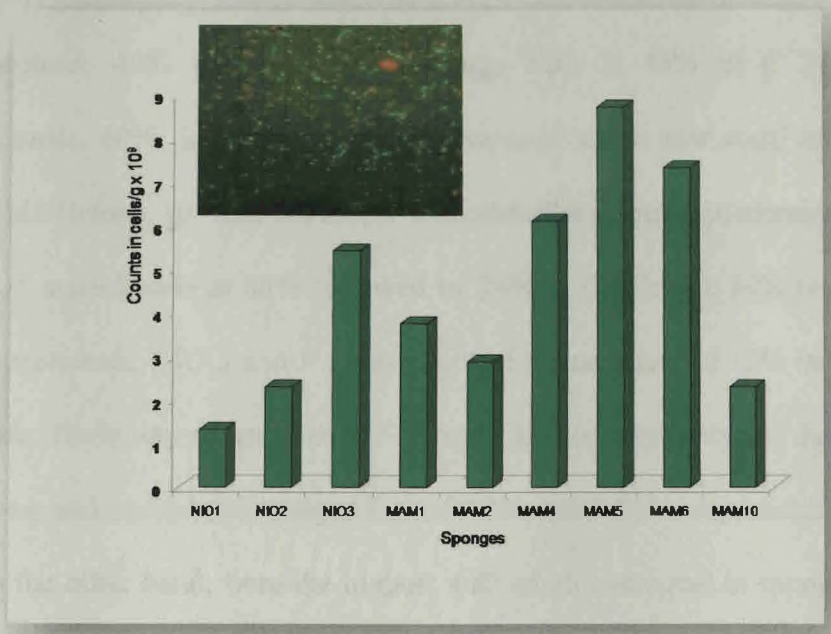


Figure 2.3 The total direct counts of bacteria associated with the sponge samples determined by AODC.

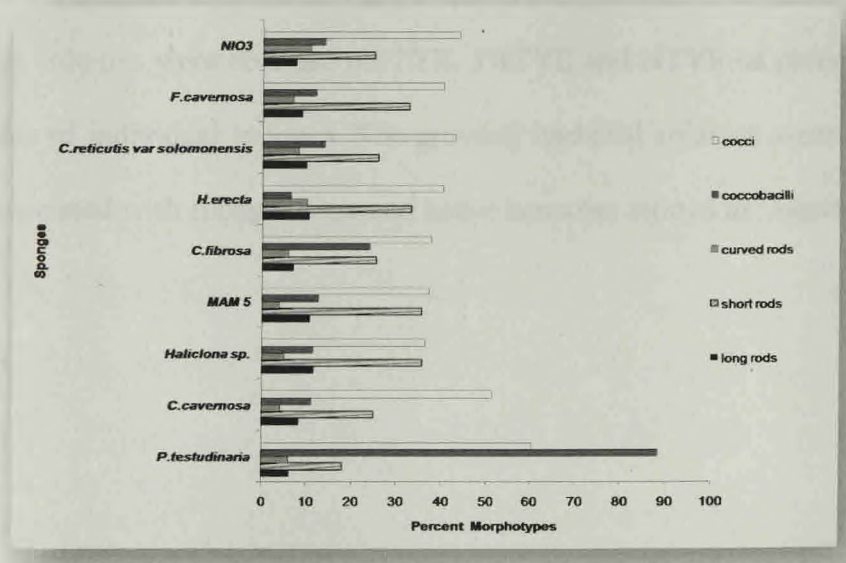
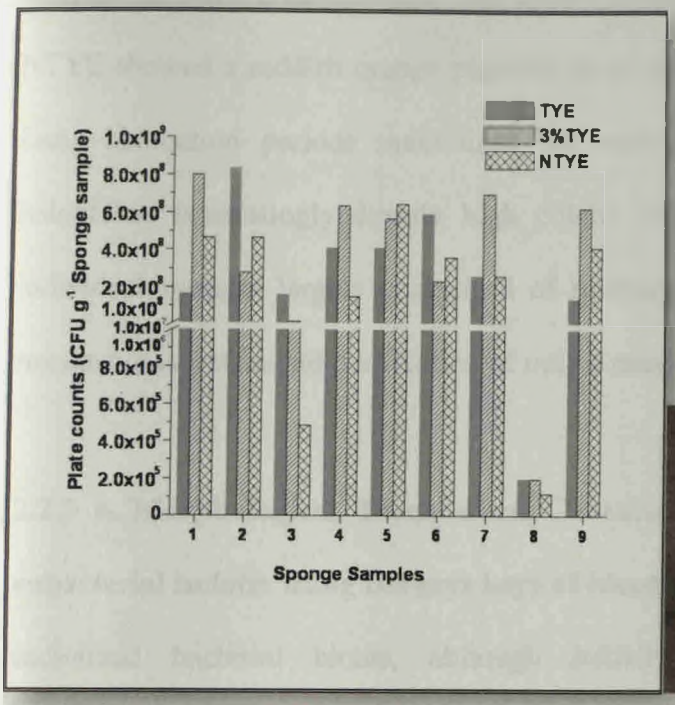


Figure 2.4 Percent Morphotypes of bacterial bionts revealed by Acridine Orange Direct Count in sponge tissue of Mandapam-India

The coccal forms were a maximum of 61% in *P. testudinaria* followed by 51% in *C. cavernosa,* 44% in Unidentified sponge NIO 3, 43% in *C. reticutis var solomonensis,* 40% in *H. erecta* and *F.cavernosa* and a near equal of 38% in *C. fibrosa, Haliclona sp.* and MAM 5. Coccobacillary forms predominated in the sponge *P. testudinaria* at 88% followed by 24% in *C. fibrosa*, 14% in *C. reticutis var solomonensis,* NIO 3 and *F.cavernosa* and a near equal of 12% in sponges *C. cavernosa, Haliclona* sp. and MAM 5. Among the rod morphotypes, the proportion of the long and curved rods ranged from 6-12% and 3-10% respectively. The short rods, on the other hand, were the highest with an almost equal in sponges MAM 5 and *Haliclona* sp. at 36% followed by 33% in *H. erecta* and *F. cavernosa,* 25% in sponges *C. fibrosa, C.reticutis var solomonensis,* NIO 3 and *C. cavernosa.*

2.2.1.1 Retrieval and isolation of pure bacterial bionts

Bacterial colonies were obtained on TYE, 3%TYE and NTYE on plating of tissue macerates of individual sponges. The growing bacterial colonies were considered to be associated with sponge tissue and hence hereafter termed as '**bionts**'



1:*Petrosia testudinaria*(MAM1)
2:Cinachyra cavernosa(MAM2)
3: Haliclona sp. (MAM4)
4.Unidentified (MAM5)
5.Callyspongia fibrosa(MAM6)
6. *Heteronemaerecta*(MAM10)
7.Callyspongia reticutisvarsolomonensis(NIO1)
8.Fasciospongia cavernosa(NIO2)
9.Unidentified (NIO3)

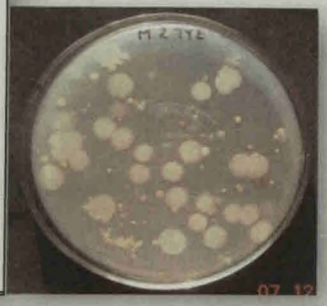


Figure 2.5 Total retrievable counts associated with sponges TYE,3%TYE and NTYE in cfu/g sponge

As seen in Figure 2.5, total viable counts on TYE ranged from $2x10^5$ - 8.32 x 10^8 cfu/g in all the sponges. The counts on TYE averaged at 3.31 ± 2.60 x 10^8 at cfu/g. The total viable counts on 3% TYE on the other hand, ranged from $2x10^5$ - 7.99 x 10^8 cfu/g with average counts of 3.83 ± 2.86 x 10^8cfu/g in all the sponges. Individual sponges gave a total viable count ranging from 1.2 x 10^5 - 6.48 x 10^8 cfu/g on NTYE. The total viable counts on NTYE averaged at 2.89 ± 2.3 x 10^8 cfu/g. The highest average counts were obtained on 3% TYE.

Greatest diversity of aerobic flora in terms of morphotypes was observed in TYE media and gradually decreased with the increasing concentration of NaCl. The number of colonies growing on TYE media consecutively decreased with the

increase of NaCl concentration. The cream coloured colonies that dominated in NTYE showed a reddish orange pigment on prolonged incubation of 20-30 days. Long incubation periods maximized the viable counts and diversity of the halophiles. Interestingly despite high colony count, the bacterial growth from individual sponges largely comprised of restricted colonial morphotypes, which resulted in isolation and purification of only a maximum of 42 different colonies.

2.2.2 A. Morphological, Biochemical Characterization and Identification of eubacterial isolates using Bergeys keys of Identification and SYSTAT

Individual bacterial bionts, although initially retrieved from growth on TYE/3%TYE/NTYE, were also able to grow at other concentrations of NaCl (Table 2.1) and hence categorized as: *marine halophiles* growing at 3% NaCl and *halotolerant or euryhaline* growing at 0-25% NaCl. 78.57% of the bionts associated with all the sponges under study were euryhaline while the remaining 21.42% were marine halophiles. A total of 42 bacterial bionts were obtained in pure form from the nine different sponges.

Of the total isolates, 61.9% were Gram positive, and the remaining 38.09% of the isolates were Gram negative. Based on the morphological and biochemical characteristics (Table 2.1) all the 42 isolates were identified upto the generic level using Bergeys keys of Systematic Bacteriology (Smibert and Krieg, 1994; Holt *et al.*, 1994). The Gram positive isolates belonged to the genera *Bacillus*, *Enterococcus*, *Corynebacterium*, *Pontibacillus* and *Planococcus* while the Gram negative isolates belonged to the genera *Loktanella*, *Enterobacter*, *Chromohalobacter* and *Pseudomonas*.

Table 2.1 Biochemical characteristics and tentative identification of dominant bacterial bionts retrieved and purified from sponges of Mandapam- India

Sponges	Isolates	Retrieval on TYE with NaCl (%)	Pigmentation	Morphotypes/ spores	Gram Character	Motility	Oxidase	Catalase	Glucose	Sucrose	Lactose	Indole	Methyl Red	VP	Citrate	NR	Growth in NaCl (%)			Genera
																	0	3	25	
MAM 1 *Petrosia estidunaria*	GUVFPM-1	25	cream	R	-	+	+	+	-	-	-	+	-	-	-	+	+	+	+	***Chromohalobacter* sp.**
	GUVFPM-2	3	cream	SR	+	-	+	+	+	-	-	+	+	-	-	+	+	+	-	***Corynebacterium* sp.**
	GUVFPM-3	3	orange	RS	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	***Bacillus* sp.**
	GUVFPM-4	3	yellow	RS	+	+	+	+	-	-	-	-	-	+	-	-	+	+	+	***Bacillus* sp.**
	GUVFPM-5	0	white	SR	-	+	-	+	+	-	-	-	+	+	-	-	+	+	-	***Enterobacter* sp.**
	GUVFPM-6	0	beige	SRS	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	***Pontibacillus* sp.**
	GUVFPM-7	0	yellow	SR	-	+	-	+	+	-	-	+	+	-	-	+	+	+	-	***Enterobacter* sp.**
	GUVFPM-8	0	beige	RS	+	+	-	+	+	-	-	+	-	-	-	-	+	+	+	***Pontibacillus* sp.**
MAM 2 *Cinachyra*	GUVFCCM-1	25	CL	SLR	-	-	+	+	-	+	+	+	-	-	-	+	+	+	+	***Pseudomonas sp.***
	GUVFCCM-2	25	cream	R	*	+	+	+	-	+	+	+	+	-	-	+	+	+	+	**Unidentified**
	GUVFCCM-3	0	yellow	RS	+	+	+	+	+	-	-	-	+	-	-	+	+	+	+	***Bacillus* sp.**

cavernosa	GUVFCCM-4	0	cream	R	+	-	+	+	+	-	-	+	+	-	-	-	+	+	+	***Corynebacterium* sp.**
	GUVFCCM-5	0	orange	RS	+	+	+	+	+	-	-	+	+	+	-	-	+	+	-	***Bacillus* sp.**
	GUVFCCM-6	0	yellow	RS	+	+	+	+	-	-	-	+	-	-	-	-	+	+	-	***Bacillus* sp.**
	GUVFCCM-7	0	white	RS	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+	***Pontibacillus* sp.**
	GUVFCCM-8	0	cream	C	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	***Enterococcus* sp.**
	GUVFCCM-9	0	white	RS	+	+	+	+	-	-	-	+	-	-	-	+	+	+	-	***Bacillus* sp.**
	GUVFCCM-10	25	cream	R	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	***Chromohalobacter* sp.**
MAM4	GUVFHM-1	25	cream	RS	+	+	+	+	-	-	-	-	+	-	-	-	+	+	+	***Bacillus* sp.**
Haliclona sp	GUVFHM-2	25	cream	R	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	***Chromohalobacter* sp.**
MAM5			Pale																	
Unidentified	GUVFUM-1	3	orange	R	+	-	+	+	-	-	-	+	-	-	-	-	+	+	-	***Corynebacterium* sp.**
sponge	GUVFUM-2	0	orange	RS	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	***Bacillus* sp.**
MAM6	GUVFCFM-1	0	cream	RS	+	+	+	+	-	-	-	+	-	+	-	+	+	+	+	***Bacillus* sp.**
Callyspongia	GUVFCFM-2	0	cream	RS	+	+	+	-	-	-	-	+	+	-	-	-	+	+	+	***Bacillius* sp.**
fibrosa	GUVFCFM-3	0	cream	SR	+	-	+	+	+	-	-	-	+	-	-	+	+	+	+	***Corynebacterium* sp.**
	GUVFCFM-4	0	cream	RS	+	+	+	+	-	-	-	+	-	+	-	+	+	+	+	***Bacillus* sp.**
MAM10	GUVFHEM-1	3	cream	R	+	-	+	+	-	-	-	+	-	+	-	+	+	+	+	***Corynebacterium* sp.**
Heteronema	GUVFHEM-2	3	cream	SLR	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	***Loktanella* sp.**
erecta	GUVFHEM-3	0	cream	SLRS	+	+	+	+	-	+	+	-	-	-	-	-	+	+	-	***Bacillus* sp.**

	GUVFHEM-4	25	CL	SLR	-	-	+	+	-	+	+	+	-	-	-	+	+	+	+	***Pseudomonas* sp.**
	GUVFHEM-5	0	cream	RS	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	***Bacillus* sp.**
	GUVFHEM-6	0	cream	SR	+	-	+	+	-	-	-	+	-	-	-	-	+	+	+	***Corynebacterium* sp.**
	GUVFHEM-7	0	cream	C	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	***Planococcus* sp.**
NIO1	GUVFCM-1	25	orange	R	*	+	+	+	+	+	+	-	-	-	-	+	+	+	+	**Unidentified**
Callyspongia	GUVFCM-2	0	cream	RS	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	***Bacillus* sp.**
reticutis var																				
olomonensis	GUVFCM-3	0	CL	SLR	-	-	+	+	-	+	+	+	-	-	-	+	+	+	+	***Pseudomonas* sp.**
NIO2	GUVFFM-1	25	orange	R	*	+	+	+	+	+	+	-	-	-	-	+	+	+	+	**Unidentified**
Fasciospongi	GUVFFM-2	25	orange	R	*	+	+	+	+	+	+	-	-	-	-	-	+	+	+	**Unidentified**
a cavernosa	GUVFFM-3	3	cream	R	-	+	+	+	-	-	-	+	-	-	+	+	+	+	+	***Chromohalobacter* sp.**
NIO3	GUVFSM-1	25	cream	R	-	+	+	+	-	-	-	-	-	-	-	+	+	+	-	***Chromohalobacter* sp.**
Unidentified	GUVFSM-2	0	cream	RS	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	***Bacillus* sp.**
sponge	GUVFSM-3	0	CL	SLR	-	-	+	+	-	+	+	+	-	-	-	+	+	+	+	***Pseudomonas* sp.**

GC, Gram character;**CL**, colourless; **R**, Rods; **RS**, Rods with spores; **SR**, Short rods; **SLR**, Slender rods; **SRS**, Short rods with spores; **SLRS**, Rods fine slender with spores; **C**, Cocci; **VP**, Voges Proskauer; **NR**, Nitrate reduction; **(+)**, present; **(-)**,absent;* could not be discerned.

SYSTAT analysis further sorted the Gram positive and Gram negative bionts based on their biochemical characteristics (Table 2.1) using distance matrix and UPGMA clustering into 4 and 1 **Phena** and 1 and 3 **Phena,** respectively as represented in the phenogram (Figure. 2.6 a and b). Thus confirming the identification resolved to generic level, for each retrieved bacterial biont (Table 2.1) according to keys of Bergey's Systematic Bacteriology (Smibert and Krieg, 1994; Holt *et al.*, 1994). Accordingly the 4 and 1 **Phena** under Gram positive cluster corresponds to 4 Genera under Class Bacilli and 1 Genus under Class Actinobacteria belonging to Phylum Firmicutis (Figure 2.6a). The retrieved bionts were distributed as in:

Phenon 1 ≈ Genus *Enterococcus* - GUVFCCM-8 a motile cocci and catalase negative

Phenon 2 ≈ Genera *Pontibacillus* - GUVFPM-6, GUVFPM-8 and GUVFCCM-7 are sporulating motile rods, catalase positive and oxidase negative

Phenon 3 ≈ Genus *Planococcus* - GUVFHEM-7 a motile cocci, catalase and oxidase positive

Phenon 4 ≈ Genera *Bacillus* - GUVFPM-3, GUVFPM-4,GUVFCCM-3, GUVFCCM-5, GUVFCCM-6, GUVFCCM-9, GUVFHM-1, GUVFUM-2, GUVFCFM-1, GUVFCFM-2, GUVFCFM-4, GUVFHEM-3, GUVFHEM-5, GUVFCM-2 and GUVFSM-2 are sporing rods, oxidase and catalase positive

Phenon 5≈ Genera *Corynebacterium* - GUVFPM-2, GUVFCCM-4, GUVFUM-1, GUVFCFM-3, GUVFHEM-1 and GUVFHEM-6 are non motile rods, catalase and oxidase positive.

Similarly the 1 and 3 **Phena** obtained under Gram negative cluster corresponds to the 3 Genera under Class A- proteobacteria and 1 Genus under Class

Gammaproteobacteria belonging to Phylum Proteobacteria (Figure 2.6 b). The retrieved bionts distributed as in:

Phenon 1 ≈ Genus *Loktanella* - GUVFHEM-2 a non motile rod, oxidase and catalase positive, methyl red negative and did not reduce nitrate to nitrite

Phenon 2 ≈ Genera *Enterobacter* - GUVFPM-5 and GUVFPM-7 are motile rods, lactose non fermentors, oxidase negative and catalase positive

Phenon 3 ≈ Genera *Chromohalobacter* - GUVFPM-1, GUVFSM-1, GUVFCCM-10, GUVFHM-2 and GUVFFM-3 are motile rods, oxidase and catalase positive, indole positive

Phenon 4 ≈ Genera *Pseudomonas* - GUVFCCM-1, GUVFHEM-4, GUVFCM-3 and GUVFSM-3are non motile rods, oxidase and catalase positive, did not produce acid from glucose

These bionts of the nine Genera showed varied distribution in sponges. The sponge *P. testudinaria* showed the presence of 62.5% of euryhaline and 37.5% of marine halophilic bionts. As detailed in Table 2.1, the euryhaline bacteria retrieved from *P.testudinaria* were identified as *Chromohalobacter* sp., *Bacillus* sp. and *Pontibacillus* sp. while the marine halophiles were identified as *Corynebacterium* sp. and *Enterobacter* sp. 70% of isolates associated with sponge *C.cavernosa* were euryhaline bacteria, identified as *Pseudomonas* sp., *Chromohalobacter* sp., *Bacillus* sp., *Corynebacterium* sp., *Pontibacillus* sp. and *Enterococcus* sp. while the remaining 30% were marine halophiles belonging to the Genus *Bacillus* sp. All the isolates associated with sponge *Haliclona* sp. were euryhaline bacteria identified as *Bacillus* sp. and *Chromohalobacter* sp. One of the isolates retrieved from the unidentified sponge MAM 5 was an euryhaline bacterium identified as

Bacillus sp. while the other, was a marine halophile identified as *Corynebacterium* sp.

All the isolates retrieved from sponge *C. fibrosa* were euryhaline bacteria belonging to the Genus *Bacillus* sp. and *Corynebacterium* sp. 85.7% of the isolates associated with sponge *H. erecta* were euryhaline bacteria identified as *Corynebacterium* sp., *Loktanella* sp. , *Pseudomonas* sp., *Bacillus* sp. and *Planococcus* sp. while the only marine halophile belonged to the Genus *Bacillus*. Isolates GUVFCM-2 and GUVFCM-3 from *C. reticutis var solomonensis* and isolates GUVFSM-2 and GUVFSM-3 from Unidentified sponge NIO 3 were euryhaline bacteria belonging to the Genus *Bacillus* sp. and *Pseudomonas* sp. Isolate GUVFFM-3 from sponge *F.cavernosa* belonged to the Genus *Chromohalobacter* sp and was a euryhaline bacterium. Four isolates namely GUVFCM, GUVFFM-1, GUVFFM-2 and GUVFCCM-2 (Table 2.1) could not be identified by this scheme.

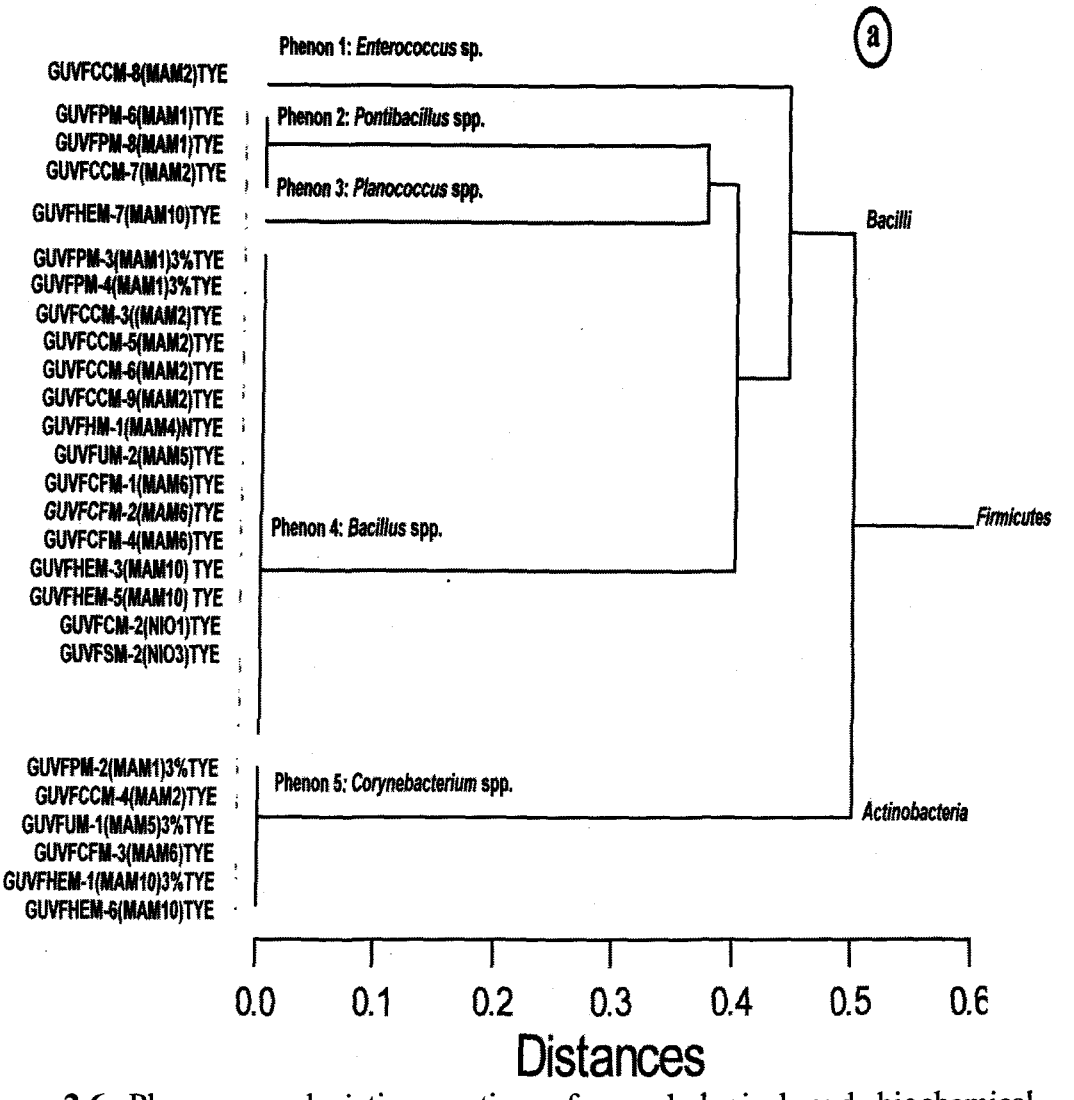


Figure 2.6 Phenogram depicting sorting of morphological and biochemical characteristics of eubacterial bionts retrieved from sponges of Mandapam-India using keys of Bergey's Systematic Bacteriology and **SYSTAT v.12.01.** (a) Gram positive bacteria ≈ **Cluster A** (Phylum: Firmicutes) with **Subcluster IA** (Class: Bacilli) with 4A **Phena** (Genera: *Enterococcus; Pontibacillus; Planococcus* and *Bacillus*) and **Subcluster IIA** (Class: Actinobacteria) with 1A **Phenon** (Genera: *Corynebacterium*).

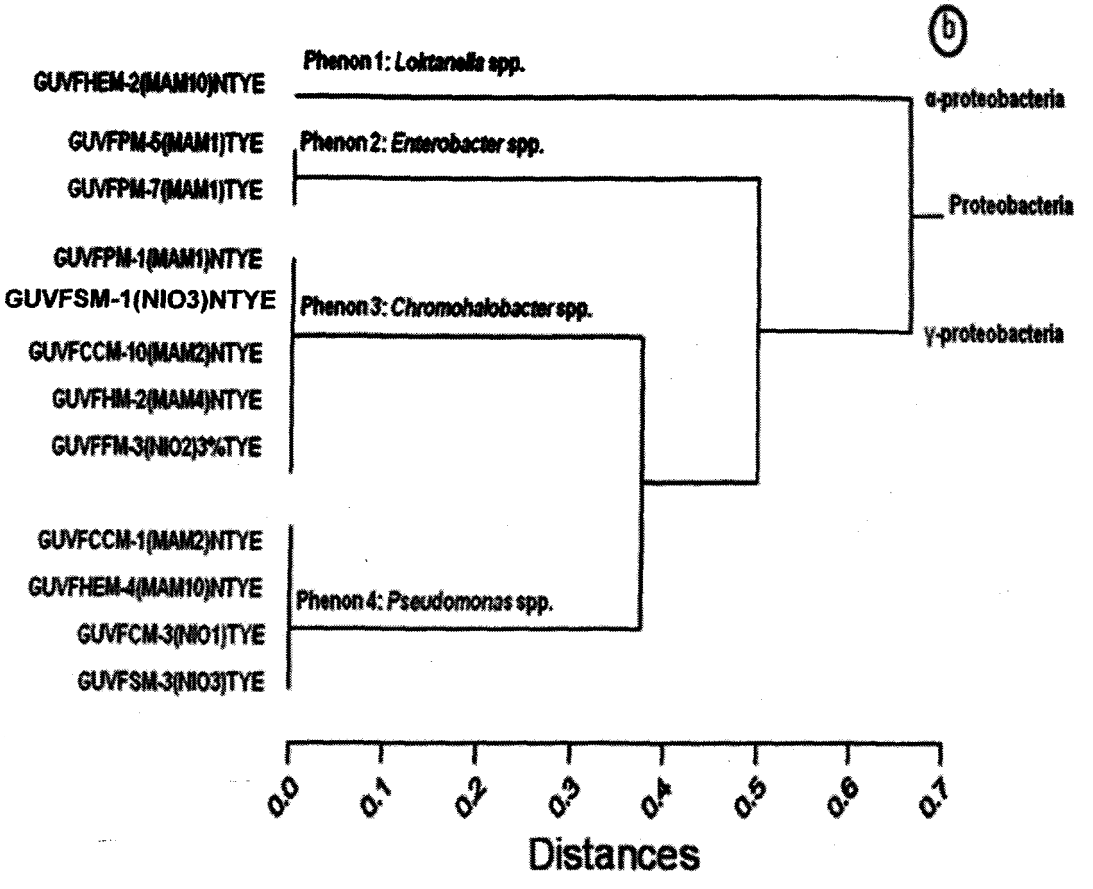


Figure 2.6 Phenogram depicting sorting of morphological and biochemical characteristics of eubacterial bionts retrieved from sponges of Mandapam-India using keys of Bergey's Systematic Bacteriology and **SYSTAT v.12.01.**

(b) Gram negative bacteria ≈ **Cluster B** (Phylum: Proteobacteria) with **Subcluster IB** (Class: A- proteobacteria) with 1B **Phenon** (Genera: *Loktanella*) and **Subcluster IIB**: (Class: Gammaproteobacteria) with 3B **Phena** (Genera: *Enterobacter; Chromohalobacter* and *Pseudomonas*).

2.2.3 A. Characterization and Identification of isolates GUVFCM, GUVFFM-1, GUVFFM-2 and GUVFCCM-2

Isolate GUVFCM from the sponge *C. reticutis var solomonensis,* GUVFFM-1 and GUVFFM-2 from the sponge *F.cavernosa* were isolated from NTYE medium as orange colored colonies. The single isolate GUVFCCM-2 from *C. cavernosa* was isolated as a cream coloured colony. The identity of all these isolates could not be discerned as their Gram character could not be distinguished, and hence the identity using Bergeys keys of Systematic Bacteriology to determine generic affiliation could not be determined. However, using the modified Gram staining technique the Gram character was determined, and all the isolates were recorded as Gram negative rods. Scanning electron microscopy revealed the cellular morphology to be rods, having dimensions in the range 0.6-1.3 μm long and 2-3 μm wide (Figure 2.7). All the isolates were susceptible to an osmotic shock when exposed to water. All the isolates could grow at salinities ranging from 0-35% NaCl in NTYE media pH 7. The growth profile of the cultures in TYE, NTYE, TYE and NTYE supplemented with 700U penicillin media are depicted in Figure 2.8 a.

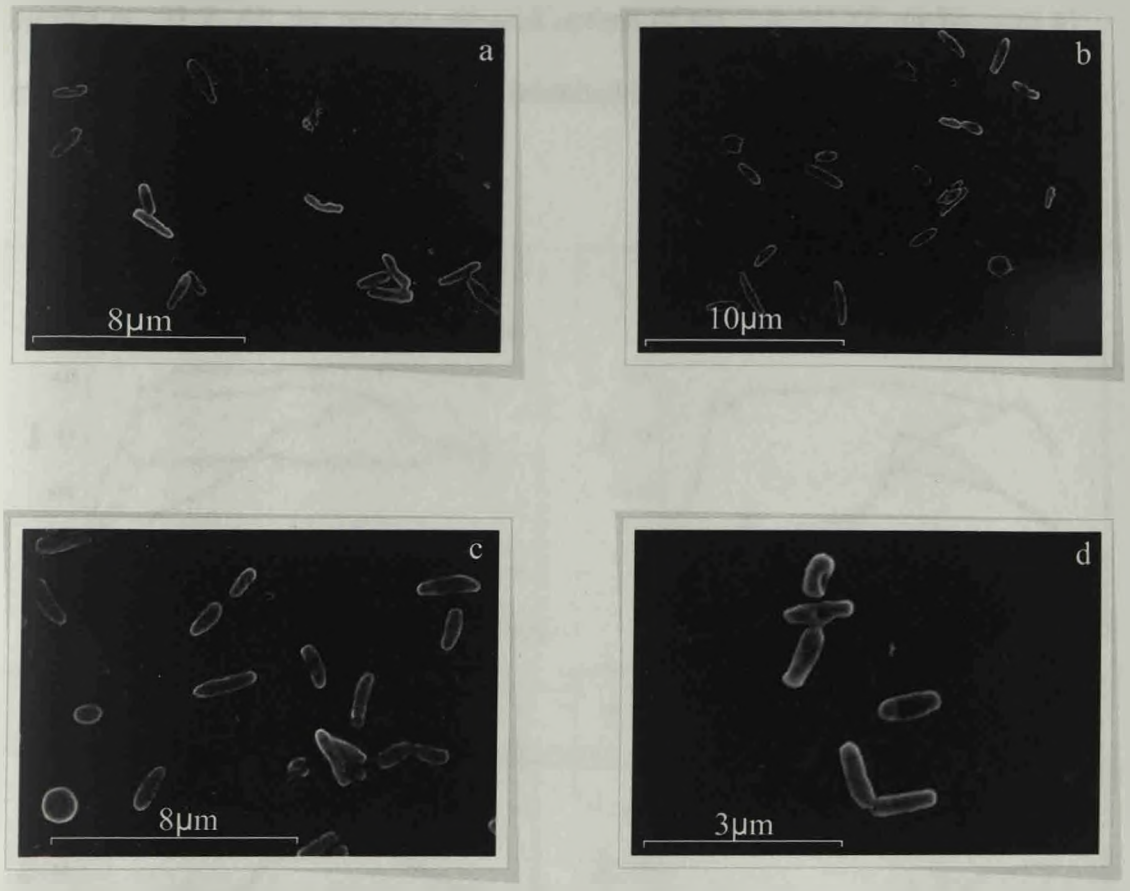


Figure 2.7 Scanning electron micrographs of isolates GUVFCM (a), GUVFFM-1 (b), GUVFFM-2 (c) and GUVFCCM-2 (d)

The cultures could withstand a wide range of pH 5-10.5 and could also grow at magnesium concentrations of 0.07mM-81mM in TYE media supplemented with 25% NaCl. The growth profiles of cultures GUVFCM, GUVFFM-1, GUVFFM-2 and GUVFCCM-2 at varying pH and high magnesium concentration (Figure 2.9 b) while the growth curves of GUVFCM, GUVFFM-1, GUVFFM-2 and GUVFCCM-2 at varying pH and low magnesium concentration (Figure 2.8 c). Culture GUVFCM, GUVFFM-2 and GUVFCM showed optimum growth in pH 6 in TYE media with 0.07mM of magnesium while culture GUVFFM-1 showed at

optima at pH 7. All the isolates showed optima of pH 7 in NTYE media with 81 mM of magnesium. The isolates were inhibited by sodium taurocholate salts.

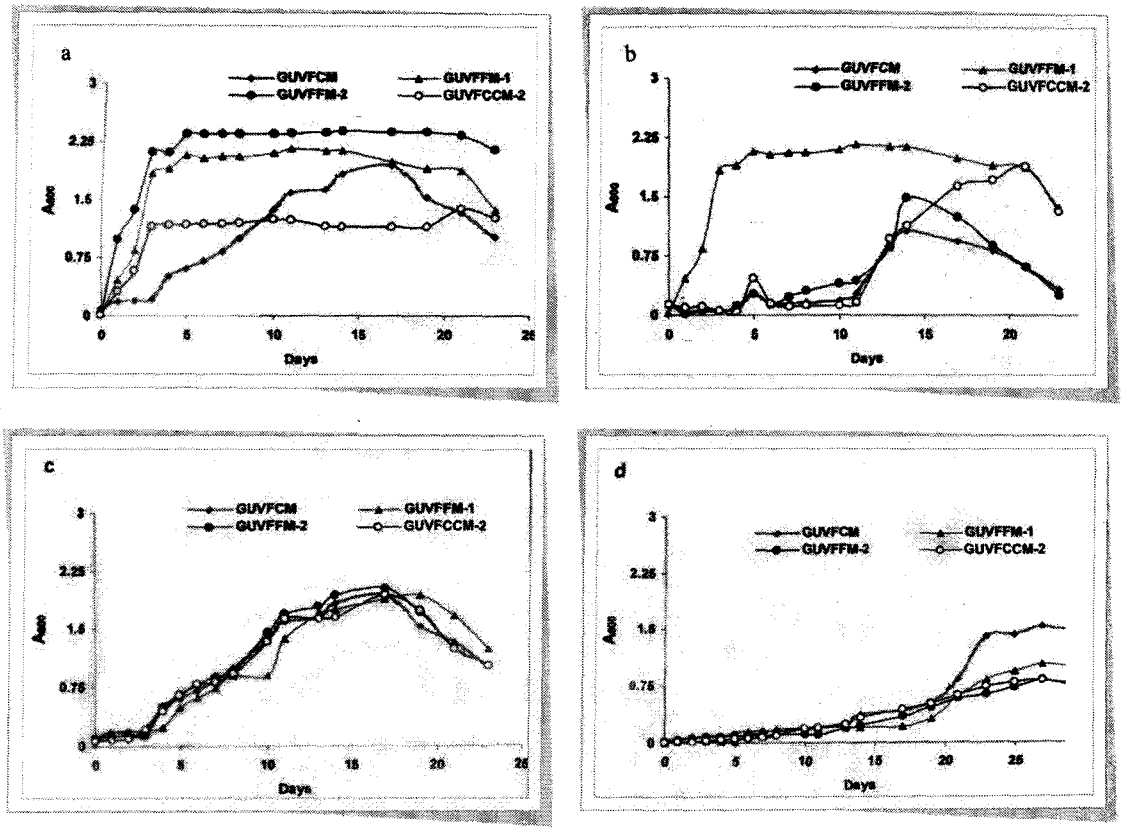


Figure 2.8 a Growth curves of isolates following up to 30 days of incubation at R.T. a) TYE medium; b) TYE medium supplemented with 700 U of penicillin; c)NTYE medium; d)NTYE medium supplemented with 700 U of penicillin

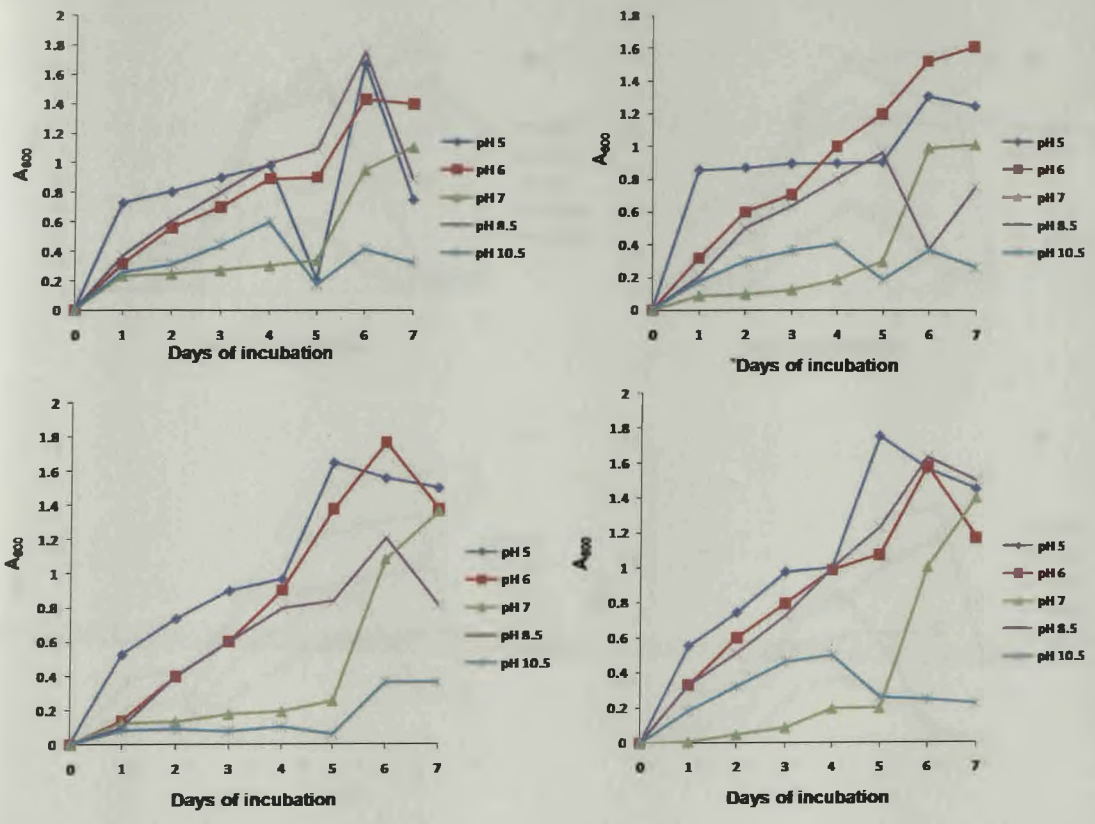


Figure 2.8 b Growth curves of isolates GUVFCM (a), GUVFFM-1 (b), GUVFFM-2 (c) and GUVFCCM-2 (d) followed up to 7 days in NTYE medium with 0.07 mM Mg^{+2} at R.T.

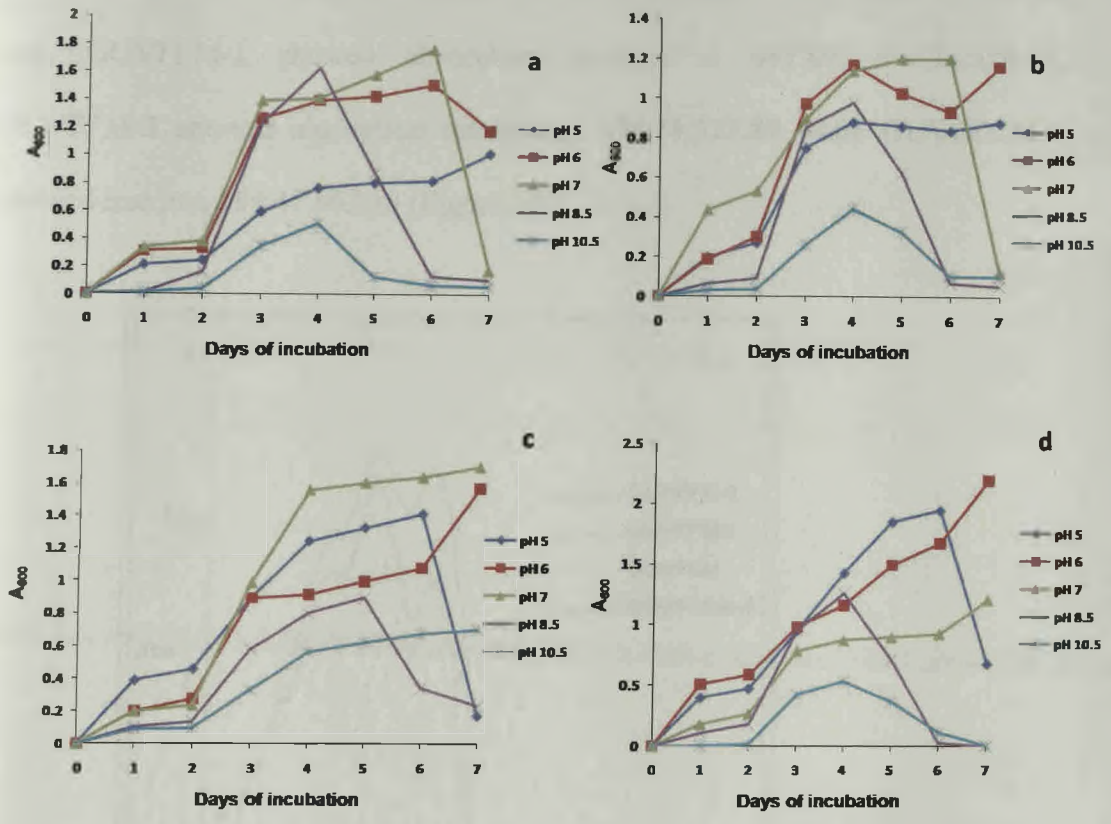


Figure 2.8 c Growth curves of isolates GUVFCM (a), GUVFFM-1 (b), GUVFFM-2 (c) and GUVFCCM-2 (d) followed up to 7 days in NTYE medium with 81 mM Mg^{+2} at R.T.

Pigment from the isolate GUVFCM extracted with acetone (Kushwaha 1974) showed absorption maxima at 467.89, 491.58, 126.32 nm, GUVFFM-1 showed absorption maxima at 467.89, 494.74,529.47, GUVFFM-2 showed absorption maxima at 494.74,527.89 while GUVFCCM-2 showed maxima 589.47,662.10 (Figure 2.9).

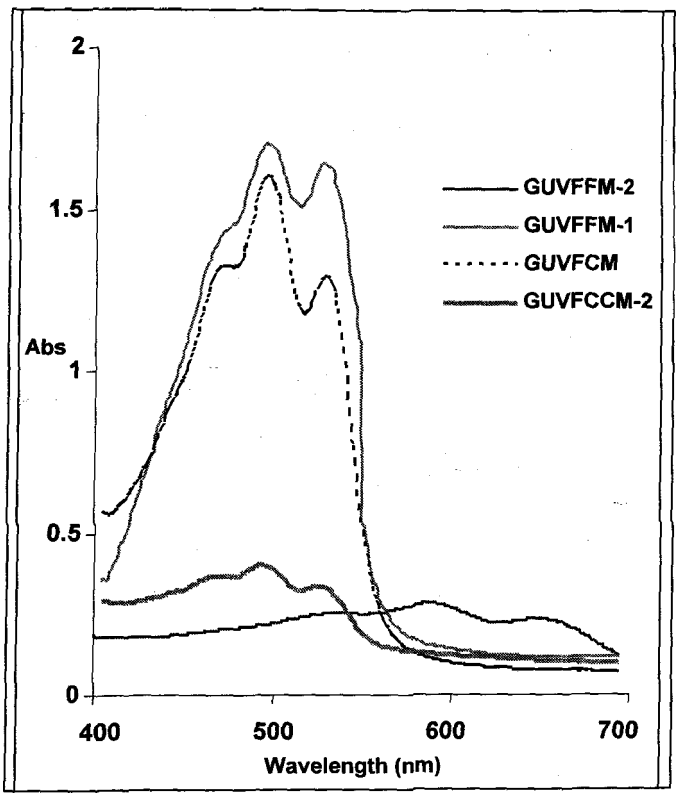


Figure 2.9 Pigment profiles of isolates GUVFCM, GUVFFM-1, GUVFFM-2, and GUVFCCM-2. Pigments were extracted by sonicating cells in acetone and recorded for absorbance.

Analysis of DAP acid on the other hand, revealed its absence with no spot at a Rf corresponding to that of standard DAP at 0.27.

Analysis of the core diether lipids showed that the strains possessed glycerol diether moieties with Rf of 0.22 (Figure 2.10). The hexane extract cultures, separated by thin layer chromatography, resolved into glycerol dietherlipid moieties (GDEM) of *Rf* 0.2, whichis not seen in the eubacterial cultures such as *Escherichia coli.* The FT-IR spectra of the cultures also revealed the presence of characteristic diether moieties with peaks in the range of 1000-1750 cms^{-1} (Figure 2.11 a,b,c,d).

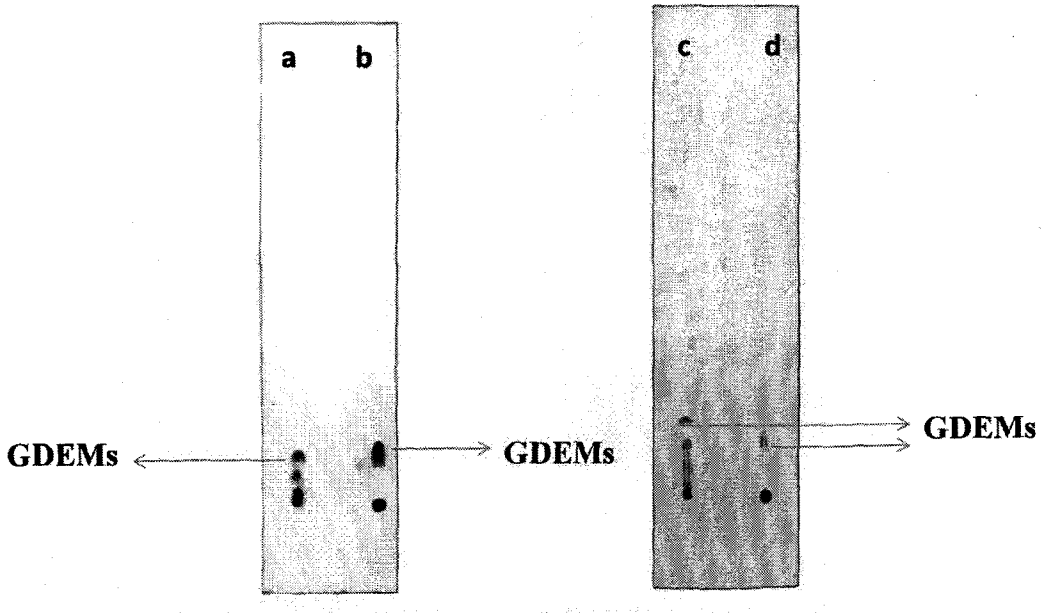


Figure 2.10 Presence of glycerol diether moieties in cell walls of GUVFCM (a), GUVFFM-1 (b), GUVFFM-2 (c) and GUVFCCM-2 (d).

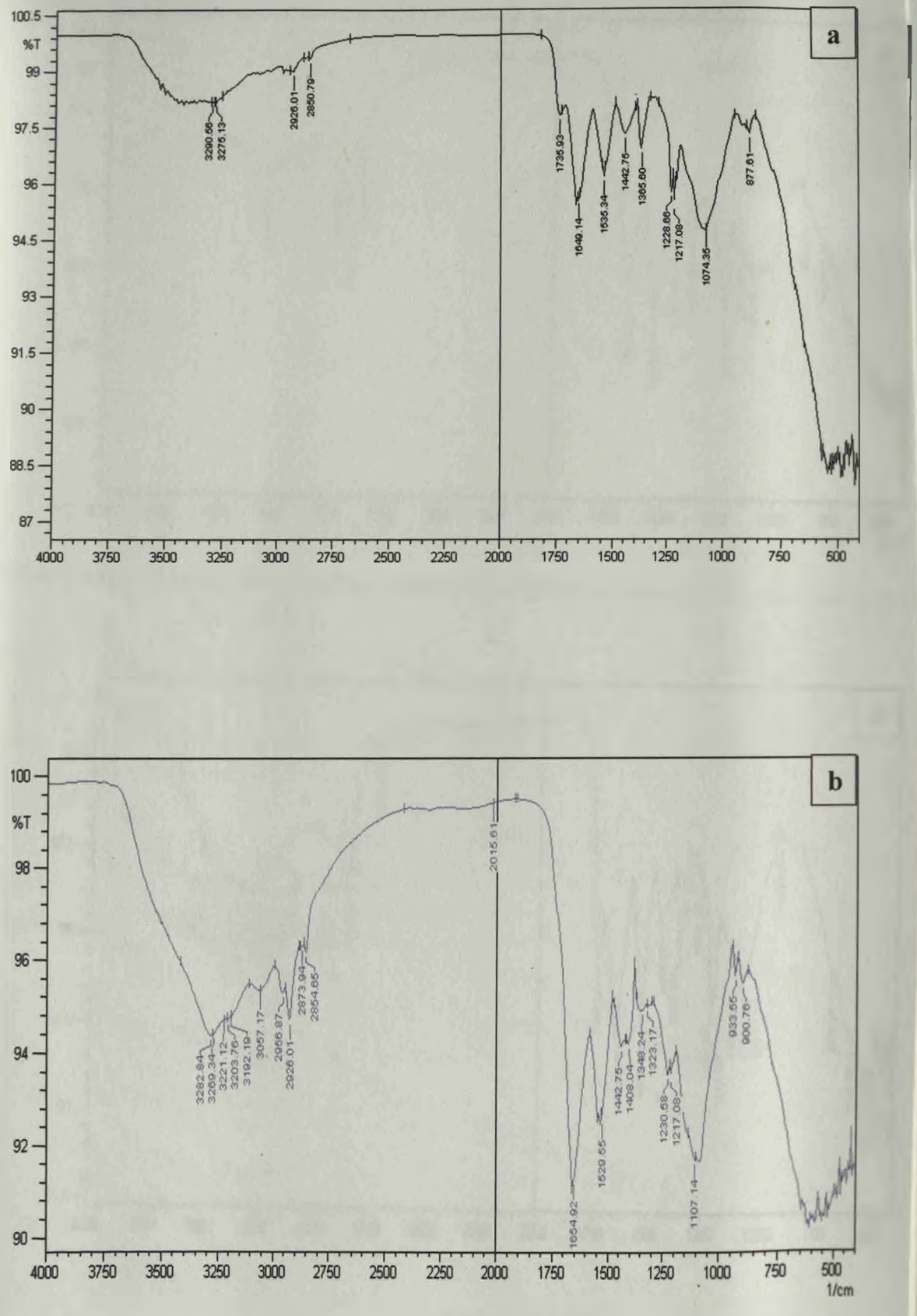


Figure 2.11a FT-IR spectra of whole cells of (a) GUVFCM, (b) GUVFFM-1

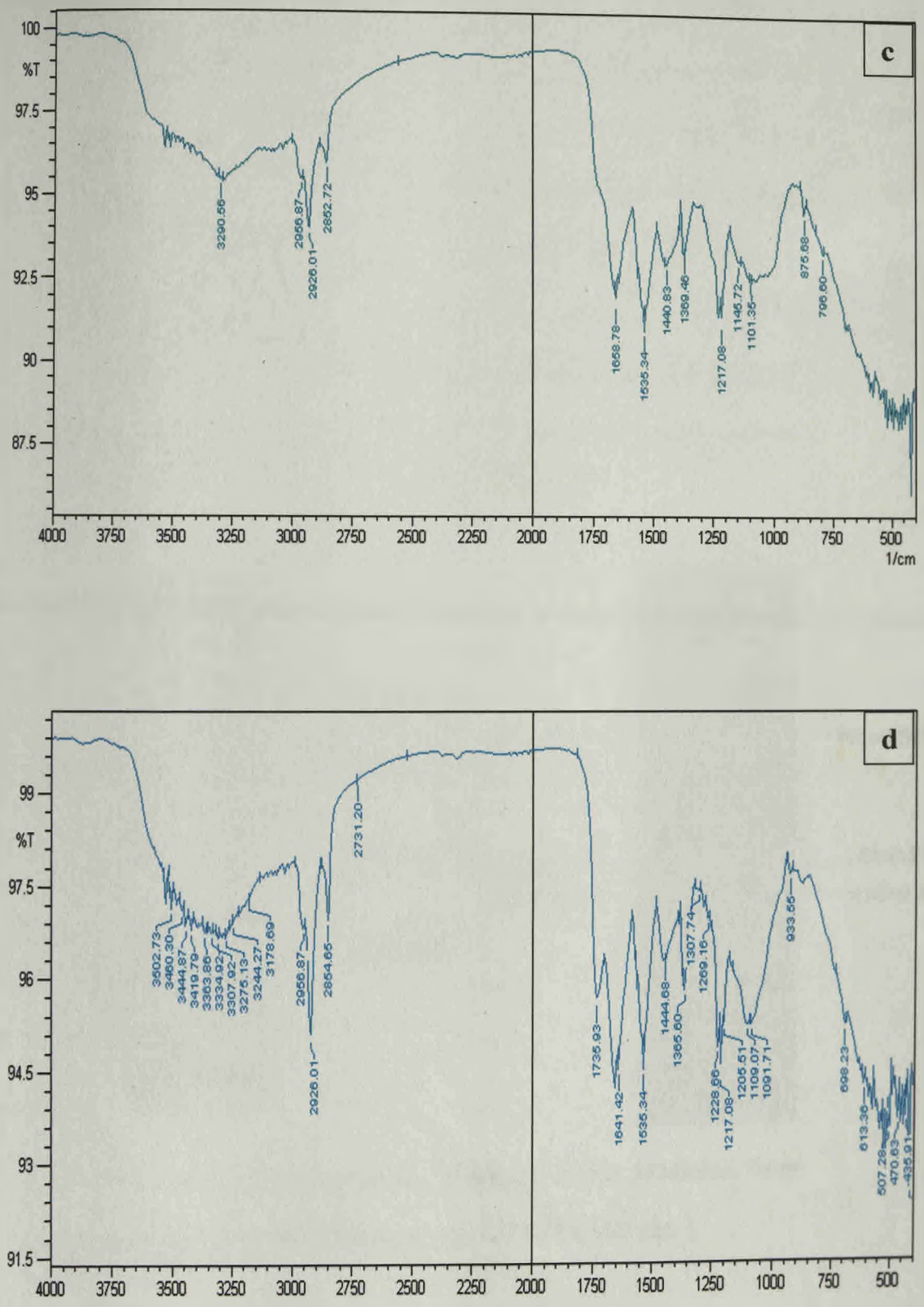


Figure 2.11b FT-IR spectra of whole cells of (c) GUVFFM-2 and (d) GUVFCCM -2.

The presence of phospholipids in culture GUVFCM was revealed by spots at Rf of 0.269, 0.476, 0.809, 0.873. The culture GUVFFM-1 showed lipids spots at Rf of 0.1587, 0.317, 0.523, 0.7923 and 0.873. Culture GUVFFM-2 on the other hand, showed spots with Rf of 0.45, 0.59 and 0.983. Phospholipids of culture GUVFCCM-2 showed spots with Rfs of 0.45, 0.51 and 0.983 (Figure 2.12 a). Analysis of glycolipids revealed a single spot at Rf 0.5 for the culture GUVFCM while culture GUVFFM-1 and GUVFFM-2 showed characteristic spots with Rf 0.4607 and 0.428 respectively while culture GUVFCCM-2 showed a single spot at Rf 0.428 (Figure 2.12 b).

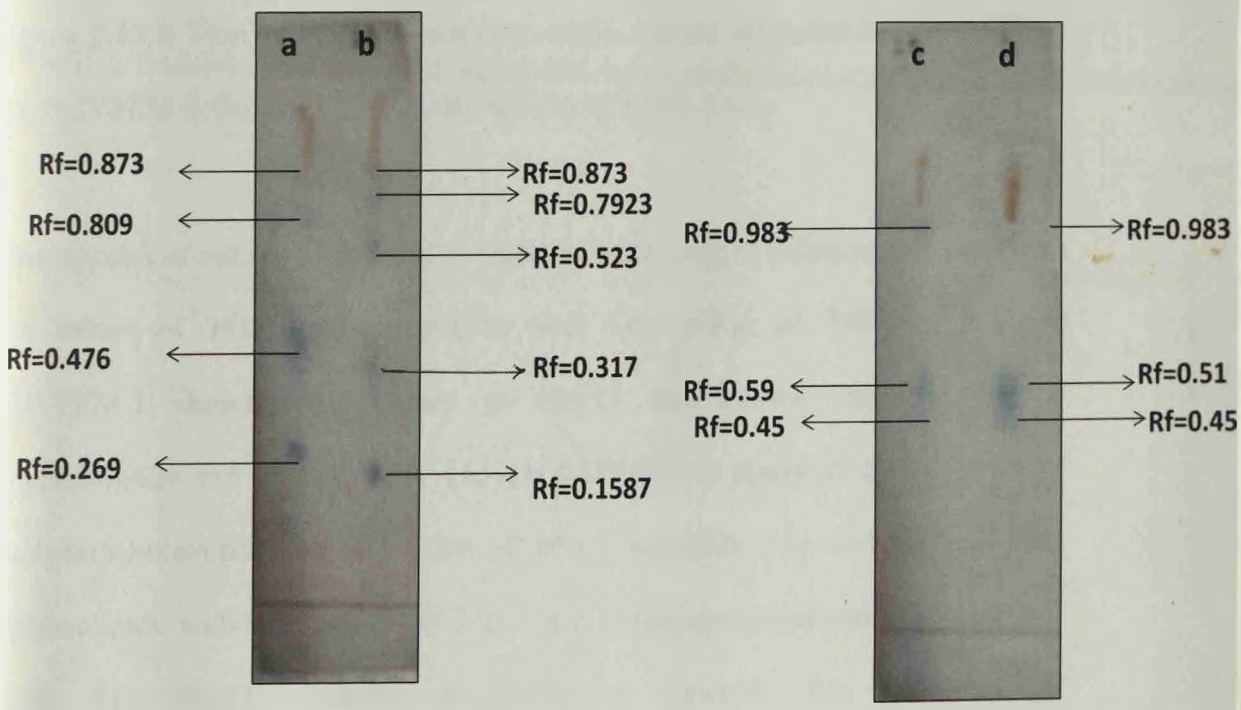


Figure 2.12 a Thin layer chromatograms of a) phospholipids extracted from GUVFCM (a), GUVFFM-1 (b), GUVFFM-2 (c) and GUVFCCM-2 (d).

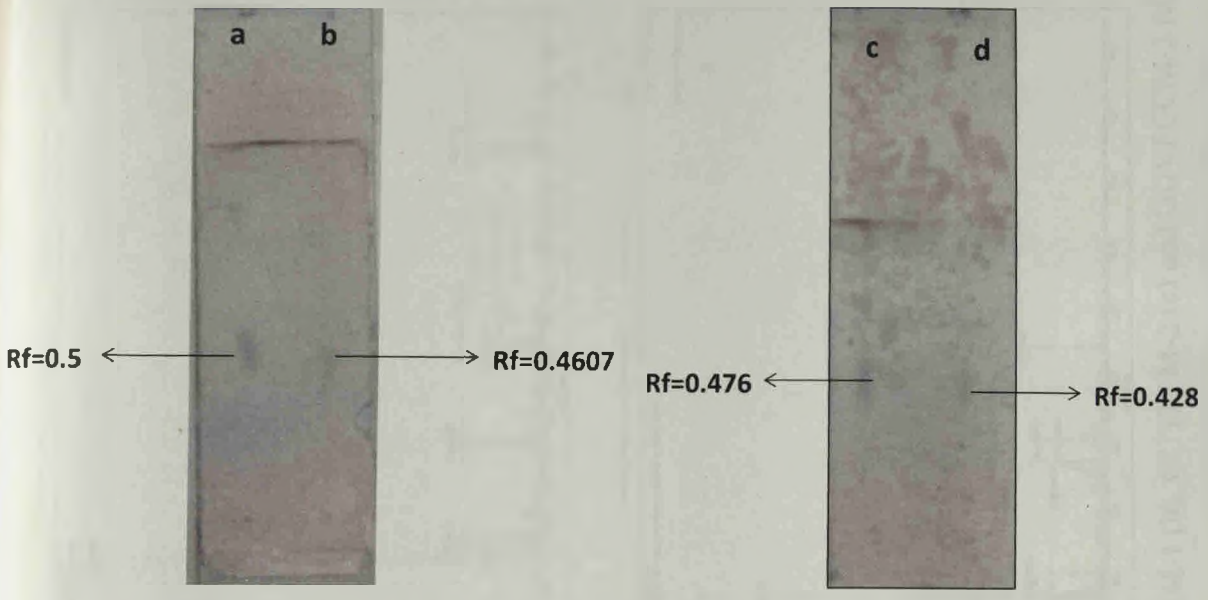


Figure 2.12 b Thin layer chromatograms ofglycolipids extracted from GUVFCM (a), GUVFFM-1 (b), GUVFFM-2 (c) and GUVFCCM-2 (d).

Mass spectra of culture GUVFCM revealed the presence of sulphonoquinones with m/z values of 341.1 and glycolipids with m/z values of 798.1193. Culture GUVFFM-1 showed m/z values at 664.27 and 780.14 corresponding to sulphonolipids and plastocyanin. Culture GUVFFM-2 revealed the presence of methylethanolamine with m/z value of 163, glycolipids with m/z vale of 180, sulphonolipids with m/z values of 258.7 and trianhydrobacterioruberin with m/z value of 666.27. Culture GUVFCCM-2 showed the presence of phosphatidylcholine with m/z value of 189 and sulpholipids with m/z value of 422.99. (Lattanzio *et al.*, 2009) (Figure 2.13 a,b,c,d).

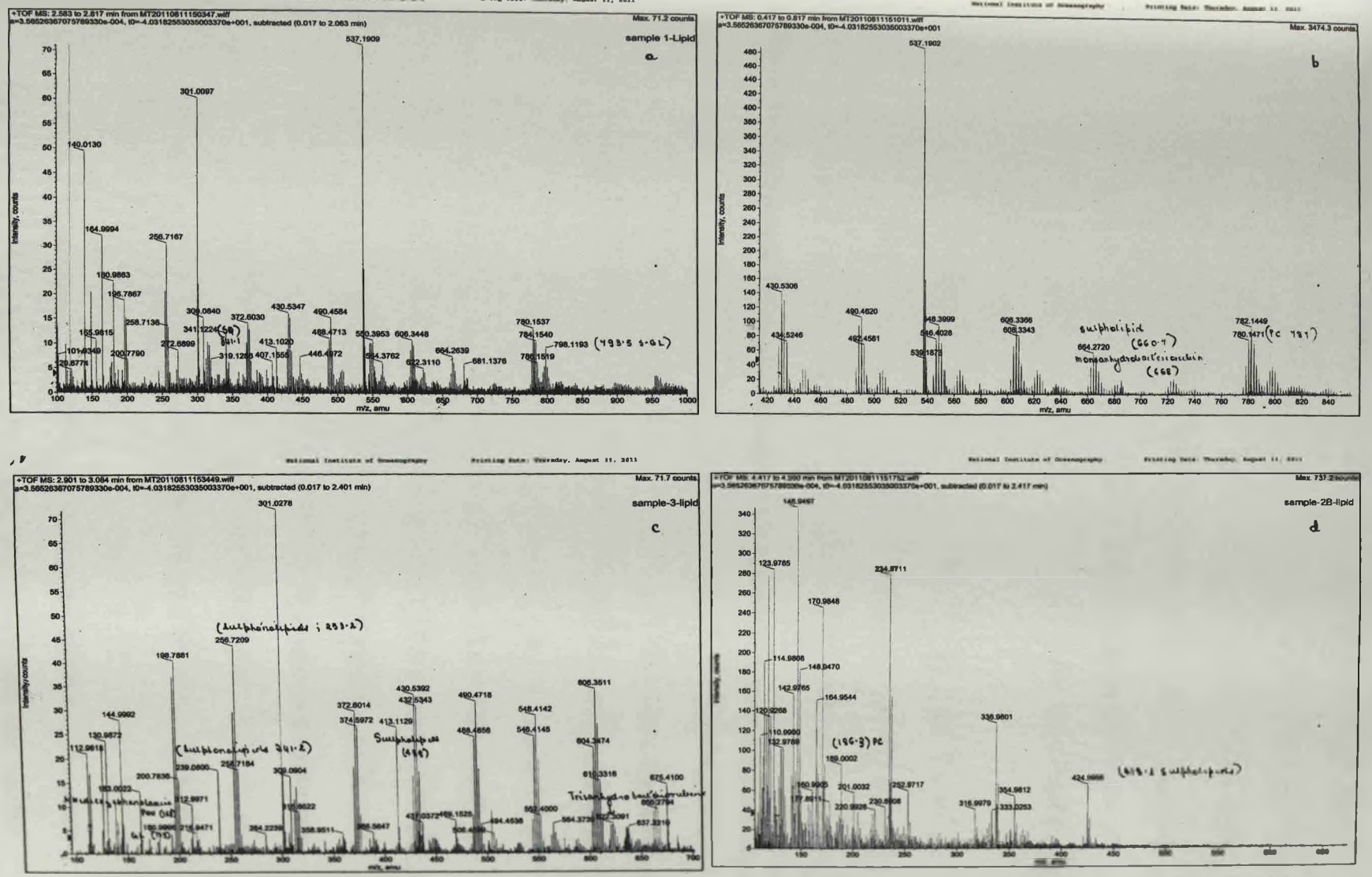


Figure 2.13 Mass spectra of lipids extracted from GUVFCM (a), GUVFFM-1 (b), GUVFFM-2 (c) and GUVFCCM-2 (d).

The cells of isolate GUVFCM, GUVVFM-1 and GUVFFM-2; were cream, pleomorphic rods and had cellular dimensions in the range of 0.7 x 1-2 μm. The isolates showed a bright red pigmentation on prolonged incubation. The NaCl range for growth is 1·5 M to saturation, with an optimum in the range 4 M. They were all Gram-negative motile rods. The optimal temperature for growth is 30-40°C, with a maximum at 50°C. The pH range for growth is pH 5-10.5, with an optimum in the range pH 5-7.

The isolates were all aerobic, catalase and oxidase positive. All these 4 isolates showed a negative methyl red and Vogues Proskauer reaction. All the isolates excluding GUVFFM-2 could not reduce nitrate to nitrite. All the isolates could not use

glucose,lactose,sucrose,sorbitol,mannitol,mannose,fructose,galactose,ribose,arabinose,maltose, pyruvate, lactate and xylose as sole sources of carbon when amended in the mineral medium. All the isolates excluding GUVFFM-2 utilized citrate as the sole source of carbon. Malate was utilised as the sole source of carbon by all the four isolates. GUVFFM-1 was the sole isolate incapable of utilizing formate as the sole source of carbon. Casein, tributyrin and cellulose were not utilized by all the 4 isolates. Based on biochemical characteristics, phenotypic characters, presence of fatty acid methyl esters, diaminopimelic acid, bacterioruberin pigments analysis following Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994),the isolates could be phenotypically affiliated to the genus *Natrialba* (Hezayen *et al.*, 2001).

The single isolate from the sponge *Cinachyra cavernosa* was ascribed to the genus *Halorubrum* as they were rod-shaped cells motile, aerobic, oxidase and catalase positive. Growth occurred in NTYE medium containing 1.5 - 5.2 M NaCl; with an

optimum concentration of 3.5-4.5 M NaCl, The temperature rangefor growth is 30-56°C with optima at 50°C. Enzyme tests were negative for gelatinase, and amylase while positive for desulphurase. The bacterium did not produce indole, could reduce nitrate to nitrite, and showed a positive methyl red and Vogues Proskaueur reaction. It produced acid from sucrose, lactose, maltose, mannose, fructose xylose, ribose, galactose but didn't produce acid from arabinose and glucose (Mc Genity and Grant, 1995).

Growth at 25% NaCl concentration, resistance to antibiotics viz. penicillin, bacitracin, absence of growth on Mac Conkey's media directed us to the conclusion that these isolates were haloarchaea. The presence of Glycerol diether moieties which is absent in the eubacterial cultures such as *Escherichia coli*, presence of absorption maxima

at 500, 470 and corresponding to bacterioruberin types of pigment, absence of Diamino pimelic acid, further confirmed the identification as the key chemotaxonomic markers for ascribing the isolates to the third domain Haloarchaea.

As recorded in Figure 2.14, bionts of genera *Bacillus,* followed by those of genera *Corynebacterium* predominated 88.9% and 55.6% of sponge samples respectively. *F.cavernosa* is the only sponge having *Chromohalobacter* as its lone Genera.

The sponges *Cinachyra cavernosa, Callyspongia reticutis var solomonensis* and *Fasciospongia cavernosa* harboured Haloarchaeal cultures.

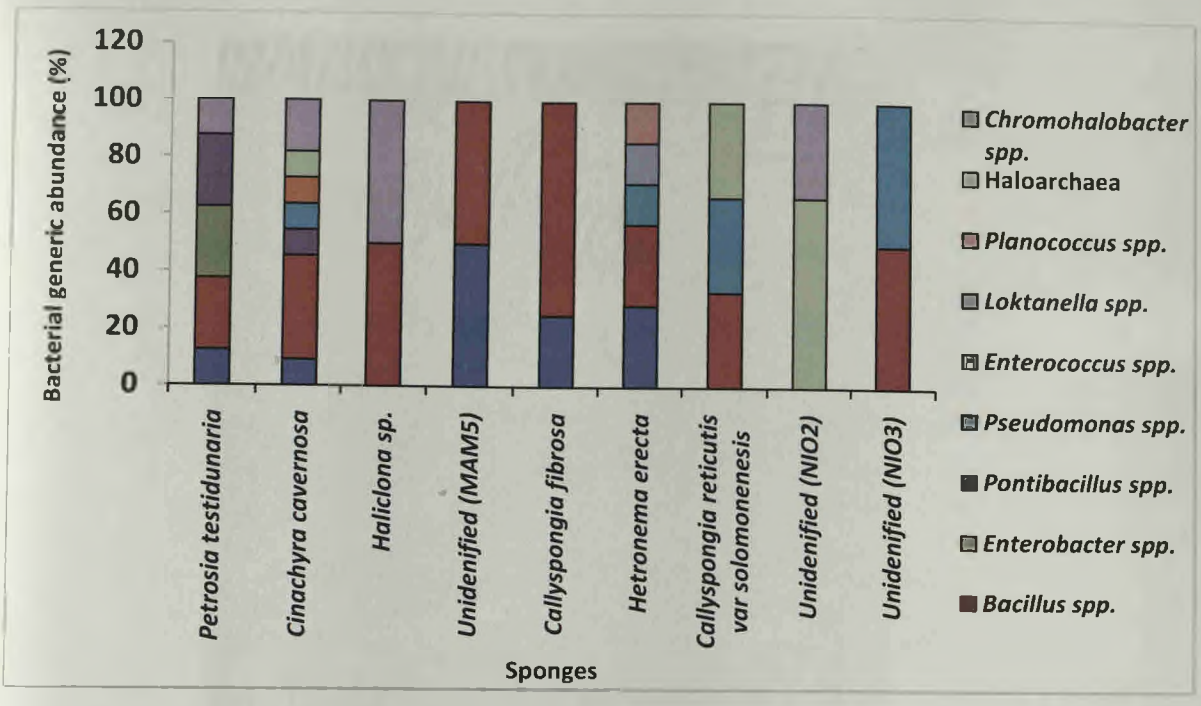


Figure 2.14 Dominant Genera of culturable bacterial bionts from different sponges.

2.2.4 A. Identification of bacterial isolates using 16S rRNA sequencing

The DNA extracted from the bacterial cultures was checked for its purity on a 0.8% (w/v) agarose gel (Figure 2.15). The genomic DNA of individual cultures was seen as discrete bands just below the wells they were loaded (Figure 2.15). The average A_{260}/A_{280} was 1.79, which suggests pure DNA free of contaminants. The average DNA yield from the isolates was 85.5 µg of DNA. PCR amplification of 16S rDNA gene yielded amplicons at 1.5 kb.

Figure 2.15 Agarose gel electrophoresis of genomic DNA from sponge bionts **(Lane 1: GUVFCFM-3; Lane 2: GUVFFM-1; Lane 3: GUVFPM-1; Lane 4: GUVFHEM-1; Lane 5: GUVFSM; Lane 6: GUVFHEM-4; Lane 7: GUVFHM-2; Lane 8: GUVFCCM-4)**

PCR amplification and sequencing of 16S rDNA gene of sponge bionts namely GUVFHM-2, GUVFCCM-4, GUVFHM-2, GUVFCFM-3 (Figure 2.16a) on (BLAST)n search at the NCBI site and subsequent alignment with ClustalW aligned the their sequences with 99% identity to *Chromohalobacter israelensis* strain Ba 1 isolated from the Dead Sea by Rafaeli-Eshkol (1968) with an accession number NR 025431.1. Blast analysis of sponge biont GUVFPM-1 revealed that this strain is a close relative with 99% similarity to *Chromohalobacter canadensis* strain ATCC 43984 NR_025430.1 isolated from the Dead Sea. Sponge

biont GUVFHEM-4 showed 95% similarity to *Chromohalobacter sarecensis* strain LV4 16S isolated from the saline Andean region of Bolivia (accession number (NR_025430.1) and 89% similarity to *Chromohalobacter nigrandesensis* strain Taxon 4 (NR_042011.1) from Lake Tebenquiche on the Atacama Saltern, Chile respectively. Amplification and sequencing of 16S rDNA gene of sponge biont GUVFHEM-1 yielded an amplicon which on (BLAST)n search at the NCBI site and subsequent alignment with ClustalW aligned the sequence with 93% identity to *Burkholderia* sp. strain R-16017 (NR_042636.1). Sponge biont GUVFSM showed similarity to *Psychrobacter okhotskensis* strain MD17 (NR_024806.1) isolated from the Monbetsu coast of the Okhotsk Sea; in Hokkaido, Japan by 91%. The sequences of all the isolates are tabulated in Table 2.2 and are deposited in the Genebank under the accession codes indicated in Table 2.2

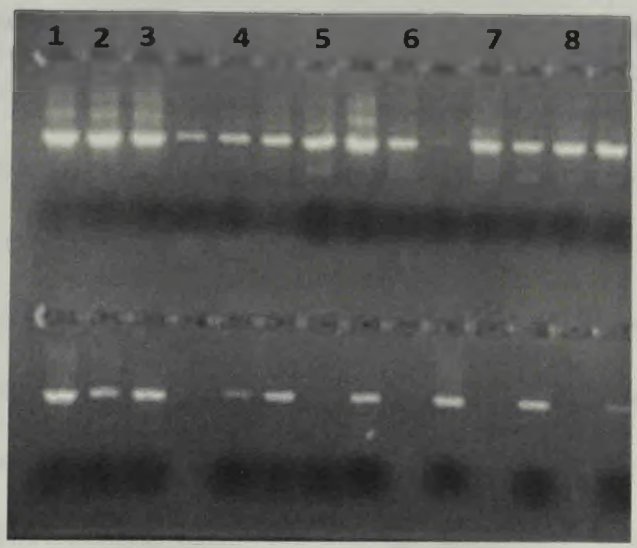


Figure 2.16 a Agarose gel electrophoresis of PCR products of 16S rDNA gene obtained using bacterial primers and DNA of individual sponge bacterial bionts **(Lane 1: GUVFCFM-3; Lane 2: GUVFFM-1; Lane 3: GUVFPM-1; Lane 4: GUVFHEM-1; Lane 5: GUVFSM; Lane 6: GUVFHEM-4; Lane 7: GUVFHM-2; Lane 8: GUVFCCM-4)**

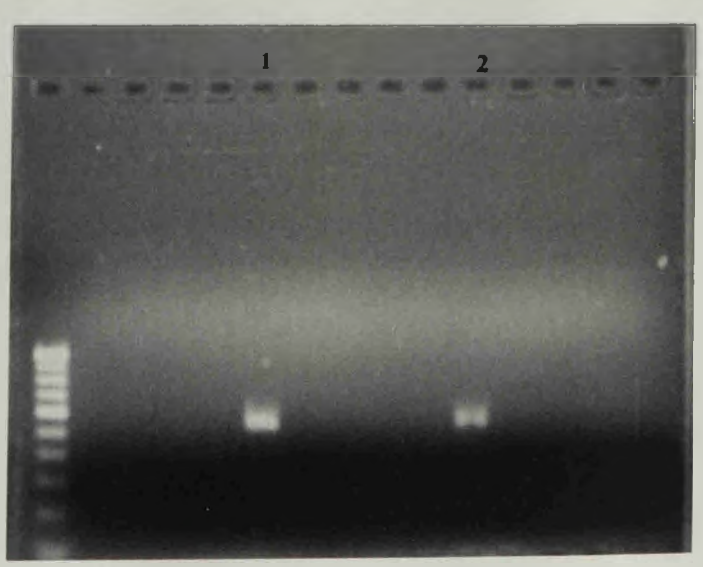


Figure 2.16 b Agarose gel electrophoresis of PCR products of 16S rDNA gene obtained using archaeal primers and DNA of bacterial biont GUVFCVVM-2 **(Lane 1 and 2: GUVFCCM-2).**

Phylogram indicating the placement and relatedness of the isolates is indicated in Figure 2.17 a.

PCR amplification of 16S rDNA gene (Figure 2.16 b) sequencing followed by NCBI-BLAST search of the isolate GUVFCCM-2 led to its identification as *Halorubrum* sp. (accession number yet to be received) as it showed 92% similarity to *Halorubrum* sp. CH3 16S ribosomal RNA gene, partial sequence (GenBank accession number FJ686129.1).The phylogram Figure 2.18 b indicated the placement and Phylogenetic relatedness of the bacterium *Halorubrum* sp. The sequence of the isolate is tabulated in Table 2.2.

Table 2.2 16S rRNA sequences of bacterial bionts associated with sponges

CULTURES	SEQUENCE	IDENTITY	ACCESSION NUMBER
GUVFCFM-3	5'ACGGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGC TTGCTGGACGCTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCC AGTCGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACG GGAGAAAGCGGGGGCTCTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGA TTAGCTGGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCT GAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG AGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGC CGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAGTGGGGAAGAAG GCTTGTCGGCCAATACCCGGCAAGAGCGACATCACCCACAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGG AATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGAAAGC CCCGGGCTCAACCTGGGAACGGCATCCGGAACGGGCAGGCTAGAGTGCAGGA GAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGA ATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAA GCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG TCGACTAGCCGTTGGGTCCCTTGAGGACTTAGTGGCGCAGTTAACGCGATAAG TCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTTCAAATGAATTGA 3'	*Chromohalobacter israelensis*	JX839255
GUVFHEM-1	5'GCCTTACACATGCGAGTCGAACGGCAGCATGGGTTGCTTGCACCTGGTGGCG AGCCGTTAGAGGGGGGGGAGTAATACATCGGAACATGTCCTGTATTCCCGTTG ATAACCCGGCGAAAGCCGGATTAATTCCGCATACGATCTACGGATGAAAGCGG GGGACCTTCCGGCCTCGCGCTTTCGGGTTGGCCGATGGCTGATTAGCTAGTTGG TGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGAC	*Burkholderia* sp.	JX839259

	CAGCCACACTGGGACTGAGACACGACCCACACTCCTACGGGAGGCAGCAGTG GGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGAGTGTGTGAA GAAGGCCTTCGGGTTGTAAAGAACTTTTGTCCGGAAAGAAATCCTTGGCTCTA ATACAGTCGGGGGATGACGGAACCGGAAGAATAAGCACCGACTAACTACGTG CCAGCAACCGCGGTAATACGTAGGGAGCGAGCGTTAATCGGAATTACTGGGC GTAATGCGTGCGCAGGCGGTTTGCTAAGACCGATGTGAAATCCCCCGGGCTCA ATCTGGGGATCTGCATTGGGTGACTGGCAGGCTAGAGTATGGGTAGAGGGGG GGTAGAATTTCCACGTGCAGCAGTGAAAATGGC 3'		
GUVFFM-1	5'CGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTGGA CGCTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTG GGGGATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAA AGCGGGGGCTCTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCT GGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGG ATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGT GTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAGTGGGGAAGAAGGCTTGTC GGCCAATACCCGGCAAGAGCGACATCACCCACAGAAGAAGCACCGGCTAACT CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT GGGCGTAAAGCGCGCGTAGG 3'	*Chromohalobacter israelensis*	JX839260
GUVFHM-2	5'GCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTGGACG CTGACGACCGTCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGG GATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGC GGGGGCTCTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGT TGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATG ATCAGCCACACTGGGACTGAGACACGGCCCATACTCCTAC 3'	*Chromohalobacter israelensis*	JX839262
	5'CGATAATGGAACGCCTGGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAA ACGATCCTAGCTTGCTAGGAGGCGTCCAGCGGCGGACGGGTGAGTAATGCATA GGAATCTACCCAGTCGTGGGGGATAACCTGAGGAAACTCAGGCTAATACCGCA	*Chromohalobacter*	JX839267

GUVFPM-1	TACGTCCTACGGGAGAAAGCGGGGGCTCTTCGGACCTCGCGCGATTGGATGAG CCTATGTCGGATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATCC GTAGCTGGTCTGAGAGGATGATCAGCCACACCGGGACTGAGACACGGCCCGG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGA TCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAG CGAGGAAGAAAGCGTGCCGGTCAATACCCGGTACGGACGACATCACTCGCAG AAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA AGCGTTAATCGGAATTACTGGGCGTAAGGCGCGCGTAGGCGGCGTGTCACGCC GGGTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACGGGCAGGC TAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAG AGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACG CTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCAC GCCGTAAACGATGTCGACTAGCCGTTGGGTCCCTTTGAG 3’	*canadensis*	
GUVFHEM-4	5’CAGGCCTAACACATGCAAGTCGAGCGGAAACAATCCCAGCTTGCTAGGCGG CGTCGAGCGTTTGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGCT GGATAACCTGAGGAAACTCAGGCTAATCCCGCATACGTCCTACGGGAGAAAG CGGGGGCTCTTCGGACCTCGCG 3’	*Chromohalobacter* *sarecensis*	Not assigned
GUVFSM	5’GACGCTGGCGGCAGGCTTACACATGCAAGTCGAGCGGAAACGATGGGAGCT TGCTCCCAGGCGTCCATCGGGGGACGGGTGAGTAATACTTAGGAATCTACCTA GTAGTGGGGGATAGCTCGGGGAAACTCGAATTAATACCGCATACGTCCTGCGG GAGAAAGGGGGCAGTTTACTGCTCTCTCTATAAGATGAGCCTAAGTCGGATTA GCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAG ATGATGATCAGTCACCCCATGACTGAGAGACGGCCCGGACTCCTACCCCCCGT CGCGGTAGCGAATATTGCACAATGCCAGAAACCCTTCTCCACCCATCCCCCCC GTGTGCCGCACATCTTATGGTT 3’	*Psychrobacter* *okhotskensis*	JX839275
	5’ACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCT TGCTGGACGCTGACCAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCCA GTCGTGGGGGATAACCTGGGGAAACCCAGGCTAATGCCGCATACGTCCTACGG	*Chromohalobacter*	JX839278

GUVFCCM-4	GAGAAAGCGGGGGCTCTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGAT TAGCTGGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTG AGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCC GCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAGTGGGGAAGAAGG CTTGTCGGCCAATACCCGGCAAGAGCGACATCACCCACAGAAGAAGCACCGG CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAG CGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGG GTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACGGGCAGGCTA GAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGTGGGTGAAATGCGTAGA GATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACGCT GAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCCTGGTAGTCCAC GCCGTAAACGATGTCGACTAGCCGTTGGGTCCCCTTGAGGGACTTAGTGGCGC AGTTAACGCGATAAGTCGACCGCCTGGGGGAGTACGGCCGCAAGGTTAAAAC TCA 3’	*israelensis*	
GUVFHM- 2A	5’ACCGGGCAGATGACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTA ACAGGTCCAGCTTGCTGGACGCTGACCAGCGGGGGACGGGTGAGTAATGCAT GGAATCTACCCAGTCGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGC ATACGTCCTACGGGAGAAAGCGGGGGCTCTTCGGACCTCGCGCGATTGGATGA GCCTATGTCGGATTAGCTGGTTGGTGGGGTAACGGCTCACCAAGGCGACGATC CGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCA GACTCCTACGGGAGGCTGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG ATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCA GTGGGGAAGAAGGCTTGCCGGCCAATACCCGGCAAGAGCGACATCACCCACA GAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG 3’	*Chromohalobacter* *israelensis*	JX839279
	5’ATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC GGTAACAGGTCCAGCTTGCTGGACGCTGACGAGCGGCGGACGGGTGAGTAAT GCATAGGAATCTACCCAGTCGTGGGGGATAACCTGGGGAAACCCAGGCTAAT	*Chromohalobacter*	JF330126

GUVFFM-3	ACCGCATACGTCCTACGGGAGAAAGCGGGGGCTCTTCGGACCTCGCGCGATTG GATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTAACGGCTCACCAAGGCGA CGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGG CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAG CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACT TTCAGTGGGGAAGAAGGCTTGTCGGCCAATACCCGGCAAGAGCGACATCACCC ACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CGCGTAGGCGGCTTGTCACGCCGGGTGTGAAAGCCCCGGGCTCAACCTGGGAA CGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCG GTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCGG CCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGG ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGTC CCTTGAGGACTTAGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTA CGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAACCGGTG GAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACCCTTGACAT CCTGCGAACCCGGAAGAGATTCCGGGGTGCCTTCGGGAGCGCAGAGACAGGG CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGTAACG AGCGCAACCCTTGTCCCTATTTGCCAGCGATTCGGTCGGGAACTCTAGGGAGA CTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGG CCCTTACGGGTAGGGCTACACACGTGCTACAATGGCCGGTACAAAGGGTTGCG AAGCCGCGAGGTGAAGCCAATCCCAGAAAGCCGGCCTCAGTCCGGATTGGAG TCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGCATCAGAATG GCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG AGTGGACTGCACCAGAAGTGGTTAGCCTAACTTCGGAGGGCGATCACCACGGT GTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCG GCTGGATCACC	*salexigens*	

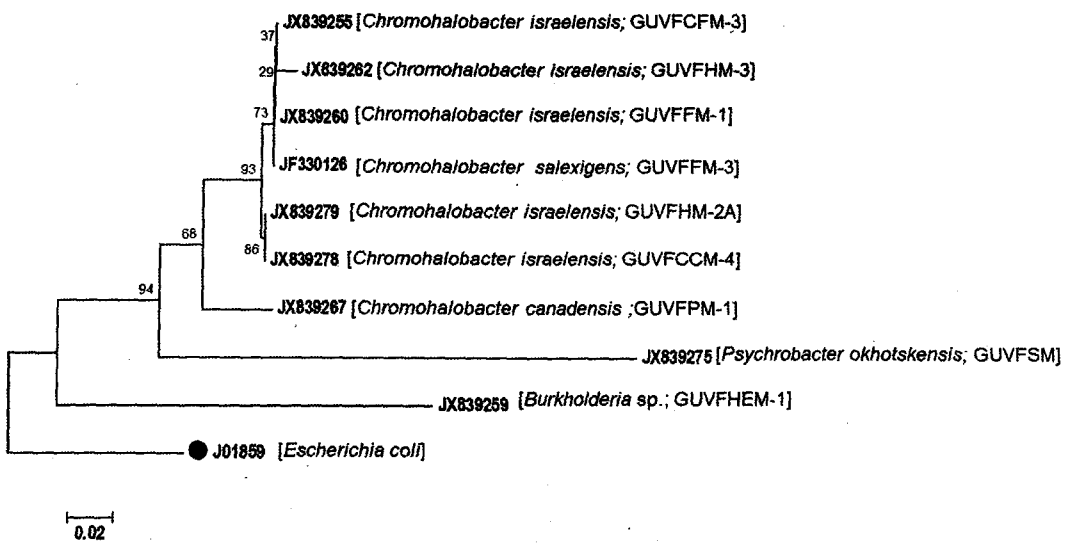


Figure 2.17 b. Bootstrap consensus (50% cut-off) rooted neighbor-Joining tree inferred from 500 replicates is shown to represent the phylogenetic position of sponge isolates. The tree is drawn to scale, the evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis was conducted in MEGA5. The outgroup used is *Escherichia coli* (J01859). Numbers given at branch nodes indicate (%) boothstrap values. Bar, 0.01 substitutions per 100 nucleotide positions

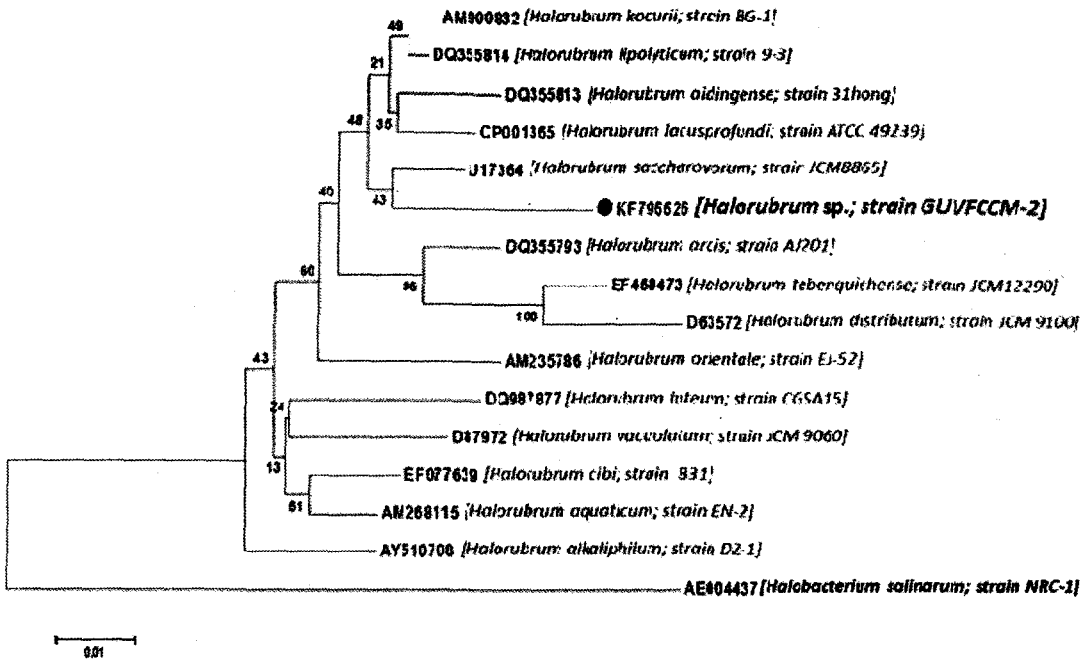


Figure 2.17 b.

Bootstrap consensus (50% cut-off) rooted neighbor-Joining tree inferred from 500 replicates is shown to represent the phylogenetic position of haloarchaeal strain GUVFCCM-2. The tree is drawn to scale, the evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences and was conducted in MEGA5. The outgroup used is Halobacterium salinarium (AE004437). Numbers given at branch nodes indicate (%) boothstrap values. Bar, 0.01 substitutions per 100 nucleotide positions

2.2.5 A. Statistical measures of diversity

PRIMER v.5 showed highest Genera richness (*d*) and diversity (*H'*) in sponge *C.cavernosa* followed by *Heteronema erecta* and *Petrosia testudinaria* (Figure 2.18 a and c). Interestingly the bacterial bionts were evenly distributed in sponges namely *Haliclona* sp., Unidentified sponge (MAM 5), *Callyspongia reticutis var solomonensis, Fasciospongia cavernosa* and Unidentified sponge (NIO 3) as seen in Figure 2.18.

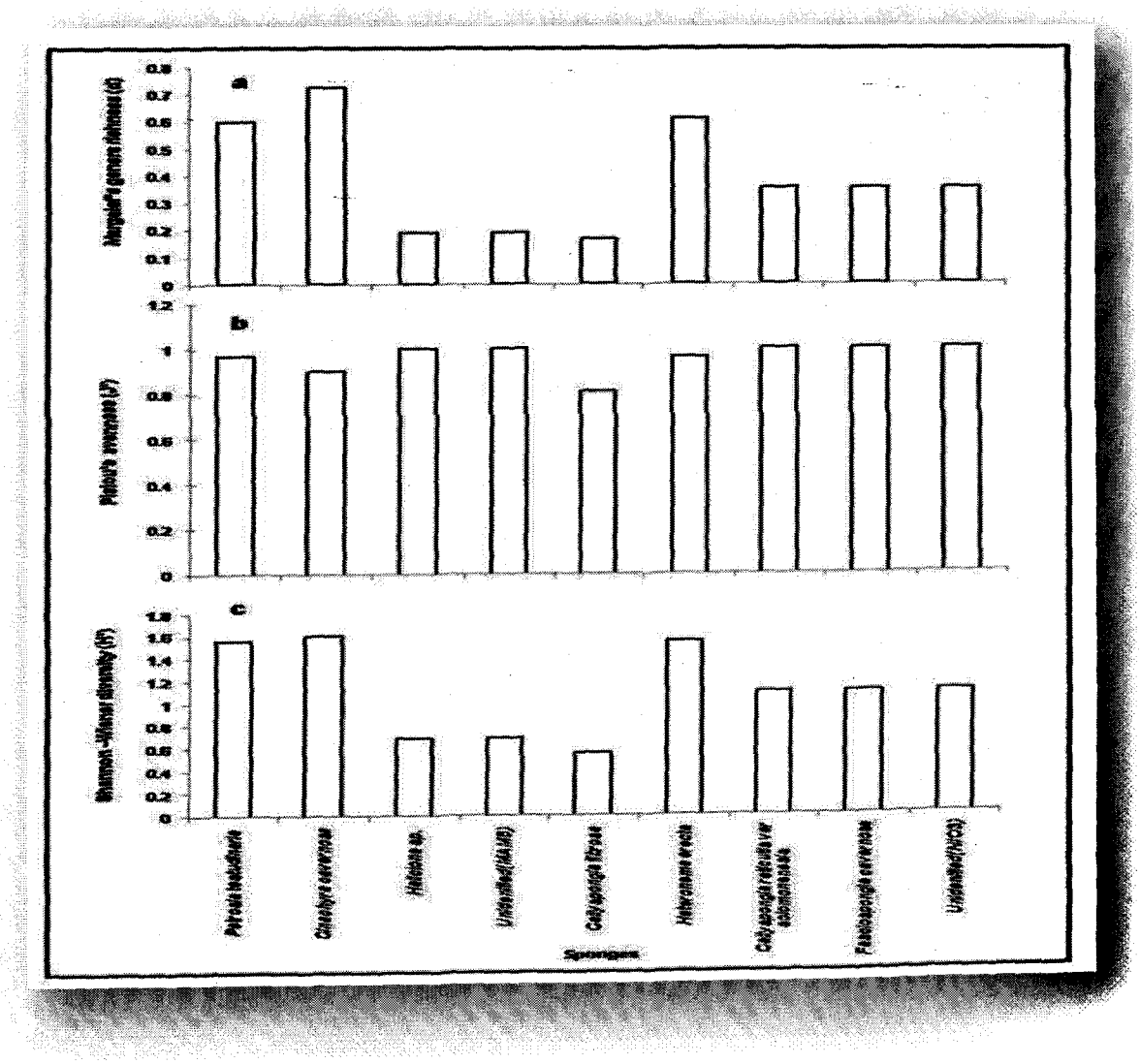


Figure 2.18 (a) Genera richness, (b) Pielou's evenness and (c) Shannon-Wiener diversity of the eubacterial community in the different sponges.

B. Bacteriology of Corals

2.2.1 B. Retrieval and isolation of pure bacterial bionts

Enumeration of bacteria from coral tissue was obtained as bacteria growing in TYE, TYE with 3% NaCl and NTYE. The total viable counts of retrieved non halophilic bacteria growing on TYE was highest in coral MAM 6 at 4.102 x 10^{11} cfu/g, MAM 4 at 1.59 x 10^{11} cfu/g, MAM 9 at 7.32 x 10^{9} cfu/g and a near equal of 3.40 x 10^{9} cfu/g 6.7 x 10^{9} cfu/g in coral MAM 7 and MAM 3 (Figure 2.19). The highest count of marine halophiles growing on 3% TYE was in coral MAM 9 with a count of 1.01 x 10^{11} cfu/g, near equal counts of 8.34 x 10^{9}cfu/g in MAM 3, 5.67 x 10^{9} cfu/g in MAM 6, 1.78 x 10^{8} cfu/g in MAM 4 and a low of 8.57 x 10^{7} cfu/g in coral MAM 7.

Highest count of extreme halophiles was observed by growth on NTYE in coral MAM 6 with a count of 6.48 x10^{11} cfu/g, followed by a near equal count of 8.81 x 10^{10} cfu/g and 1.30x 10^{10} cfu/g in coral MAM 3 and MAM 9. MAM 4 and MAM 7 had near equal counts of 4.87 x10^{9} cfu/g and1.34 x 10^{9} cfu/g in coral MAM 4 and MAM 7.

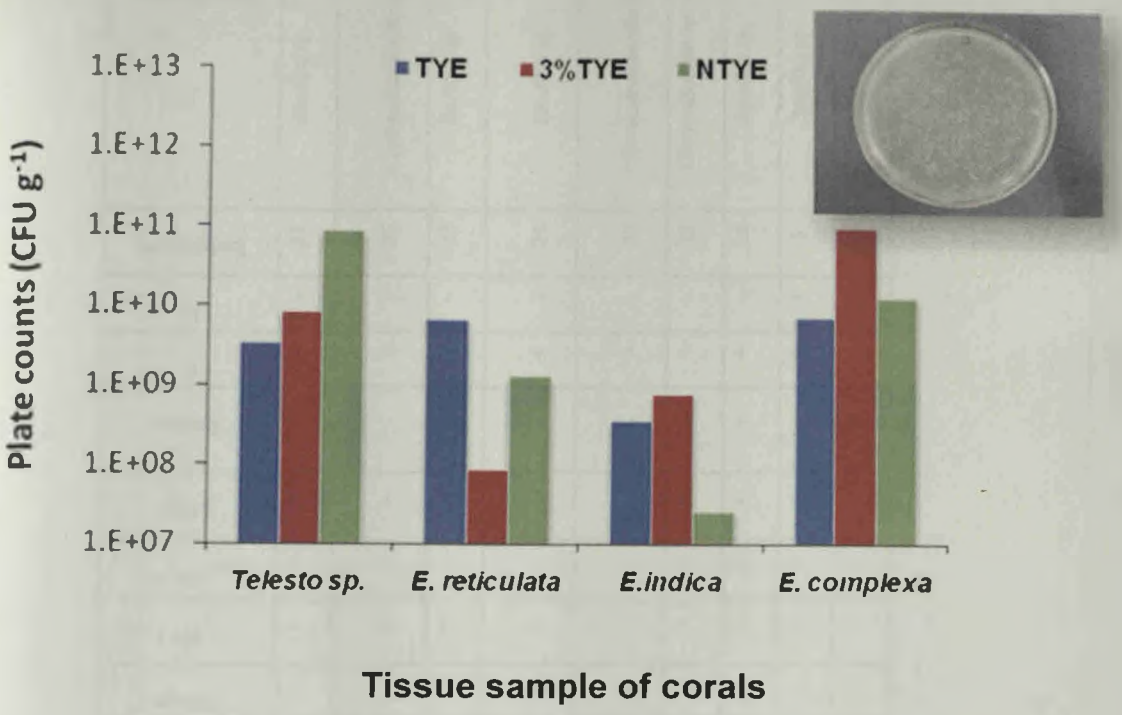


Figure 2.19 Total retrieval bacterial counts in cfu/g on TYE, 3% TYE and NTYE associated with corals.

2.2.2 B. Morphological, Biochemical Characterization and Identification of eubacterial isolates using Bergeys keys of Identification and SYSTAT

Based on morphological and biochemical characteristics (Table 2.3), all the isolates were identified up to generic level using Bergeys keys of Systematic Bacteriology. The Gram positive isolates belonged to the genera namely *Bacillus*, *Pontibacillus*, *Planococcus* and *Corynebacterium* while the Gram negative isolates were assigned to the genera *Chromohalobacter, Pseudomonas, Alteromonas, Alcaligens, Vibrio* and *Psychrobacter.*

Table 2.3 Biochemical potentials and tentative identification of dominant bacterial bionts retrieved and purified from corals of Mandapam- India

Coral	Designation	Morphology	Pigment	GC	motility	oxidase	cat	glu	suc	lac	I	MR	VP	citrate	Nitrate	0%	3%	25%	Spores	P	M	Isolated On	Identification
Telesto sp. MAM 3	GUVFTM-1	SL	orange	+	M	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	25	*Bacillus* sp.
	GUVFTM-2	R	cream	+	M	+	+	+	-	-	+	+	-	-	-	+	+	+	-	+	-	25	*Chromohalobacter* sp.
	GUVFTM-3	SR	cream	+	M	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	25	*Bacillus* sp.
	GUVFTM-4	R	cream	+	M	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	25	*Bacillus* sp.
	GUVFTM-5	SR	orange	-	M	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+	-	25	*Chromohalobacter* sp.
	GUVFTM-6	R	cream	+	M	+	+	+	-	-	+	+	-	-	-	+	+	+	-	+	-	25	*Chromohalobacter* sp.
	GUVFTM-7	R	cream	+	M	+	+	-	-	-	+	-	-	-	+	+	+	+	+	+	-	25	*Bacillus* sp.
	GUVFTM-8	SR	orange yellow	+	M	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	3	*Bacillus* sp.
	GUVFTM-9	R	cream	+	M	+	+	+	-	-	+	+	-	-	-	+	+	+	-	+	-	25	*Chromohalobacter* sp.

	GUVFTM-10	R	brown	+	M	+	+	+	-	-	-	+	-	-	-	+	+	-	+	-	-	3	*Bacillus* sp.
	GUVFTM-11	LR	cream	-	M	+	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+	3	***Vibrio* sp.**
	GUVFTM-12	SR	yellow	+	M	+	+	+	-	-	+	-	-	+	+	+	+	+	+	-	+	0	***Bacillus* sp.**
	GUVFTM-13	R	cottony white	+	M	+	+	-	-	-	+	-	-	-	-	+	+	+	+	+	-	0	***Bacillus* sp.**
	GUVFTM-14	FR	orange	+	NM	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	+	0	***Corynebacterium* sp.**
	GUVFTM-15	R	cream	-	M	+	+	+	-	-	-	+	-	-	+	+	+	+	-	-	+	0	***Aeromonas* sp.**
Echinogorgia reticulata MAM 7	GUVFERM-1	SLR	cream	+	NM	+	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	25	*Corynebacterium*
	GUVFERM-2	SR	cream	-	M	+	+	+	-	-	-	+	-	-	+	+	+	+	-	+	-	25	*Chromohalobacter* sp.
	GUVFERM-3		cream	+																+	-	25	lost
	GUVFERM-4	R	yellow	-	M	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	-	25	*Pseudomonas* sp.
	GUVFERM-5	R	white	+	M	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	0	*Bacillus* sp.

	GUVFERM-6	R	white	+	M	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	0	*Bacillus* sp.
	GUVFERM-7	SLR	orange	-	M	+	+	-	+	+	+	-	-	-	-	+	+	+	-	-	+	0	*Alcaligens* sp.
	GUVFERM-8	R	orange	-	M	+	+	-	+	+	-	+	-	-	-	+	+	+	-	+	+	0	*Alteromonas* sp.
	GUVFERM-9	SR	cream	+	M	+	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	0	*Bacillus* sp.
	GUVFERM-10	R	yellow	-	M	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	-	25	*Pseudomonas* sp.
	GUVFERM-11	R	cream	+	M	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	0	*Bacillus* sp.
	GUVFERM-12	SR	cream	-	M	+	+	+	-	-	-	+	+	-	-	+	+	-	-	-	+	0	*Vibrio* sp.
	GUVFERM-13	Rod	cream	+	M	+	+	-	-	-	+	-	-	-	+	+	+	-	+	-	+	0	*Bacillus* sp.
Echinomuricea indica MAM 8	GUVFEIM-1	SLR	cream	+	M	+	+	+	+	+	-	+	-	-	-	+	+	+	-	-	+	25	*Chromohalobacter* sp.
	GUVFEIM-2	R	cream	+	M	+	+	-	-	-	-	-	-	+	-	+	+	+	+	+	-	3	*Bacillus* sp.
	GUVFEIM-3	SLR	cream	-	M	+	+	+	-	-	+	+	-	-	+	+	+	+		+	+	3	*Chromohalobacter* sp.

GUVFEIM-4	R	orange	-	M	+	-	-	+	+	-	-	+	-	-	+	+	+	-	-	-	3	*Pontibacillus* sp.
GUVFEIM-5	R	orange	-	M	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	3	*Alteromonas* sp.
GUVFEIM-6	SR	brown	+	M	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	3	*Bacillus* sp.
GUVFEIM-7	R	cream	+	M	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	3	*Chromohalobacter* sp.
GUVFEIM-8	R	cream	+	M	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	3	*Chromohalobacter* sp.
GUVFEIM-9	SR	cream	+	M	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	-	3	*Psychrobacter* sp.
GUVFEIM-10	SLR	cream	-	M	+	+	+	-	-	+	+	-	-	+	+	+	+		+	+	3	*Chromohalobacter* sp.
GUVFEIM-11	SR	white	+	M	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	-	3	*Bacillus* sp.
GUVFEIM-12	SR	cream	+	M	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	-	3	*Psychrobacter* sp.
GUVFEIM-13	SR	cream	+	M	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	-	3	*Psychrobacter* sp.
GUVFEIM-14	R	cream	+	M	+	+	-	-	-	+	-	-	-	+	+	+	+	+	-	-	0	*Bacillus* sp.

	GUVFEIM-15	C	cream	+	M	-	+	-	-	-	+	-	-	-	+	+	+	+	-	-	-	0	*Planococcus* sp.
	GUVFEIM-16	R	cream	+	M	+	+	+	-	-	+	+	-	-	+	+	+	+	+	-	-	0	*Bacillus* sp.
	GUVFEIM-17	R	cream	+	NM	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	0	*Corynebacterium* sp.
	GUVFEIM-18	SR	cream	+	M	+	+	-	-	-	+	-	-	-	+	+	+	+	+	-	-	0	*Bacillus* sp.
	GUVFEIM-19	SR	cream	+	NM	+	+	-	-	-	-	+	-	-	-	+	+	+	-	+	+	0	*Corynebacterium* sp.
	GUVFEIM-20	C	cream	+	M	+	+	-	-	-	+	-	-	-	-	+	+	+	-	-	+	0	*Planococcus* sp.
	GUVFEIM-21	R	translucent	+	M	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	0	*Bacillus* sp.
	GUVFEIM-22	SLR	translucent	+	M	+	+	-	-	-	+	-	-	-	+	+	+	+	+	-	-	0	*Bacillus* sp.
	GUVFEIM-23	SR	translucent	+	M	+	+	-	-	-	+	-	+	-	+	+	+	+	+	-	-	0	*Bacillus* sp.
Echinigorgia complexa MAM 9	GUVFECM-1	C	yellow	+	M	+	-	+	-	-	-	+	-	-	+	+	+	-	-	-	+	0	*Planococcus* sp.
	GUVFECM-2	R	creamyyellow	-	M	+	+	+	+	-	-	-	+	-	-	+	+	+	-	+	-	25	*Chromohalobacter* sp.

GUVFECM-3	C	cream	-	NM	+		-	-	-	+	-	-	-	+	+	+	+	-	+	-	25	unidentified
GUVFECM-4	R	orange	-	M	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	-	25	*Alteromonas* sp.
GUVFECM-5	R	translucent	+	M	+	+	-	-	-	+	+	-	-	+	+	+	+	+	+	-	25	*Chromohalobacter* sp.
GUVFECM-6	C	white	+	NM	+	+	-	-	-	-	-	-	+	+	+	+	+	-	+	-	25	*Chromohalobacter* sp.
GUVFECM-7	R	yellow	-	M	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	-	25	*Pseudomonas* sp.
GUVFECM-8	R	cream	+	M	+	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	3	*Bacillus* sp.
GUVFECM-9	LR	orange yellow	+	M	+	+	+	-	-	-	+	-	+	-	+	+	+	+	+	+	3	*Chromohalobacter* sp.
GUVFECM-10	C	yellow	+	M	+	-	+	-	-	-	+	-	-	+	+	+	-	-	-	+	0	*Planococcus* sp.
GUVFECM-11	R	cream	+	NM	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	0	*Corynebacterium* sp.
GUVFECM-12	C	cream	+	M	+	-	+	-	-	-	+	-	-	+	+	+	-	-	-	+	0	*Planococcus* sp.

GC, Gram character, **mot**, motility,**oxi**, oxidase, **cat**, catalase, **glu**, glucose, **lac**, lactose, **VP**, Voges Proskauer; **MR**, Methyl red **N**, Nitrate reduction, **P**,Penicillin, **M**,Mac Conkey (+), present; (-),absent

SYSTAT analysis further sorted the Gram positive and Gram negative bionts based on their biochemical characteristics (Table 2.3) using distance matrix and UPGMA clustered the Gram positive bacteria into 4 phena and the Gram negative bacteria into 7 phena respectively as represented in the phenogram (Figure. 2.20 a and b). Thus confirming the identification resolved to generic level, for each retrieved bacterial biont (Table 2.3) according to keys of Bergey's Systematic Bacteriology (Smibert and Krieg, 1994; Holt *et al.,* 1994).

Accordingly the 4 and 1 **Phena** under Gram positive cluster corresponds to 4 Genera under Class Bacilli and 1 Genus under Class Actinobacteria belonging to Phylum Firmicutis (Figure 2.20 a). The retrieved bionts were distributed as in:

Phenon 1 ≈ Genera *Planococcus* -GUVFEIM-15, GUVFEIM-20, GUVFECM-1, GUVFEIM-10, GUVFEIM-12 are motile cocci, catalase and oxidase positive.

Phenon 2 ≈ Genera *Bacillus* - GUVFTM-1,GUVFTM-3,GUVFTM-4,GUVFTM-7,GIVFTM-8,GUVFTM-10,GUVFTM-12,GUVFTM-3,GUVFERM-5,GUVFERM-6,GUVFERM-9,GUVFERM-11,GUVFERM-13,GUVFEIM-2,GUVFEIM-6,GUIVFEIM-11,GUVFEIM-14,GUVFEIM-16,GUVFEIM-18,GUVFEIM-21,GUVFEIM-22,GUVFEIM-23 and GUVFECM-8 are sporing rods, oxidase and catalase positive

Phenon 3 ≈ Genus *Pontibacillus* -GUVFEIM-4 are sporulating motile rods, catalase positive and oxidase negative

Phenon 4≈ Genera *Corynebacterium* -GUVFTM-14, GUVFERM-1, GUVFEIM-17, GUVFEIM-19 and GUVFECM-11 are non motile rods, catalase and oxidase positive.

Similarly all the **Phena** obtained under Gram negative cluster corresponds to the 6 Genera under Class gammaproteobacteria and 1 Genus under Class betaproteobacteria belonging to Phylum Proteobacteria (Figure 2.19 b). The retrieved bionts were distributed as in:

Phenon 1 ≈ Genera *Vibrio*-GUVFTM-11 and GUVFERM-12 curved rods, motile, catalase positive,oxidase positive,citrate positive,Vogues proskauer positive and indole positive.

Phenon 2 ≈ Genus *Aeromonas* – GUVFTM-15 rods,catalase positive,oxidase positive, indole negative, citrate negative, Vogues proskauer negative,indole negative and methyl red positive.

Phenon 3 ≈ Genera *Chromohalobacter*-GUVFTM-2,GUVFTM-5,GUVFTM-6,GUVFTM-9,GUVFEIM-1,GUVFEIM-3,GUVFEIM-7,GUVFEIM-8,GUVFEIM-10,GUVFECM-2,GUVFECM-5,GUVFECM-6 and GUVFECM-9are motile rods, oxidase and catalase positive, indole positive

Phenon 4 ≈ Genera *Pseudomonas*-GUVFERM-4 and GUVFECM-7 are non motile rods, oxidase and catalase positive, did not produce acid from glucose.

Phenon 5≈ Genus *Alcaligens*- GUVFERM-7 motile rods, non nitrate reducing, catalase oxidase positive and citrate positive.

Phenon 6≈ Genera *Alteromonas*-GUVFERM-8, GUVFEIM-5 and GUVFECM-4 motile rods, catalase negative, oxidase positive, non nitrate reducing, yellow pigmented.

Phenon 7≈ Genera *Psychrobacter* GUVFEIM-9, GUVFEIM-2 and GUVFEIM-13 non motile coccobacilli, catalase and oxidase positive.

These bionts of the eleven genera showed varied distribution in corals. As detailed in Table 2.3, the genera retrieved from *Telesto* sp. were identified as

Chromohalobacter sp., *Bacillus* sp., *Vibrio* sp.,*Corynebacterium* sp. and *Aeromonas* sp. Genera *Chromohalobacter* sp., *Bacillus* sp., *Alcaligens* sp.,*Alteromonas* sp., *Vibrio* sp. and *Pseudomonas* sp. were retrieved intoculture from coral *Echinogorgia reticulata. Echinomuricea indica* showed the presence of genera namely *Chromohalobacter* sp., *Bacillus* sp., *Pontibacillus* sp., *Alteromonas* sp., *Psychrobacter* sp., *Planococcus* sp. and *Corynebacterium* sp. From the coral *Echinogorgia complexa*, genera retrieved were *Chromohalobacter* sp., *Bacillus* sp., *Alteromonas* sp., *Pseudomonas* sp., *Planococcus* sp., and *Corynebacterium* sp. (Figure. 2.20 a and b).

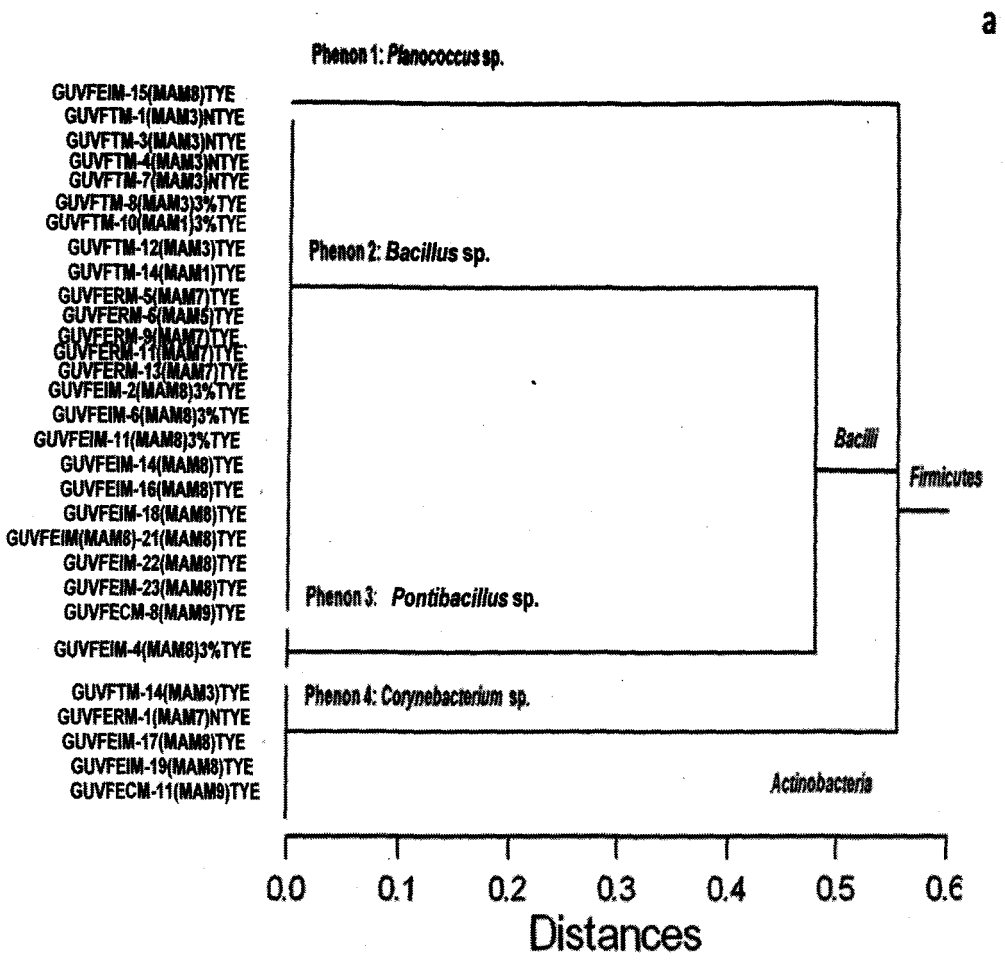


Figure 2.20 Phenogram depicting sorting of morphological and biochemical characteristics of eubacterial bionts retrieved from corals of Mandapam-India using keys of Bergey's Systematic Bacteriology and **SYSTAT v.12.01.** (a) Gram positive bacteria ≈ **Cluster A** (Phylum: Firmicutes) with **Subcluster IA** (Class: Bacilli) with 3A **Phena** (Genera: *Pontibacillus; Planococcus* and *Bacillus*) and **Subcluster IIA** (Class: Actinobacteria) with 1A **Phenon** (Genera: *Corynebacterium*).

b

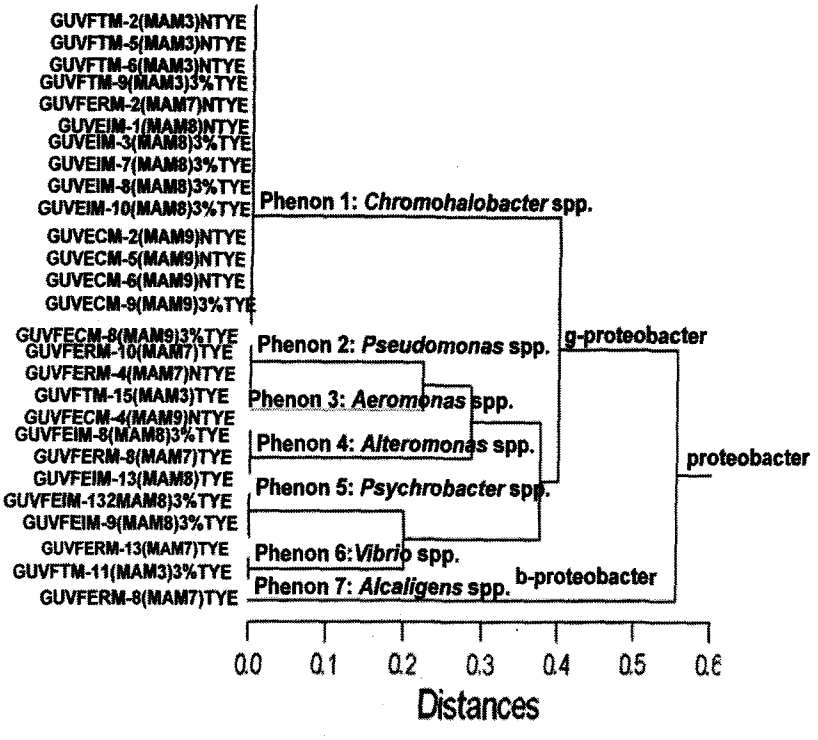


Figure 2.20 Phenogram depicting sorting of morphological and biochemical characteristics of eubacterial bionts retrieved from corals of Mandapam-India using keys of Bergey's Systematic Bacteriology and **SYSTAT v.12.01.**

(b) Gram negative bacteria ≈ **Cluster B** (Phylum: Proteobacteria) with **Subcluster IB** (Class: betaproteobacteria) with 1B **Phenon** (Genera: *Alcaligens*) and **Subcluster IIB**: (Class: Gammaproteobacteria) with 7B **Phena** (Genera: *Alteromonas*, *Chromohalobacter*, *Vibrio*, *Psychrobacter*, *Aeromonas*, *Alcaligens* and *Pseudomonas*).

The dominant bacterial genera associated with corals are represented in (Figure 2.21) As recorded in Figure 2.21, bionts of genera *Bacillus* sp. predominated corals *Telesto* sp., *E.reticulata*, *E.indica* and *E.complexa*. The coral *E. complexa* was the only coral predominated by bacteria of the genera *Chromohalobacter* sp.

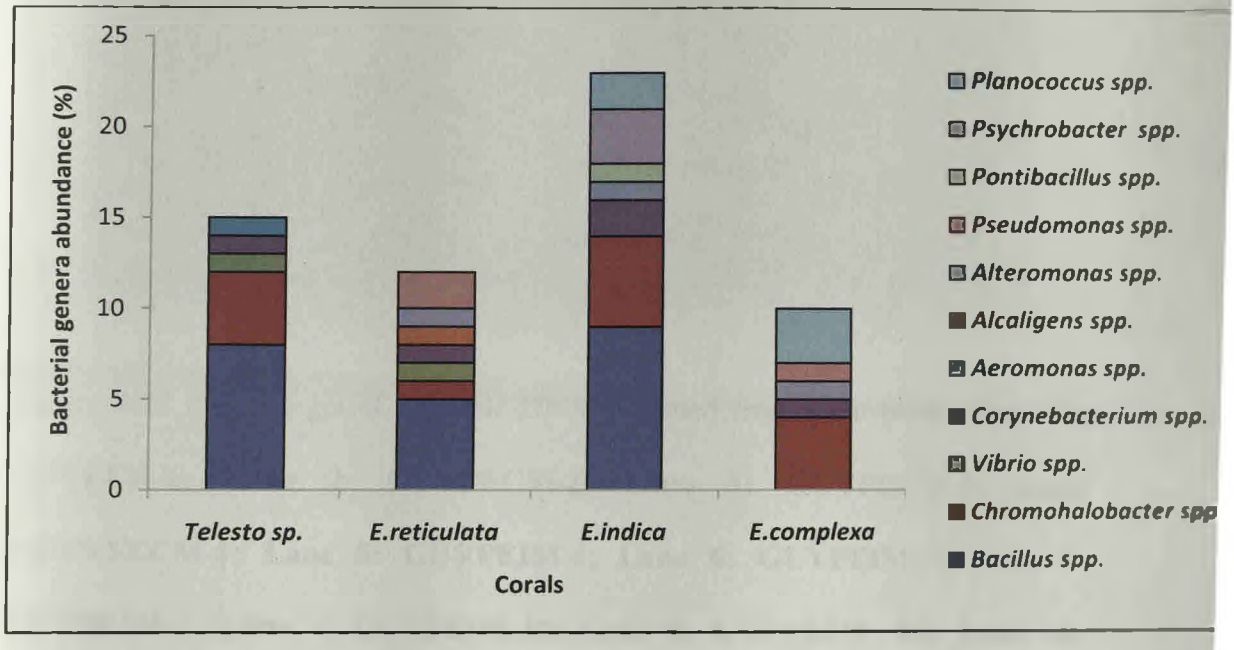


Figure 2.21 Dominant bacterial genera associated with corals.

2.2.3 B. Identification of bacterial isolates using 16S rRNA sequencing

The DNA extracted from the bacterial cultures was checked for its purity on a 0.8% agarose gel (Figure 2.22). The genomic DNA of individual cultures was seen as discrete bands just below the wells they were loaded (Figure 2.22). The average A_{260}/A_{280} was 1.6, which suggests pure DNA free of contaminants. The average DNA yield from the isolates was 76.42 µg of DNA. PCR amplification of 16S rDNA gene, yielded amplicons at 1.5kb (Figure 2.23).

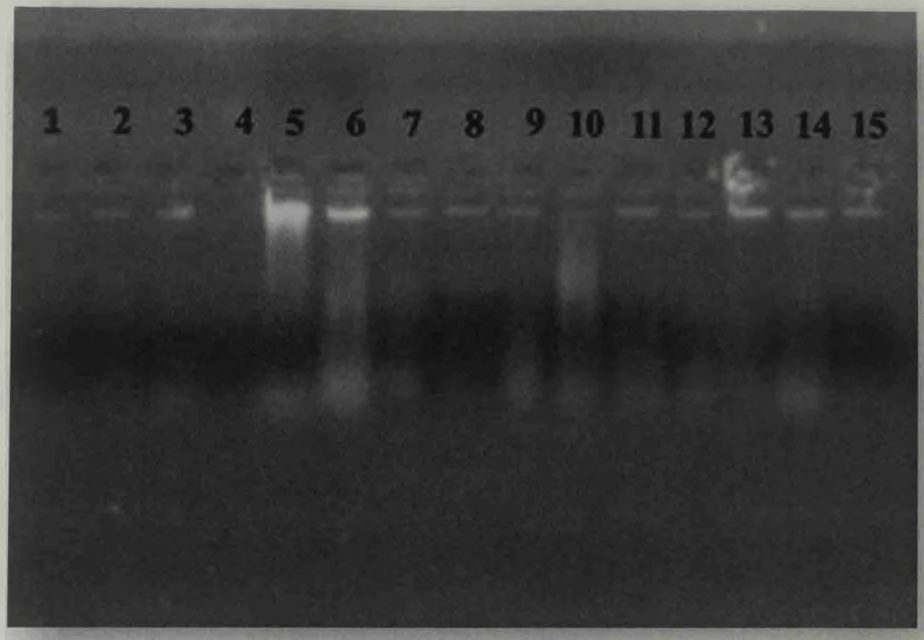


Figure 2.22 Agarose gel of genomic DNA extracted from coral bionts **(Lane 1: GUVFTM-6; Lane 2: GUVFECM-2; Lane 3: GUVFECM-6; Lane 4:GUVFECM-5; Lane 5: GUVFEIM-4; Lane 6: GUVFEIM-7; Lane 7: GUVFEIM-1; Lane 8: GUVFEIM-10; Lane 9: GUVFEIM -12; Lane 10: GUVFTM-2; Lane 11: GUVFEIM-3; Lane 12: GUVFTM-4; Lane 13: GUVFECM-6; Lane 14: GUVFECM-8 Lane 15: GUVFECM-5).**

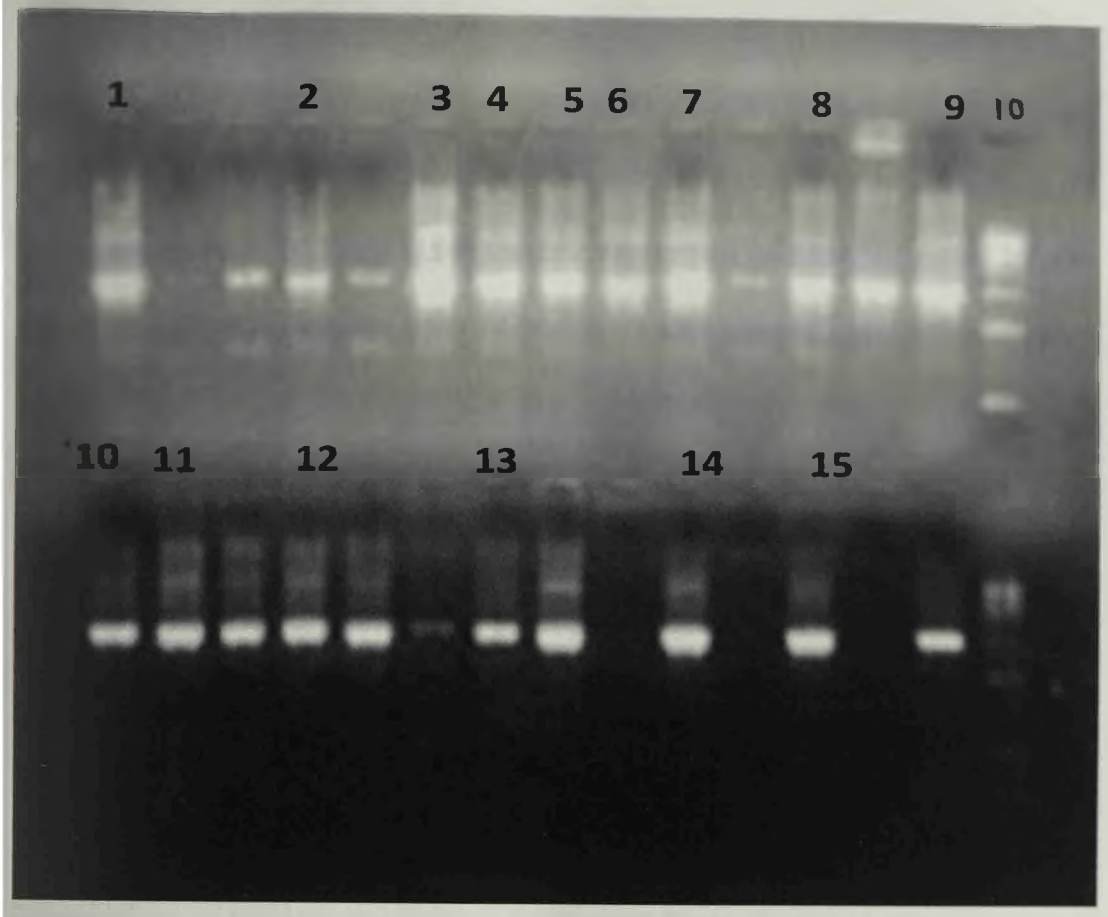


Figure 2.23 Agarose gel electrophoresis of PCR products of 16S rDNA gene obtained using bacterial primers and DNA of individual coral bacterial bionts **(Lane 1: GUVFTM-6; Lane 2: GUVFECM-2; Lane 3: GUVFECM-6; Lane 4: GUVFECM-5; Lane 5: GUVFEIM-4; Lane 6: GUVFEIM-7; Lane 7: GUVFEIM-1; Lane 8: GUVFEIM-10; Lane 9: GUVFEIM-12; Lane 10: GUVFTM-2; Lane 11: GUVFEIM-3; Lane 12: GUVFTM-4; Lane 13: GUVFECM-6; Lane 14: GUVFECM-8; Lane 15: GUVFECM-5).**

PCR amplification and sequencing of 16S rDNA gene of coral bionts namely GUVFTM-2, GUVFEIM-3, GUVFEIM-10, GUVFECM-2, GUVFEIM-7,

GUVFEIM-1, GUVFTM-4, GUVFECM-6 (Figure 2.24) on (BLAST)n search at the NCBI site and their subsequent alignment with ClustalW aligned the sequences with 99% identity to *Chromohalobacter israelensis* strain Ba 1 isolated from the Dead Sea by Rafaeli-Eshkol (1968) with an accession number NR 025431.1 The coral bionts GUVFEIM-12, GUVFECM-5 were identified as *Psychrobacter celer.* (BLAST)n search at the NCBI site and subsequent alignment with Clustal W aligned the sequences with 99% strain *Psychrobacter celer* SW-238 NR_043225.1 isolated from seawater of the South Sea in Korea.

Blast analysis of coral biont GUVFERM-2 revealed that this strain is a close relative with 99% similarity to *Chromohalobacter canadensis* strain ATCC 43984 NR_025430.1 isolated from the Dead Sea. Coral biont GUVFTM-6 89% similarity to *Chromohalobacter nigrandesensis* strain Taxon 4 (NR_042011.1) from Lake Tebenquiche on the Atacama Saltern, Chilerespectively. Similarly, amplification of 16S rDNA gene and sequencing of coral biont GUVFEIM-4 yielded amplicons which on (BLAST)n search at the NCBI site and subsequent alignment with ClustalW aligned the sequences 96% identity to *Virgibacillus salexigens* strain C-20Mo (NR_044921.1) respectively. Amplification of 16S rDNA gene of coral biont GUVFEIM-4 yielded an amplicon which on (BLAST)n search at the NCBI site and subsequent alignment with ClustalW aligned the sequences with 96% identity to *Virgibacillus salexigens* strain C-20Mo (NR_044921.1).The phylogram representing the Phylogenetic relatedness of coral bionts is indicated in Figure 2.25. The sequences of all the isolates are tabulated in Table 2.4 and are deposited in the Genebank under the accession codes as indicated in Table 2.4.

Table 2.4 16S rDNA Sequences of coral bionts

CULTURES	SEQUENCES	IDENTIFICATION	ACCESSION NUMBERS
GUVFTM-6	5'ATTGTACGCTGTGCGGCAGGCCTGACACATGCAAGTCGAGCGGAAACAATCCTAGCT TGCTAGGTGGCGTCTCCCTTATAAAGGGGTGAGTAATGCATAGGAATCTACCCAGTCG TGGGGGATAACCTGAGGAAACCCTGGCTAATACCGCATACGT 3'	*Chromohalobacter nigrandesensis*	Not assigned
GUVFECM-2	5'TGGCGGCAGGATACCATGCAGTCGAGCGGTAACAGGTCCAGCTTGCTGGACGCTGA CGAGCCTTTGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGGGATAACCT GGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGCTCTTCGG ACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTAACGGCTCA CCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGA CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAGGGGCGAAAG CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAG TGGGGAAGAAGGCTTGCCGGCCAATACCCGGCAAGAGCGACATCACCCACAGAAGAA GCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG GAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGAAA3'	*Chromohalobacter israelensis*	JX839256
GUVFECM-5	5'CGAGCGGAAACGATGGGAGCTTGCTCCCAGGCGTCCCCTTTGCGGAGGGGTGAGTA ATACTTAGGAATCTACCTAGTAGTGGGGGATAGCTCGGGGAAACTCGAATTAATACCG CATACGTCCTACGGGAGAAAGGGGGCAGTTTACTGCTCTCGCTATTAGATGAGCCTAA GTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTC TGAGAGGATGATCAGCCACACCGGGACTGAGACACGGCCCGGACTCCTACGGGAGGC AGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTGT	*Psychrobacter celer*	JX839257

	GAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCAGTGAAGAAGACCTAACGGTTAAT ACCCGTTAGCGATGACATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGC CGCGGTAATACAGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCG TAGGTGGCTTGATAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCTG ATACTGTTAAGCTAGAGTAGGTGAGAGGGAAGTTAGATTTCCTGGTGTAGCGGTGAAA TGCGTAGAGATCTGGAGGAATACCGATGGCCAAAGGCAGCTTCCTGGCATCCTACTGA CACTGAGGCTTCGAAAGCG3'		
GUVFECM-6	5'GCCTAACACATGCAAGTCGAGCGGAAACGATCCTAGCTTGCTAGGAGGCGTCGCGC GGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGGGATAACCTGAGGA AACTCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGCTCTTCGGACCTCG CGCGATTGGATGAGCCTATGTCGGATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGG CGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACCGGGACTGAGACACGGC CCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGAT CCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAGCGAGGA AGAAAGCGTGCCGGTCAATACCCGGTACGGACGACATCACTCGCAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTA CTGGGCGTAAAGCGCGCGTAGGCGGCGTGTCACGCCGGGTGTGAAAAGCCCCGGGCT CAACCTGGGAACGGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAG3'	*Chromohalobacter canadensis*	JX839258
GUVFEIM-4	5'CTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCGGGAAGCAGGCAGAACTCCTTC GGGAGTGATGCCTGTGGAACGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGC CTGTAAGACTGGGATAACCCCGGGAAACCGGGGCTAATACCGGATAACACTTTTTGGT ACATGCCAAGAAGTTGAAAGGCGGCCTTTTTGGCTGTCATTTACA3'	*Virgibacillus salexigens*	JX839261

GUVFEIM-7	5'GCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTGGA TGCTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGGGA TAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGCT CTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTAAC GGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGA CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG CGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCAC TTTCAGTGGGGAAGAAGGCTTGTCGGCCAATACCCGGCAAGAGCGACATCACCCACA GAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG TTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGAA AGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAGA GGAAGGTAGAATTCCCGGTGTAGC3'	*Chromohalobacter israelensis*	JX839263
GUVFEIM-1	5'TGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTGGACG CTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGGGATA ACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGCTCT TCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTAACGG CTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTG AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGA AAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTT CAGTGGGGAAGAAGGCTTGCCGGCCAATACCCGGCAAGAGCGACATCACCCACAGAA GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGAAAGC CCCGGGCTCAACCTGGGAACGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAGAGGA AGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAGTGG	*Chromohalobacter israelensis*	JX839264

	CGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAA CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACCGATGTCGACTAGCCCGTTGGGT CCCTTGGAGGACTTAGTGGGCGCAATTAACGCGATAAGT3'		
GUVFEIM-10	5'CGGCAGGCCTAAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTGGACGCT GACGAGCCGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGGGATAAC CTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGCTCTTC GGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTAACGGCT CACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAA AGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTC AGTGGGGAAGAAGGCTTGTCGGCCAA3'	*Chromohalobacter israelensis*	JX839265
GUVFEIM-12	5'TTTGCAGCTGACGCTGGCGCAGCTACCATGCAGTCGAGCGGAACGATGGAGCTGCTA CAGGCGTGCGTGGCAGAGACAGGTGAGTATACTTAGGAATCTACCTAGTAGCCGTGGG GATAGCTCGGGGAAACTCGAATTAATACCGCATACGTCCTACGGGAGAAAGGGGGCA GTTTACTGCTCTCGCTTTTAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAG GCCTACCAAGGCGACGATCTGTAGCTGGTCTGGGAGGATGATCAGCCACACCGGGACT GAGACACGGCCCGGGCTCCTACGGGAGGCAGCAGTGTGGAAAGACGAAAAATGGGGG AAACCCTGACCCAGCCATGCCTCTTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTT A3'	*Psychrobacter okhotskensis*	JX839266
GUVFTM-2	5'CTGGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTGGA CGCTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGGGA TAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGCT CTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTAAC	*Chromohalobacter israelensis*	JX839268

	GGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGA CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG CGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCAC TTTCAGTGGGGAAGAAGGCTTGCCGGCCAATACCCGGCAAGAGCGACATCACCCACA GAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG TTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGAA AGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAGA GGACGGTAGACTTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAG TGGCGAAGGCGGC3'		
GUVFECM-6A	5'ACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTTGCTG GACGCTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGG GATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGG CTCTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGGGGTA ACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGG GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG GGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGC ACTTTCAGTGGGGAAGAAGGCTTGTCGGCCAATACCCGGCAAGAGCGACATCACCCAC AGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGC GTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGA AAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAG AGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCA GTGGCGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAG CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTGGG GTCCCTTGAGGACTTAGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACG	*Chromohalobacter israelensis*	JX839274

	GCCGCAAGGTTAAAACTCATATGAATTGGCGGGGGCCCGCACAAGCGGGTGGGAGCA AGTGGTTTAA3'		
GUVFECM-8	5'ATCTGCATCATCAGTCACTTCGGCGCTGGCTCAAAGGTACTCACCGACTTCGGGTGT TACAATAAAATGAGATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT3'	*Lysinibacillus boronitolerans*	Not assigned
GUVFTM-4	5'GATGACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGTCCAGCTT GCTGGACGCTGACCAGTTGGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGT GGGGGATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCG GGGGCTCTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTAGCTGGTTGGTGG GGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACA CTGGGACTGAGACACGGCCCAGACTCCTCCGGGAGGAAACAGTGGGGAATATTGGAC AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTG3'	*Chromohalobacter israelensis*	JX839276
GUVFEIM-3	5'CCAAGCTGATCATCTGGCGGCATGCCTAACACATGCAAGTCGAGCGGTAACAGGTCC AGCTTGCTGGATGCTGACCCTTAGGAGGACGGGTGAGTAATGCATAGGAATCTACCCA GTCGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGA AAGCGGGGGCTCTTCGGACCTCTCGCGAGTGGATGAGCCTATGTGGGATTAGCTGGTT GGTGGGGTAACGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAG CCACACTGGGACTGAGACTCGGTCCAGTCTCCTACTGGAGGCAGGTAGGGGGGAATAT TGT3'	*Chromohalobacter israelensis*	JX839277

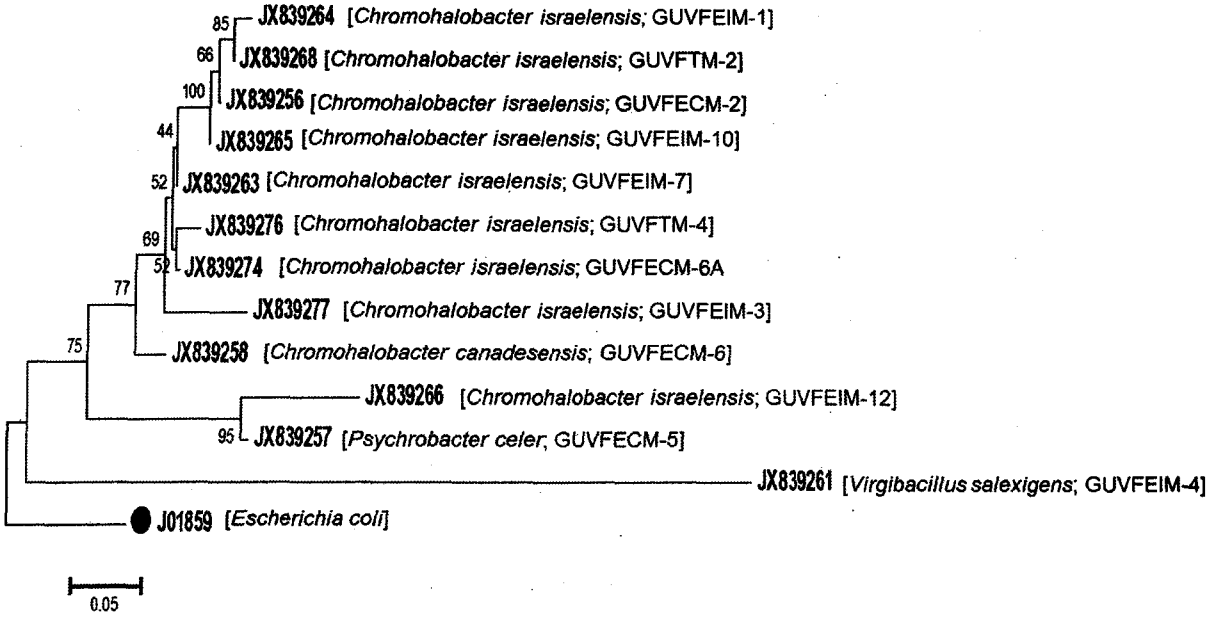


Figure 2.25 Bootstrap consensus (50% cut-off) rooted neighbor-Joining tree inferred from 500 replicates is shown to represent the phylogenetic position of coral isolates. The tree is drawn to scale, the evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis was conducted in MEGA5. The outgroup used is *Escherichia coli* (J01859). Numbers given at branch nodes indicate (%) boothstrap values. Bar, 0.01 substitutions per 100 nucleotide positions

2.2.4 B. Statistical measures of diversity

PRIMER v.5 showed highest Genera richness (*d*) and diversity (*H'*) in *E. reticulata.* Interestingly the bacterial bionts were evenly distributed in sponges namely *E. reticulata*, *E.indica* and *E. complexa* as seen in (Figure 2.24).

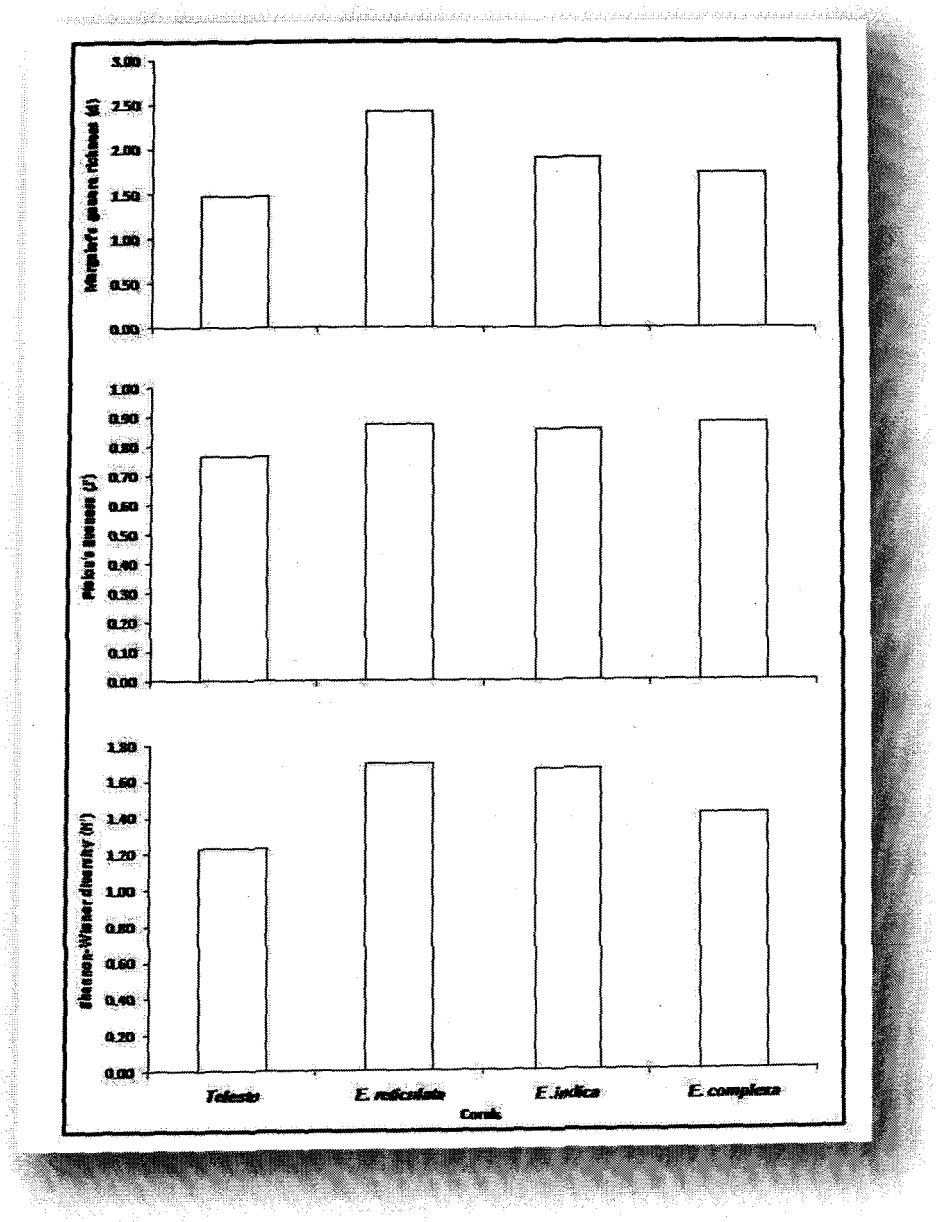
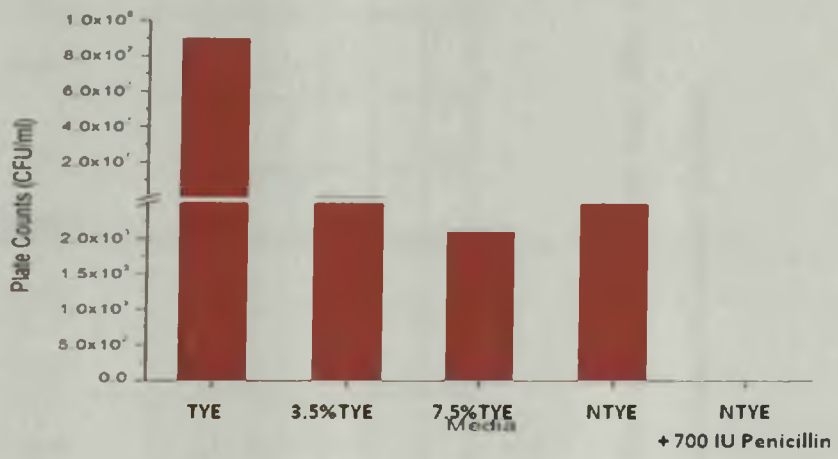


Figure 2.24 (a) Genera richness, (b) Pielou's evenness and (c) Shannon-Wiener diversity of the bacterial community in the different corals.

C. Bacteriology of Bivalve

2.2.1 C. Retrieval and isolation of pure bacterial bionts

The single bivalve *Perna viridis* sampled in this study showed the highest count in TYE medium at 8.961x 10^7 cfu/g, followed by a count of 2.486 x10^5 cfu/g and a near equal count of 2.1 x 10^3 and 3.17 x 10^3 cfu/g in 7.5% NTYE and NTYE



respectively .

Figure 2.25 Total viable counts of bacteria associated with *Perna viridis*

2.2.2 C. Morphological, Biochemical Characterization and Identification of isolates using Bergeys keys of Identification

Six distinct colonial morphotypes were selected for identification and their identities along with their biochemical potential are presented in Table 2.5. The isolates were identified using Bergeys keys of Systematic Bacteriology. The isolates from the single bivalve were identified as *Planococcus* sp., *Psychrobacter* sp., *Staphylococcus* sp. and *Bacillus* sp.

Table 2.5 Biochemical potentials and tentative identification of dominant bacterial bionts retrieved and purified from bivalve of *Mandapam- India*

Sponges	Isolates	Retrieval on TYE with NaCl (%)	Pigmentation	Morphotypes/ spores	Gram Character	Motility	Oxidase	Catalase	Glucose	Sucrose	Lactose	Indole	Methyl Red	VP	Citrate	NR	Growth in NaCl (%)			Genera
																	0	3	25	
	GUVFPVM-1	0	Cream	C	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	***Planococcus* sp.**
'ernia viridis	GUVFPVM-2	3	orange	RS	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	***Bacillus* sp.**
	GUVFPVM-3	3	Yellow	RS	+	+	+	+	-	-	-	-	-	+	-	-	+	+	+	***Bacillus* sp.**
	GUVFPVM-4	3	Yellow	RS	+	+	+	+	-	-	-	-	-	+	-	-	+	+	+	***Bacillus* sp.**
	GUVFPVM-5	0	Cream	R	-	-	+	+	+	-	-	-	+	-	-	+	+	+	+	***Psychrobacter* sp.**
	GUVFPVM-[illegible]	3	Yellow	C	+												+	+	+	***Staphylococcus sp.***

GC, Gram character;**CL**, colourless; **R**, Rods; **RS**, Rods with spores; **SR**, Short rods; **SLR**, Slender rods; **SRS**, Short rods with spores; **SLRS**, Rods fine slender with spores; **C**, Cocci; **VP**, Voges Proskauer; **NR**, Nitrate reduction; **(+)**, present; **(-)**,absent.

2.2.3 C. Identification of bacterial isolates using 16S rRNA sequencing

Amplification and sequencing of 16S rDNA gene of bivalve biont GUVFPVM-3 and GUVFPVM-6 yielded amplicons which on (BLAST)n search at the NCBI site and subsequent alignment with ClustalW aligned the sequences with 99% and 100% identity to *Salinicoccus* sp. with an accession number of JQ 269701.1 and *Psychrobacter* sp.with an accession number of JN602216.1. Bivalve biont GUVFPVM-8 showed 97% similarity to *Staphylococcus* sp. KC113044.1 while isolate GUVFPVM-5 showed 100% similarity to *Psychrobacter* sp. JN602216.1. Phylogram representing the phylogentic relatedness of bivalve bionts is represented in Figure 2.26. The sequences of all the isolates are tabulated in Table 2.6 and are deposited in the Genebank under the accession codes as indicated in Table 2.6.

Table 2.6 16S rRNA sequences of bivalve bionts

CULTURES	SEQUENCES	IDENTITY	ACCESSION NUMBER
GUVFPVM-5	5'GGCAGGCTTACACATGCAAGTCGAGCGGAAACGATGGGAGCTTGCTCCCAGGC GTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCTAGTAGTGGGGGATA GCTCGGGGAAACTCGAATTAATACCGCATACGTCCTACGGGAGAAAGGGGGCAGT TTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAA GGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACCGG GACTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA ATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTG TAAAGCACTTTAAGCAGTGAAGAAGACCTAACGGTTAATACCCGTTAGCGATGAC ATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAG AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGTAGGTGGCTTGA TAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCTGATACTGTTA AGCTAGAGTAGGTGAGAGGGAAGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA GAGATCTGGAGGAATACCGATGGCGAAGGCAGCTTCCTGGCATCATACTGACACT GAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGTCTACTAGTCGTTGGGTCCCTTGAGGACTTAGTGACG3'	*Psychrobacter celer*	JX839269
GUVFPVM-8	5'ACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGATAAGGAGCTTG CTCCTTTGAAGTTAGCTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATA AGACTGGAATAACTTCGGGAAACCGGAGCTAATGCCGGATAACATTTGGAACCGC ATGGTTCTAAAGTAAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTA TTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGA	*Staphylococcus equorum*	JX839270

	GAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTG AGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGT AAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGT GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGT AAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGT GGAGGGTCATTGGAAACTGGGAAACTTGAGTACAGAAGAGGAAAGTGGAATTCC ATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGA CTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACACGATT AGATACCCTGGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTTGTTAGGGGGCT TCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGGCACTCCGCCTGGGGAGTACGA CCGCAAGGTTGAAACTCAGAGGGAATTGAACGGGGACCCCGCACAAGGCGGGTG CAGCATGTGGCTTTAGTACGAAGGCAACCCGAAGAAACGTTAACCGAATTCTGGA CATCCCTTTGAAA3’		
GUVFPVM-6	5’GGCAGGCTTACACATGCAAGTCGAGCGGAAACGATGGGAGCTTGCTCCCAGGC GTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCTAGTAGTGGGGGATA GCTCGGGGAAACTCGAATTAATACCGCATACGTCCTACGGGAGAAAGGGGGCAGT TTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAA GGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACCGG GACTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA ATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTG TAAAGCACTTTAAGCAGTGAAGAAGACCTAACGGTTAATACCCGTTAGCGATGAC ATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAG AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGTAGGTGGCTTGA	*Psychrobacter celer*	JX839271

	TAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCTGATACTGTTA AGCTAGAGTAGGTGAGAGGGAAGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA GAGATCTGGAGGAATACCGATGGCGAAGGCAGCTTCCTGGCATCATACTGACACT GAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGTCTACTAGTCGTT3'		
GUVFPVM-1	5'CTGAGCGGCGTGCCTAATACATGCAAGTCGAACGCGAGAGGAGGGAGCTTGCT CCCTCTGAGGCGTTAGAAGGACGGGTGAGTAACACGTAGGCAACCTGCCCATCAC CTCCGGGATAACCGCGGGAAACCGTGGCTAATACCGGATGATCCTCTCCACCGCG GGGTGGGGAGTTGAAAGACGGTTTCGGCTGTCGCTGATGGATGGGCCTGCGGCGC ATTAGCTGGTTGGCGGGGTAAGGGCCCACCAAGGCAACGATGCGTAGCCGACCTG AGAGGGTGATCGGCCACACTGGGACTGAGACGCGGCCCAGACTCCTACGGGAGG CAGCAGTAGGGAAGCTTCCCCAAG3'	*Salinicoccus luteus*	JX839272

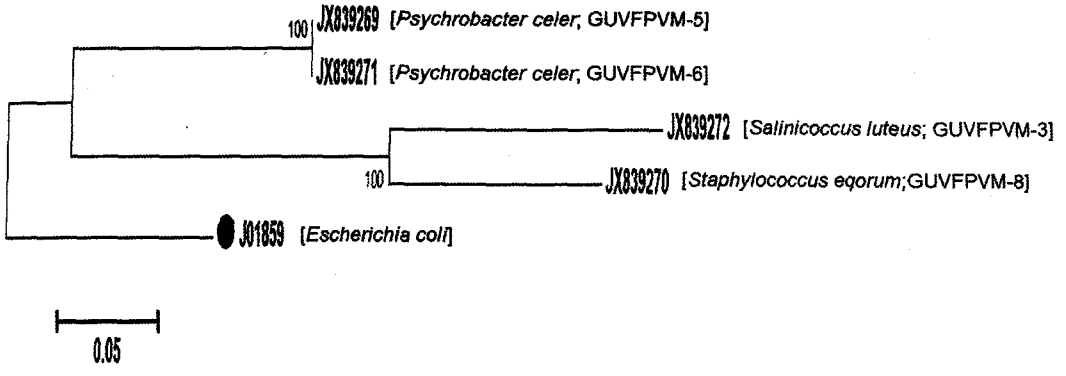


Figure 2.26 Bootstrap consensus (50% cut-off) rooted neighbor-Joining tree inferred from 500 replicates is shown to represent the phylogenetic position of bivalve isolates. The tree is drawn to scale, the evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis was conducted in MEGA5. The outgroup used is *Escherichia coli* (J01859). Numbers given at branch nodes indicate (%) boothstrap values. Bar, 0.01 substitutions per 100 nucleotide positions

2.3 Discussions

Section A: Bacteriology of Sponges

The total bacterial counts determined using AODC method, averaged at, $5.20 \pm 2.2 \times 10^9$ cells/g and are comparable with counts $2.8 \pm 0.4 \times 10^9$ cells/ml reported from other marine sponges (Naglaa *et al.*, 2008). This result clearly supports the finding that sponges provide an econiche wherein several bacterial types dock for safety against predators or harsh environmental conditions and for nutrition (Bathel and Gutt, 1992; Hill, 1996; Taylor *et al.*, 2007).

Even though our sponges were washed repeatedly with 3% NaCl to get rid of the surface associated bacteria the total viable counts of intimate associates of sponges are 10^3 times higher in comparison to the earlier reports from sponges, of this

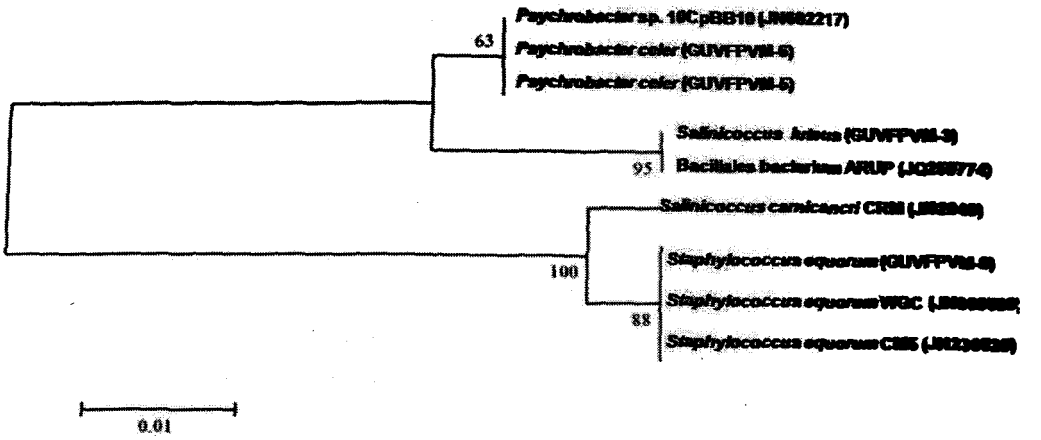


Figure 2.26 Phylogram indicating the placement and relatedness of the bivalve bionts.Numbers given at branch nodes indicate (%) boothstrap values. Bar, 0.01 substitutions per 100 nucleotide positions.

2.3 Discussions

Section A: Bacteriology of Sponges

The total bacterial counts determined using AODC method, averaged at, 5.20 ± 2.2 x 10^9 cells/g and are comparable with counts 2.8 ± 0.4 x 10^9 cells/ml reported from other marine sponges (Naglaa *et al.,* 2008). This result clearly supports the finding that sponges provide an econiche wherein several bacterial types dock for safety against predators or harsh environmental conditions and for nutrition (Bathel and Gutt, 1992; Hill, 1996; Taylor *et al.*, 2007).

Even though our sponges were washed repeatedly with 3% NaCl to get rid of the surface associated bacteria the total viable counts of intimate associates of sponges are 10^3 times higher in comparison to the earlier reports from sponges, of this

embayment (Saravanakumar *et al.*, 2011). This is also substantiated by the total bacterial counts in the same sponge samples. However, this may not be unusual as bacterial counts in marine invertebrates including sponges are reported to be 2-3 folds higher in comparison to the free living/planktonic bacteria in the ambient waters and is a pointer to the significant role played by these microorganisms in sponges (Friedrich *et al.*, 2001).

The isolates retrieved from the different sponge samples under study differed in the requirement of NaCl concentration used for their recovery and hence were either euryhaline or marine halophiles or haloarchaea. It was intriguing to note that even though culturable bacteria were obtained on all the three isolation media (TYE, 3% TYE and NTYE) from all the sponge samples under study, the euryhaline bacteria predominated the culturable microbial assemblage. Euryhaline bacteria are capable of growth over a wide range of NaCl concentration (between 0-25% NaCl) and are also referred to as 'halotolerant extremophiles' (Ventosa *et al.*, 1998).

Sponges through their filter feeding mode of nutrition (Pile *et al.*, 1996; Ribes *et al.*, 1999) accumulate large amounts of this dissolved organic matter (DOM) from the habitat waters, in their choanocytes. In recent years, assimilation of DOM is ascribed to the sponge-microbe association who participate in the sponge nutrition by secreting extracellular enzymes that act on the particulate organic matter accumulated within the sponge body, and thereby provide assimilable carbon to the sponge host (Yahel *et al.*, 2003; Dohoeij *et al.*, 2008; Feby and Nair 2010). The relenting osmolal stress owing to the increased concentration of organic compounds in the sponge interiors due to the sequestering of DOM creates a selection pressure on euryhaline bacteria. Given to understand, the tolerance of euryhaline halophiles to high or fluctuating salinities (Kunzel and Oren, 2003), it is

reasonable to consider that we found euryhaline bacteria to predominate the cultural assemblage in intertidal sponge species which face abrupt and short term salinity fluctuations.

The physiological flexibility of these isolates (Ventosa *et al.,* 1998) and their ability to resist the changing osmolar conditions also adds to the selection pressure. The retrieval of euryhaline archaeafrom these sponges in our study corroborates with the findings of Martins *et al* (Martins *et al.*, 1997) who demonstrated the production of compatible solutes to cope osmotic stress that is possibly responsible for the establishment of bionts of genera that are not known to produce osmolytes.

In the present study, we have demonstrated *Bacillus* sp. and *Corynebacterium* sp., as the major culturable bacterial community in all the sponges under study. The greater predominance of Gram-positive bacteria is probably attributable to their ability to produce spores under adverse conditions; a physiological adaptation enabling eubacterial bionts to survive within the sponge interiors. Studies by Prabha *et al.,* 2010; Feby and Nair, 2010; Vasanthbharathi and Jayalakshmi, 2012 and other workers have demonstrated the presence of bacteria in Indian sponges. The single report on bacterial isolation from sponges of Mandapam is by Saravanakumar *et al.,* 2011.

The detection of the haloarchaeal colonies after long incubation periods is an observation that has been reported by earlier co-workers wherein long incubation periods have been reported for maximizing the viable counts and diversity of the haloarchaeal groups (Burns *et al.*, 2004). The ability of the cultures to grow in media with 500 IU penicillin possibly reflects the presence of a cell wall other than cross-linked peptidoglycan hence exhibiting resistance to cell wall inhibitors such as penicillin and bacitracin (Bonelo *et al.,* 1984).

The presence of Glycerol diether moieties, which is absent in the eubacterial counterparts, and the presence of bacterioruberin type of pigments are the key chemotaxonomic markers for ascribing the isolates to haloarchaea. In addition, the presence of archaeol lipids is further suggestive of the affiliation of the isolate to the haloarchaeal group. The ability of the isolates to grow at concentrations less than 15% NaCl is an indicator that there isn't an absolute requirement of the isolates for high concentration of NaCl as seen by extremely halophilicarchaeal isolates and thus this isolates are similar to those reported by Purdy *et al.*, 2004 who reports extremely halophilic archaea capable of growth at low salinities.

The order *Halobacteriales* is in the domain *Archaea* (Woese *et al.*, 1990) and contains one family, *Halobacteriaceae* Representatives of the order *Halobacteriales* (halobacteria) are the most halophilic organisms known, requiring at least 1.5M NaCI for growth. Halobacteria predominate in the aerobic zone when hypersaline waters approach saturation (Rodriguez Valera *et al.*, 1981). Occurrence of such bacteria in an econiche having salinities in the range of 3.5% is a finding which has not been previously reported and hence of much significance

A lack of pure cultures for most sponge-associated microorganisms has contributed to a paucity of knowledge about their physiological characteristics. There are very scarce reports of cultivable archaea reported from sponges and hence the presence of haloarchaea in sponges (with a salinity level of 3% –3.5% NaCl), an econiche in which they are not expected to survive, is a significant finding that reflects the physiological and ecological complexities of halophilic archaea, and furthermore, raises the question pertaining to the survival mechanism employed in overcoming adverse hyposaline conditions of such an environment.

The possible reasoning for occurrence of this extremely halophilic bacterium may

be a result of the dynamic condition of this estuarine bay with high nutrient levels, different types of pollutants and remarkable water mixture.

The diversity of these oceanic environments characterized by temperatures lower than 4^0C, pressures higher than $100x10^5$ Pa, a relatively high concentration of Na^+ ions and a nutritionally starved environment has exerted a driving force on bacterial selection leading to adaptive strategies that is reflected in their physiological and biochemical properties and this in turn has lead to the synthesis of novel metabolites many of which have therapeutic potential.

This is the first record of retrieval of euryhaline, halotolerant extremophilic eubacteria (0-20%) and haloarchaeal bionts from nine different benthic sponges of Mandapam in India. Earlier studies on Indian sponges have reported bacteria growing in 0.5-3% NaCl (Prem Anand *et al.*, 2006; Vasanthbharathi and Jayalakshmi, 2012; Saravankumar *et al.*, 2011) whereas in the present study bacterial bionts grew at 0-25% NaCl.

To the best of our knowledge eubacteria from Genera *Loktanella* sp., *Pontibacillus* sp., *Planococcus* sp., *Enterococcus* sp. retrieved by us have not been reported from Mandapam waters possibly, as they could not withstand tidal fluctuations. Retrieval of sponge bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* known for their pathogenicity to humans and others, reflects their occurrence in the habitat waters, possibly accounting to reports describing Mandapam in the GoM, as a sink, receiving high load of organic matter through the indiscriminate discharge of industrial effluents, household wastes, leachate of solid waste dumps and garbage, animal and human excreta etc. (Wild anad Maier, 2010; Ramadas and Rajeshwari, 2001; Sahayam *et al.*, 2010)

The retrieval of bacteria from nine Genera indicates that the sponge tissue provides a suitable substratum for settlement of bacteria reflecting their occurrence in habitat waters.

Section B: Bacteriology of Corals

Corals are eukaryotic hosts which provide a unique surface for microbial colonization. Culture independent studies show that the tissue harbour diverse and abundant prokaryotic communities. However, little is known about the diversity of bacteria associated with the corals of Gulf of Mannar. The present study characterized the bacterial diversity of four different corals namely namely *Telesto* sp., *Echinogorgia complexa*, *Echinogorgia reticulata*, *Echinomuricea indica*. The high counts observed in this study may be attributed to the glycopeptide rich mucus which serves as a substrate for heterotrophic bacteria (Rublee *et al.*, 1980), thus suggesting that mucus would form the basis for microbial food-chain. Mucus is utilized by the marine bacteria as a carbon and nitrogen source (Mitchell and Chet, 1975). The total viable bacterial counts of intimate associates of corals are $10^3 - 10^4$ times higher in comparison to the earlier reports from corals, of this embayment

This change in bacterial counts in different coral species could be due to the change in mucus production by different coral species (Ducklow and Mitchell, 1979) and organic carbon content in the mucus (Pascal and Vacelet, 1981). The probable finding for the increased bacterial counts is the detritus particles that enriched the reef waters due to land run-off that largely increased the distribution of organic matter (Sreepada *et al.*, 1993). The present study clearly revealed that the density of heterotrophic bacteria was much higher then in the coral reef waters

and sediments (Kannapiran *et al.*, 2008) thereby clearly supporting the finding that the internal contents of the coral tissue provides a protective niche for the microbes to nestle. Heterotrophic bacteria in turn play a pivotal role in the degradation of organic matter and nutrient regeneration. Activities and relative abundance of the heterotrophic bacteria are controlled by the hydrobiological factors and nutrient levels of the aquatic environment.

A total of 63 strains of bacteria were isolated from four coral species from which, 11 genera are recorded namely *Bacillus* sp., *Chromohalobacter* sp., *Vibrio* sp., *Corynebacterium* sp., *Aeromonas* sp., *Pseudomonas* sp., *Alcaligens* sp., *Alteromonas* sp., *Pontibacillus* sp., *Psychrobacter* and *Planococcus* sp. Among the Gram negative bacteria the genera *Pseudomonas* sp., *Aeromonas* sp. and *Alcaligens* sp., have been reported from corals in the GoM while among the Gram positive bacteria the genera *Corynebacterium* sp., *Bacillus* sp. have also been reported (Kannapiran *et al.*, 2008). However it was fascinating to note that *Pseudomonas* sp., dominated the cultural assemblage in corals in the study by Kannapiran *et al.*, 2008 however, the corals in our study were dominated by *Bacillus* sp. among the Gram positive bacteria and *Chromohalobacter* sp., among the Gram negative bacteria.

C. Bacteriology of Bivalve

Bivalves are recently been organized as potential sources of antibacterial and antifungal compounds. The potential of marine bivalves as a source of biologically active products is largely unexplored. Hence this part of the research work is based on screening the microbial bionts associated with the marine bivalve *P.viridis* sampled from Mandapam in the GoM for the presence of antimicrobial

compounds. Bivalves are characterised by the sedentary feeding habits and thus are efficient accumulators of a large number of bacteria and chemical pollutants (Bernal-Hernandez *et al.*, 2010). Cellular and humoral components of the immune system, which constitutes the first line defense and physicochemical barriers such as the cuticle, shell and mucus layer prohibits the adherence of microbial bionts (Galloway and Depledge, 2001). Only the micro-organisms that can evade the immune system of the host thrive in the host tissue.

The 2-3 folds higher bacterial counts than the ambient waters are witness to the efficient filter feeding node of nutrition in this bivalve.

The bacterial bionts found associated with the bivalve tissue *Planococcus* sp., *Psychrobacter* sp. *Staphylococcus* sp. while three were identified as ***Bacillus*** sp. The sporadic work reported from the single bivalve delved in this study makes it impossible to compare this work with any other previous reports.

2.4 Conclusions

The study clearly reveals:

(1) The presence of cohabitating marine halophiles (3% NaCl), halotolerant or euryhaline halophiles (0-25% NaCl) and extreme halophiles/haloarchaea.

(2) The predominance of euryhaline bacteria, capable of coping with osmolal stress in the host interiors, possibly due to the sequestration of DOM from the ambient waters of the sponge habitat, which in turn supports co-existence of bionts of Genera unknown to produce osmolytes.

(3) The presence of haloarchaea in marine sponges (with a salinity level of 3% – 3.5% NaCl), an econiche in which they are not expected to survive, is a significant finding that reflects the physiological and ecological complexities of halophilic archaea, and furthermore, raises the question pertaining to the survival mechanism employed in overcoming adverse hyposaline conditions of such an environment.

(4) That retrieval of bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* reflects the entry of bacteria, known for pathogenicity to humans, into waters of Mandapam, through sewage and other pollutants.

(5) Microbial pollution of Mandapam waters and *Heralds Caution* for taking measures for safeguarding the waters and ensuring sustainability of biota.

CHAPTER 3

EVALUATION OF *ACROPORA FORMOSA* FROM LAKSHADWEEP - INDIA FOR ITS BACTERIAL COMMUNITY BY DGGE

Several corals namely *Acropora latistella* sp., *Acropora digitifera, Acropora formosa, Favia palida, Subergorgia suberosa and Junceella juncea* have been mapped from the Indian subcontinent and the Indian Ocean (Babu *et al.*, 2004, Thenmozhi *et al.*, 2009, Chellaram *et al.*, 2011, Gnanambal *et al.*, 2005). Among these corals, the coral of the genus *Acropora* sp. have been studied for the antagonistic effect of the associated microflora. However the coral *Acropora formosa* from the bioreserve of GoM and the Alchipelago of Lakshadweep has not been studied for its associated microbial diversity. *A. formosa* is reported to occupy an important position in the Lakshadweep group of islands. There are limited reports on microbial studies on *Acropora* sp. although it is phylogenetically divergent and commonly involved in reef building activities (Babu *et al.*, 2004, Thenmozhi *et al.*, 2009. The attempt made to cultivate bacteria (i) culturable on the basis of its salt dependability and (ii) DGGE as community analysis tool form the content of this chapter. The coral *Acropora formosa* was sampled by co-Ph.D. student Mr. Subhajit Basu while on a Sagar Sukti 199 cruise to the Kavaratti island in Lakshadweep in 2008.

3.1 Methodology

3.1.1 Sampling Site and Collection

Samples of visibly healthy coral *Acropora formosa*were collected from Lakshadweep while on a Sagar Sukti199 cruise to the Kavaratti island in Lakshadweep in the year 2008 (Figure 3.1).

Figure 3.1 Kavarrati in Lakshadweep situated at 10.5700° N, 72.6200° E. on East Coast in the Indian Subcontinent

Fig 3.2 Coral sample *Acropora formosa* collected from Kavarrati island in Lakshadweep.

Class: Anthozoa; Order: Scleractinia; Family: Acroporidae; Genus: *Acropora* Species*: formosa*

3.1.2 Enumeration and retrieval of bacteria from coral tissue

The working sample was excised from a healthy coral, washed twice with 0.2 µm of filter sterilized and autoclaved sea water to remove any loosely associated microbes. An aseptically excised core of the coral was rewashed in a 3% (w/v) NaCl solution and crushed using a sterile mortar and pestle in 5 ml of 3% (w/v) sterile NaCl solution. 5µl of the coral macerate was plated on TYE medium, TYE medium supplemented with 3% (w/v) and 25% (w/v) of NaCl (NTYE). 200 µl of the macerate was plated onto NTYE medium and 700U of penicillin. The plates

were then incubated for a period of 10 days at R.T. Dominant colonies showing different morphology were purified by restreaking onto respective media and were maintained on agar slopes at 4^0C. All the chemicals used were from Himedia-India.

3.1.3 Isolation and Characterisation of retrieved Bacteria

Morphological and biochemical potentials of the isolates were examined using standard bacteriological procedures and individual isolates were identified up to generic level following schemes given by Smibert and Krieg, 1994; and Holt *et al.*, 1994, as discussed in detail in section 2.1.3 A and D of Chapter II. DNA of isolate GUVFAM-1 was extracted and amplified using forward and reverse universal primers 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1522R (5'AAG GAG GTG ATC CAG CCG CA 3')(Genei-India) (Hicks *et al.*, 1992) and sequenced as detailed in section 2.1.3 D. of Chapter II.

3.1.4 Extraction of total bacterial community DNA

Total DNA was extracted from pulverized coral fragments consisting of coral mucus, tissue and skeleton. Briefly, coral fragments were collected using a hammer and chisel, rinsed with filtered (0.2 μm) seawater, and frozen at -80°C before transportation to the laboratory. Small fragments were ground to a fine powder in a pre-chilled (on dry-ice) mortar and pestle, before proceeding with the extraction protocol. For the extraction of DNA from coral tissue slurries, the tissue was blasted off its skeleton with TES buffer (10 x Tris-EDTA+ 100 mM NaCl, pH 8.0) using an air-brush at 80 psi. The "blastate" was centrifuged and the supernatant discarded. Whole-community DNA was directly extracted from 2 g of

sample (fresh weight of known moisture content) using a bead-beating method (MoBio -Ultraclean soil for soil, Bio 101 Inc., USA) as per manufactures protocol. Samples were stored at -80°C prior to experimental analysis.

3.1.5 Estimation of yield of coral DNA

An aliquot of 10 ml of crude DNA extract was run on 0.8% (w/v) agarose gel at 70 V for 30 minutes in a Hoefer HE 100 Super Sub electrophoresis unit Hoefer Pharmacia Biotech in 0.5 x TAE 20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 8 and with 1 kb labda DNA digest ladder Promega as the molecule size marker. Gels were stained with Ethidium bromide 0.5µg/ml, for 30 minutes and visualised in UV light transilluminator.

3.1.6 PCR.

10 ng of extracted DNA was used as the template for PCR in which prokaryotic 16S ribosomal DNA (rDNA)-specific primers and archaeal specific primer sets were used. The PCR mixtures (20µl) contained each deoxynucleoside triphosphate at a concentration of 200 mM (Sigma), 0.65 µl of 50 mM $MgCl_2$, 0.4 µl each primer at a concentration of 5X, 1 U/ml of Taq DNA polymerase (Invitrogen), 3µl of total DNA and the PCR buffer supplied with the enzyme. The universal primer sets used were FDD2-RPP2 (FDD2: 5'CCGGATCCGTCGACAGA GTTTGATCTGGCTCAG- 3': RPP2:5'CCAAGCTTCTAGACGG5TACCTTGT TACGACTT3', GC-SRV31-SRV3-2 (GC-SRV3:5'CGCCCGCCGCGCGCGG CGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG3' and SRV3-2 5'TTACCGCGGC TGCTGGCAC3') and GC-Com1-Com 2 (GC-Com 1: 5'CCGGATCCGTCGACCGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC

ACGGGGGGCAGCAGCCGCGGTAATAC3' and Com2: 5' CCGTCAATTCC TTTGAGTTT3') while the archaeal primer sets were Arch165F-RPP2 (Arch 165F:5'TCCGGTTGATCCTGCCAG3' and RPP2: 5'CCAAGCTTCTAGACG G5TACCTTGTTACGACTT3'), Arch 344F-Arch 915R (Arch 344F : 5'CCGCGCCCGTCCCGCCGCCCCCGCCGACGGGGYGCAGCAGGCGCGA3' and Arch 915R : 5'GTGCTCCCCCGCCAATTCCT3') and GC Arch344F-Arch 915R (GC Arch344F:GC 5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCGACGGGGYGCAGCAGGCGCGA3' and Arch 915R :5'GTGCTC CCCCGCCAATTCCT3').

The PCR program included an initial denaturation at 94°C for 5 minutes followed by 30 cycles of 94 °C for 60 seconds (denaturation), annealing at 60°C (with the temperature decreasing 1°C each cycle) for 60 seconds, and extension at 72°C for 60 seconds, followed by 30 cycles of 94°C for 1 minute, and 72°C for 10 minutes. An aliquot of the PCR product was electrophoresed in a 2% (w/v) agarose gel, stained with Ethidium bromide, and quantified by using a standard (Low DNA Mass Ladder; Gibco BRL). PCR was performed in Gene amp PCR System 9700 Applied Biosystems.

3.1.7 Nested PCR

The resulting PCR products were reamplified using a nested PCR approach using two sets of primers SRV3-1 with a GC clamp 5'CGCCCGCCGCGCG CGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG3'and SRV3-2 (5'TTACCGCGGCTGCTGGCAC3') and COM-1 with GC clamp ('CCGGATCCGTCGACCGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCAGCAGCCGCGGTAATAC3') with and COM-2 (5' CCGTCAATTCCTTTGAGTTT3') respectively. The conditions were the same as mentioned in section 3.1.6. Nested PCR was performed in Gene amp PCR System 9700 Applied Biosystems.

3.1.8 DGGE analysis

DGGE analysis was performed as described by Heuer *et al.*, (2001) using DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, München, Germany). Electrophoresis was performed in a 0.75-mm-thick 6% (w/v) polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1 (w/v) submerged in TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4) at 65°C. 100 ng of nested 16S rDNA fragments were loaded onto an 8% (w/v) polyacrylamide gel, which was made with a denaturing gradient ranging from 30% (w/v) to 70% (w/v). The denaturant (100%) contained 7 M urea and 40% (w/v) formamide. Initial PCR products were visualized on an agarose gel to ensure sufficient product for the community DGGE analysis. A positive result was indicated on the agarose gel by DNA bands of uniform intensity and size (approximately 500 bp).

The products were separated by running in 1×TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) for 6 h at a constant voltage of 120 V and temperature of 65°C. A 100 bp ladder was loaded on a separate lane to determine the size of the separated bands. The gels were stained with Ethidium bromide 5μg/ml and visualized using UV radiation 360nms, dried at 37 °C and scanned as shown by Heuer *et al.*, 2001. The number of operational taxonomic units (OTUs) in each sample was defined as the number of DGGE bands. All the DGGE bands were excised from the gel reamplified and then sequenced.

3.1.9 Elution of DGGE bands

DGGE bands were sequenced subsequent to excision from the gel and reamplification using the nested primers. PCR products demonstrating amplification close to expected size i.e. 200 bp for SRV3-1 and SRV3-2 and 400 bp for COM-1 and COM-2 in agarose gel electrophoresis were excised, resuspended in 20 ml of MilliQ water and stored at 4°C overnight. An aliquot of the supernatant obtained was used for PCR reamplification with the original primer set. 30 and 50 ng of the reamplified PCR product was used for a sequencing reaction (with the corresponding forward and reverse primer) in an ABI PRISM model 3100 Genetic analyser automated sequencer (Applied Biosysytems). The sequences were compared with public DNA database sequences by using BLAST and Ez taxon server.

3.1.10 Data analysis

The DGGE bands were detected on the DGGE gel using the band-searching algorithm of BioNumerics software (BioSystematica, Tavistock, Devon, United Kingdom). After normalization of the gels, only those bands with a peak height intensity exceeding 2.0% of the strongest band in each lane were included in further analyses. Diversity indices were also calculated: richness (S) was determined from the number of bands in each lane, and the Shannon-Wiener index (H') was calculated from $H' = -\Sigma P_i \ln P_i$ (Shanon and Weaver, 1949), where P_i is the importance probability of the bands in a lane, calculated from n_i/N, where n_i is the peak height of a band and N is the sum of all peak heights in the densitometric curve. Evenness (E) was calculated as $E = H'/H'_{max}$, where $H'_{max} = \ln S$.

3.2 Results

3.2.1 Enumeration and retrieval of bacteria from coral tissue

The total viable counts of retrieved non halophilic bacteria growing on TYE was a high of 7.44×10^{13} cfu/g. The count of marine halophiles growing on 3% TYE was 8.49×10^{13}cfu/g. A low count of halophiles 8.29×10^{6} cfu/g was observed on NTYE media (Figure 3.3).

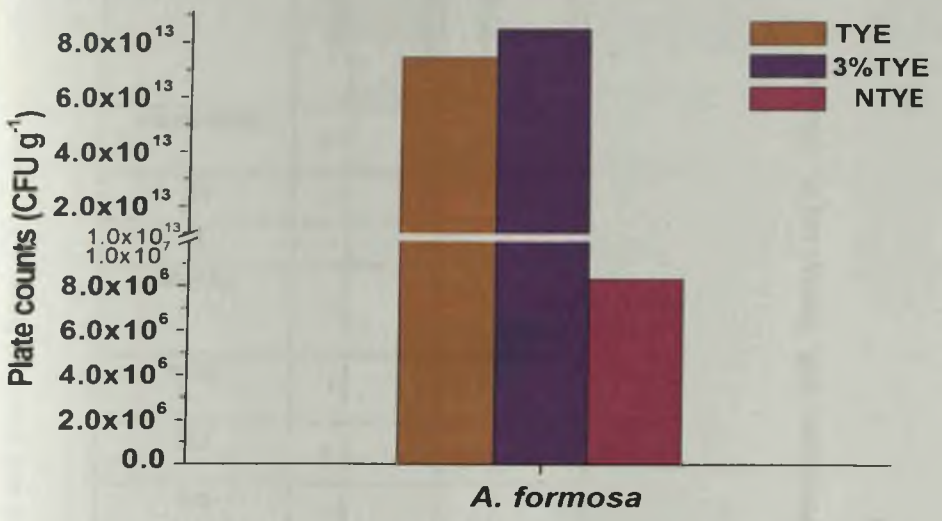


Figure 3.3 Total retrieval bacterial counts in cfu/g on TYE, 3% TYE and NTYE associated with *A.formosa*

3.2.2 Isolation and Characterisation of retrieved Bacteria

The five isolates were identified using the Bergeys Keys of Systematic Bacteriology as *Bacillus* sp., *Pontibacillus* sp., *Corynebacterium* sp. while one remained unidentified as it was lost on repeated subculture. Biochemical potentials and tentative identification of dominant bacterial bionts retrieved and purified from *A.formosa* are tabulated in Table 3.1.

Table 3.1: Biochemical characteristics and tentative identification of dominant bacterial bionts retrieved and purified from *A.formosa*

Coral	Designation	Morphology	Pigmentation	GC	mot	oxi	cat	glu	suc	lac	I	MR	VP	cit	N	0%	3%	25%	Spores	P	M	Isolated on	Identification
A.formosa	GUVFAM-1	Rods	cream	+	M	+	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	3	*Bacillus* sp.
	GUVFAM-2	Rods	orange	+	M	-	+	-	+	+	-	-	+	-	-	+	+	+	-	-	-	3	*Pontibacillus* sp.
	GUVFAM-3	Rods	cream	+	NM	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	0	*Corynebacterium* sp.
	GUVFAM-4	Rods	cream	+	M	+	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	3	*Bacillus* sp.

GC, Gram character, **mot**, motility,**oxi**, oxidase, **cat**, catalase, **glu**, glucose, **lac**, lactose, **VP**, Voges Proskauer; **MR**, Methyl red **N**, Nitrate reduction, **P**,Penicillin, **M**, Mac Conkey (+), present; (-),absent

Similarly, amplification of 16S rRNA gene of coral biont GUVFAM-1 yielded an amplicon which on (BLAST)n search at the NCBI site and subsequent alignment with ClustalW aligned the sequences with 92% *Bacillus lehensis* strain MLB2 16S (NR_036940.1), isolated from soil from Leh, India.

3.2.3 DGGE analysis

The PCR products of 16S rDNA extracted from the coral *Acropora formosa* were analysed by DGGE between the variable region 6 (V6) and variable region 8 (V8) and the DGGE patterns formed are illustrated in Figure 3.4.

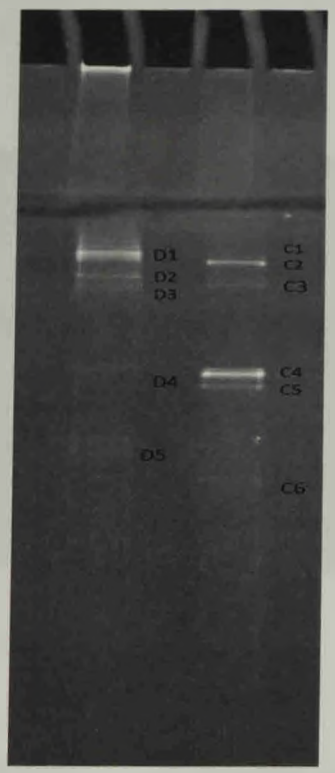


Figure 3.4 Denaturing gel electrophoresis of PCR amplified products. Lane 1 PCR products amplified with GC-COM1 and COM-2(designated D) primers and Lane 2 PCR products amplified with GC-SRV3-1 and SRV3-2(designated C).

A bacterial community profile was generated using denaturing gradient gel electrophoresis (DGGE). Lane 1 shows the PCR products amplified using GC-COM-1 and COM -2 primers (designated as D) while lane 2 shows the PCR amplified products with primers GC-SRV3-1 and SRV3-2 (designated as C). Interestingly, there were five bands obtained using GC-COM-1 and COM -2 primers and six bands obtained using primers GC-SRV3-1 and SRV3-2. All bands obtained were clear enough to be distinguished. The PCR amplified products amplified using SRV3-1 and SRV3-2 primers gave an amplicon of 200 bp (Figure3.5a) while PCR amplified using COM-1 and COM-2 primers gave amplified products with a size of 400 bp (Figure 3.5b).

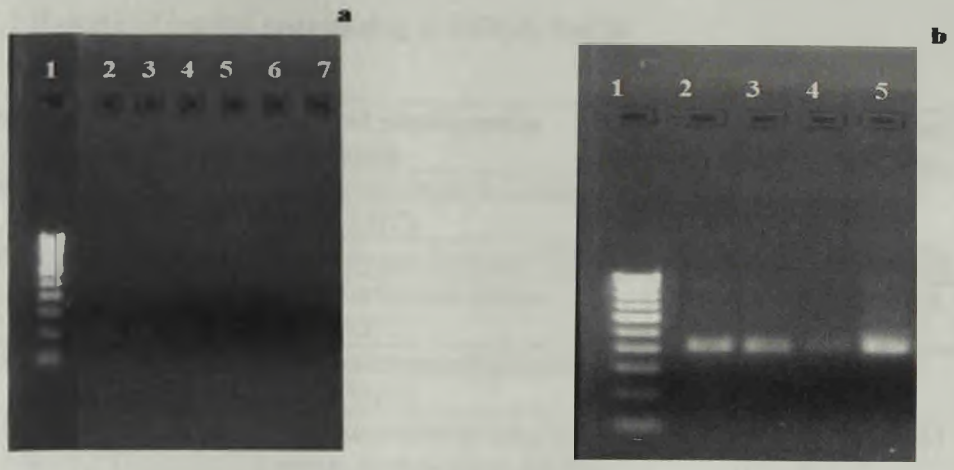


Figure 3.5 a. Agarose gel electrophoresis of bands eluted from DGGE and PCR amplified using SRV3-1 and SRV3-2 primers. Lane 1 is 100bp ladder and Lane 2 to 7 PCR amplified products with size 200bp.

Figure 3.5 b. Agarose gel electrophoresis of bands eluted from DGGE and PCR amplified using COM-1 and COM-2 primers. Lane 1 is 100bp ladder and Lane 2 to 5 PCR amplified products with size 400bp.

Sequencing analysis of the predominant bacteria, its closest phylogenetic affiliation and percent homology that represent the DGGE bands is summarized in Table 3.2.

Table 3.2 Results of partial sequencing of DGGE bands

DGGE band designation	Closest phylogenetic affiliation	Percent homology	Accession Numbers
D1 (COM-1 and COM-2)	*Brevundimonas halotolerans* MCS 24(T)	97.8	M83810
D3 (COM-1 and COM-2)	*Luteimonas lutimaris* G3(T)	85.38	GU199001
D4 (COM-1 and COM-2)	*Brevundimonas staleyi* FWC43(T)	83.84	AJ227798
D5 (COM-1 and COM-2)	*Brevundimonas halotolerans* MCS 24(T)	97.8	M83810
C1 (SRV3-1 and SRV3-2)	*Polynucleobacter necessarius subsp. asymbioticus* QLW-P1DMWA-1(T)	81.12	CP000655
C2 (SRV3-1 and SRV3-2)	*Pseudomonas toyotomiensis* HT-3(T)	78.91	AB453701
C3 (SRV3-1 and SRV3-2)	*Bacillus thermolactis* R-6488(T)	81.21	AY397764
C4 (SRV3-1 and SRV3-2)	*Lysobacter xinjiangensis* RCML-52(T)	86.9	EU833988
C5 (SRV3-1 and SRV3-2)	*Candidatus Accumulibacter phosphatis* UW-1/CP001715	85.93	AJ224937
C6 (SRV3-1 and SRV3-2)	*Lysobacter xinjiangensis* RCML-52(T)	87.58	EU833988

Once generated fingerprint similarity could be assessed to determine the structure of microbial assemblage. All the sequences obtained in this study have been submitted to the GenBank nucleic acid sequence database and accession numbers are tabulated in Table 3.2.

None of the sequences obtained in this study were closely related to planktonic bacteria having a large representation in GenBank. Most of the sequences showed less than 90% similarity to the sequences deposited in the Genebank. The band D1 was identified as *Brevundimonas halotolerans* as it showed 97.8% homology with the type strain *Brevundimonas halotolerans* MCS 24(T). Band D3 identified as

*Luteimonas lutimaris*showed 85.38% similarity to the *Luteimonas lutimaris* G3(T). Band D4 and D5 were identified as *Brevundimonas staleyi* and *Brevundimonas halotolerans* as its showed 83.84% and 97.8% to *Brevundimonas staleyi* FWC43(T) and*Brevundimonas halotolerans* MCS 24(T). Band C1 and C2 amplified by the second set of primers (SRV3-1 AND SRV3-2) were identified as *Polynucleobacter necessarius subsp. asymbioticus* and *Pseudomonas toyotomiensis* as they showed 81.12% and 78.91% similarity to the type strains *Polynucleobacter necessarius subsp. asymbioticus* QLW-P1DMWA-1(T) and *Pseudomonas toyotomiensis* HT-3(T). C3 and C4 on the other hand, showed 81.21% similarity with strains *Bacillus thermolactis* R-6488(T) and 86.9 % similarity to *Lysobacter xinjiangensis* RCML-52(T). Over 50% of the sequences were putative novel species (i.e. less than 97% similarity to GenBank entries).

One exception was a recurrent ribotype with 97% similarity to *Brevundimonas halotolerans*, a genus that belongs to the α- proteobacteria. Interestingly, this genus is prevalent in other coral species, and is known to be present in marine biofilms (Abraham, 2010).

Phylogenetic trees showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics is shown in Figure 2.28 a and b. The taxa joined together in the tree are implied to have descended from a common ancestor.

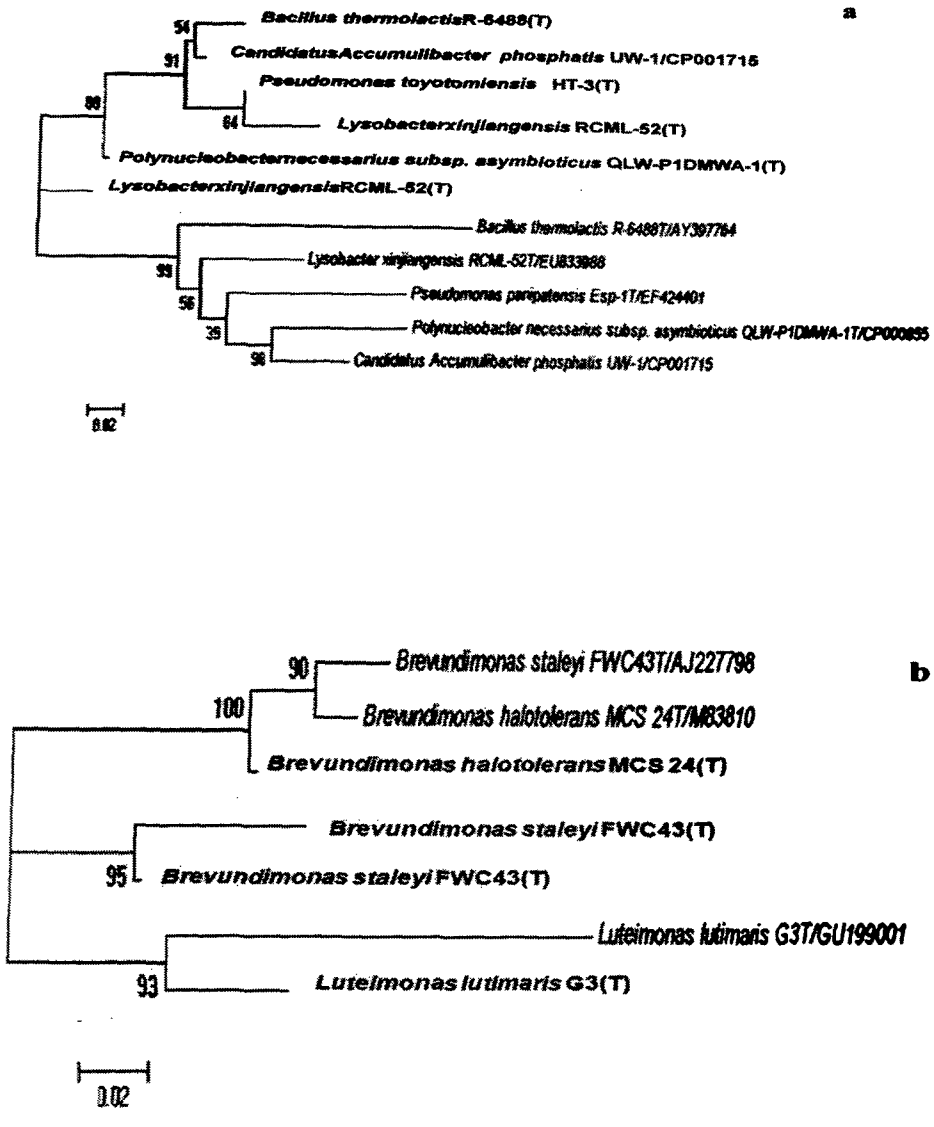


Figure 2.33 a and b Phylogram indicating the placement and relatedness of the isolates is indicated. Numbers given at branch nodes indicate (%) boothstrap values. Bar, 0.02 substitutions per 100 nucleotide positions

The diversity indices namely Diversity indices richness (*S*) Shannon-Wiener index (*H'*) Evenness (*E*) calculated calculated from the PCR-DGGE banding profiles tabulated in Table 3.3. DGGE thus provided a good estimate of the abundance of predominant microbial community members.

Table 3.3 Diversity indices namely Diversity indices richness (*S*) Shannon-Wiener index (*H'*) Evenness (*E*) calculated calculated from the PCR-DGGE banding profiles

Lanes	*H'*	H'_{max}	Evenness (*E*)
L1	1.31730149	2.944439	0.447386
L2	0.544111601	2.944439	0.184793
L3	1.885285527	2.944439	0.640287
L1	2.516455008	2.944439	0.854647
L2	1.392524911	2.944439	0.472934
L3	2.778819272	2.944439	0.943752
L4	0.750671025	2.944439	0.254945
L5	1.990361912	2.944439	0.675973

***H'*, Shannon-Wiener index, H'_{max}, maximum Shannon-Wiener index, (*E*) Evenness**

3.3 Discussions

The microbial community of *A. formosa* comprised of eight different bacterial groups with sequences, falling primarily within the Proteobacter cluster and is dominated by γ-proteobacteria. The retrieval of these closely related ribotypes, all of which fall within a particular cluster of the γ-proteobacteria, from coral specimens of different species and geographical regions (Bourne and Munn, 2005; Ainsworth *et al.,* 2006) suggests that this group of γ-proteobacteria occupies a specific niche possibly associated with reef coral tissues, and warrants a classification for this group. The consistent finding of this bacterial group intracellularly in sea slugs and anemones (Kurahasi and Yokota 2007; Schuett *et al.,* 2007) and in reef building corals and sponges suggests that these bacteria occur globally, associated with various benthic marine invertebrates, and warrant further investigations into their relationship with the host species.

Proteobacteria are common in marine environments (Sekiguchi *et al.*, 2002; Wagner-Do¨ bler *et al.*, 2002; Imhoff, 2001) and are always associated with marine plants (Weidner *et al.*, 2000) or animals (Lau *et al.*, 2002). Proteobacteria including α, h and γ-subdivisions have been found in many corals from different marine locations, and clone libraries demonstrated that the majority of retrieved sequences from coral tissue slurry libraries affiliated with *γ-Proteobacteria* (Bourne and Munn, 2005; Frias-Lopez *et al.*, 2002). Proteobacteria has been suggested to have varied effects on hosts such as nitrogen fixation (Burnett and Mckenzie, 1997) and manipulating host reproduction (Stouthamer *et al.*, 1999). Kalinovskaya *et al.*, 2004 demonstrated that Proteobacteria produce low molecular-weight biological active compounds with antimicrobial and surface-active properties. Proteobacteria was also found to produce enzymes at high levels

for degrading protein and polysaccharides (Groudieva *et al.*, 2004). In addition, a sulfate-reducing function of Proteobacteria was suggested by Hayes and Lovely (2002), and bioactive compounds have been found in *Cytophaga* isolated from the marine environment. It was interesting to find *Bacillus thermolactis* normally occurring in dairy environments and *Lysobacter xinjiangensis* isolated from an abandoned gold mine (Icoorevits *et al.*, 2011; Liu *et al.*, 2011).

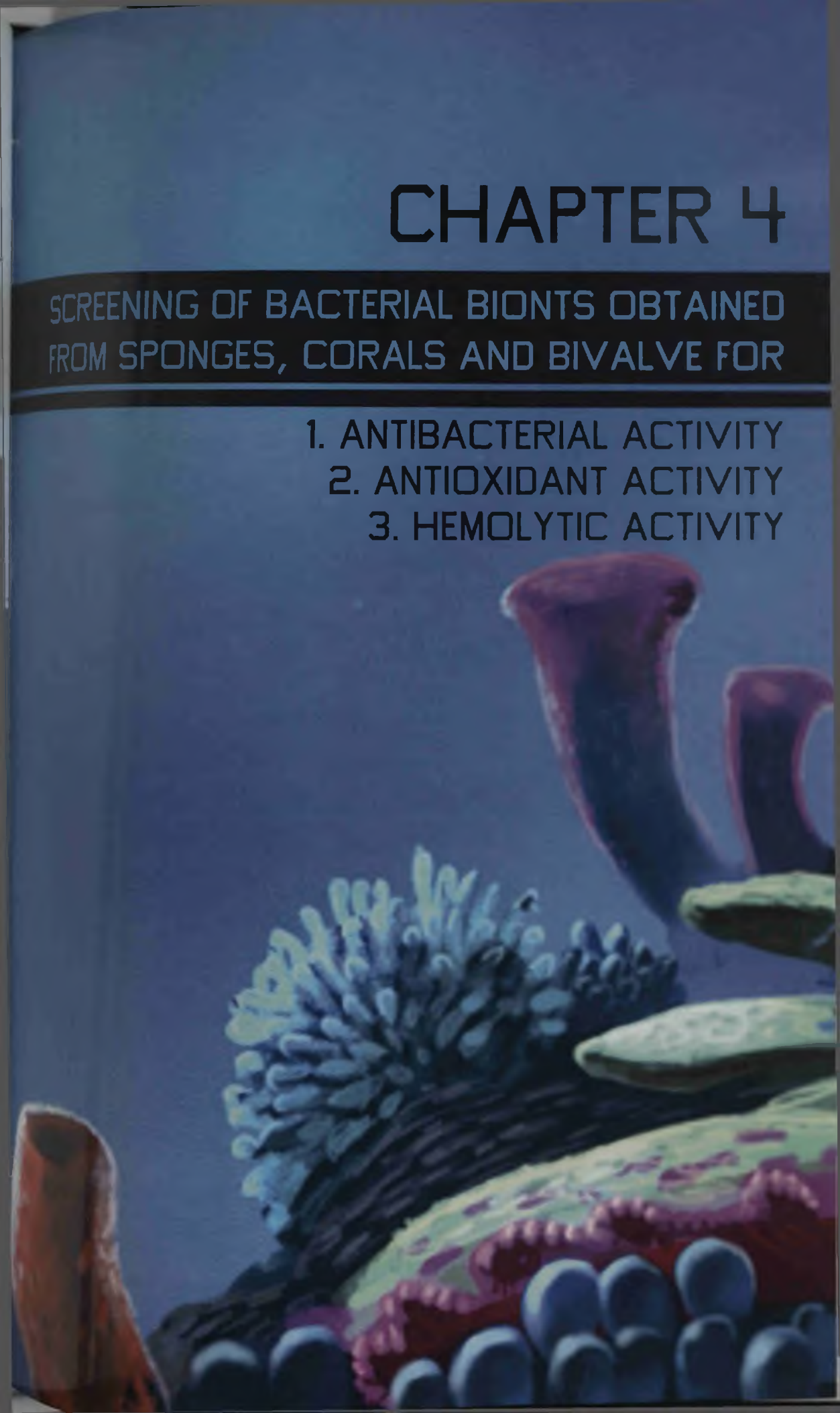
3.4 Conclusions

Thus, we conclude that the comprehensive 16S rRNA-based molecular approach to describing the bacterial community composition in *A. formosa* was valuable in revealing the diversity of bacteria associated with this coral. Thus, corals do appear to be reservoirs of currently uncultured, elusive marine microorganisms that might be a probable reservoir for production of novel pharmaceutically important compounds.

CHAPTER 4

SCREENING OF BACTERIAL BIONTS OBTAINED FROM SPONGES, CORALS AND BIVALVE FOR

1. ANTIBACTERIAL ACTIVITY
2. ANTIOXIDANT ACTIVITY
3. HEMOLYTIC ACTIVITY



Section A: Screening of bionts from Sponges, Corals and Bivalve for antibacterial activity

Soil has been widely explored as the source of micro-organisms possessing large number of bioactive molecules. However, the continual and cyclic need of new antibiotics to combat the emerging resistant forms of bacterial pathogens has led to the exploration of newer econiches and biota, thereof. Marine ecosystem and its organisms, particularly the invertebrates, such as sponges, coelenterates (sea whips, sea fans and soft corals), tunicates, molluscs (nudibranchs, sea hares, etc.), echinoderms (starfish, sea cucumbers, etc.) and bryozoans (moss animals) (Proksch *et al.*, 2002; Kijjoa and Sawangwong, 2004) are recent targets of bioprospecting and mining for a large group of structurally unique natural products encompassing a wide variety of chemical classes such as terpenes, polyketides, acetogenins, peptides and alkaloids of varying structures representing and having pronounced pharmacological activities (Wright, 1998). The diversity of marine organisms and the highly competitive environmental habitats in which access to space and nutrients are limited is responsible for this stunning variety.

One school of thought is that these marine invertebrates combat potential invaders, predators or competitors by producing secondary metabolites as chemical weaponary of their defense mechanisms (Le Kam Wah *et al.*, 2006). Several of these metabolites have been characterized to be enzymes, hemolytic factors and antibiotics. In the recent years, however, investigations reveal that these bioactive factors may not be products of the marine invertebrates but may actually be produced by the microorganisms associated and or inhabiting the sessile hosts (Proksch *et al.*, 2002; Lee *et al.*, 2001; Jajayatuake *et al.*, 1996; Mitova *et al.*, 2003; Suzumura *et al.*, 2003). Additionally it is increasingly becoming evident that

numerous natural products from marine invertebrates have striking structural similarities to metabolites of microbial origin. This poses a serious question on the role of host associated microbes: whether these are the true source of the metabolites used in defense by the host? or whether these are intricately involved in biosynthesis of the metabolites used in defense (Proksch *et al.*, 2002). Studies in these lines have demonstrated that microbes associated with invertebrates far exceed, in their bioactivity, as against that produced by free living planktonic bacteria (Boyd *et al.*, 1999; Lu *et al.*, 2009). Inspite of this, there are limited reports on studies attempting to retrieve bacteria inhabiting marine invertebrates of Indian origin, into cultures, and scrutinizing their antibacterial activity (Prabha *et al.*, 2010; Rodrigues *et al.*, 2004; Anand *et al.*, 2006)

The Gulf of Mannar, is the world's richest marine bioreserve lying between the southern tip of India, the south-eastern coast of Tamil Nadu state, and the north-west coast of Sri Lanka. It supports a diverse and productive community of marine life. The Gulf of Mannar, is reported to harbour 295 species of sponges, 106 species of corals, 466 species of molluscs including 271 gastropods, 174bivalves, 5 polyplacophorans, 16 cephalopods, 5 scaphopods, 100 species of echinoderms 180 species of marine algae, seaweeds, etc (Ramadhas *et al.*,1999).

In view of the limited reports on the antibacterials produced by bacteria isolated from marine sponges (Prabha *et al.*, 2010 and 2011; Rodrigues *et al.*, 2004; Saravanakumar *et al.*, 2011; Thakur and Anil, 2000) corals (Chinnachamy *et al.*, 2011; Gnanambar *et al.*, 2008) and bivalves (Chandran *et al.*, 2009) of Indian origin.

4.1 A.Methodology

4.1.1 A. Bacterial Bionts

100 heterotrophic, halophilic bacterial bionts isolated from 9 sponges (*Petrosia testudinaria, Cinachyra cavernosa, Haliclona* sp., *Callyspongia fibrosa,Heteronema erecta, Fasciospongia cavernosa, Callyspongia reticutis var solomonenesis* and two unidentified sponge samples), 4 corals (*Telesto* sp., *Echinogorgia reticulata, Echinomuricea indica, Echinogorgia complexa,* and the bivalve (*Perna viridis*) collected from the Mandapam in the Gulf of Mannar were screened for antagonistic activity.

4.1.2 A.Human bacterial pathogens

Human bacterial pathogens *S. typhi, E. coli, P. vulgaris, A. aerogens, S. marcesans, S. citreus* obtained from Goa Medical College, Bambolim ,the State Government hospital were pregrown separately in 5 ml Nutrient broth medium to an O. D. of 1 at 600 nms for 24 h at R.T.

4.1.3 A.Screening of bacterial bionts for antibacterial activity.

Individual bacterial bionts were inoculated into 5 ml of 3% TYE and NTYE. Culture broth showing visual growth occurring in 2/7days respectively were dispensed into agar cups borne onto Muller Hinton agar plated spread plated with the individual pregrown human bacterial pathogens.

4.1.4 A.Demonstration of cell associated antibacterial factor

Individual bacterial bionts were cultured in 3% TYE and NTYE at R.T. (22-28°C) for a minimum of 2 days and a maximum of 7 days at 150 rpm. The cell free

supernatant obtained on separation of cells by centrifuging at 12000 × g for 20 minutes at 4° was extracted three times in ethyl acetate (EA), concentrated to dryness under vacuum using rotary evaporator (Buchi-Germany). Crude extract was dissolved in methanol and used for bioactivity studies.

4.1.5 A.Quantification of antibacterial activity

Mueller Hinton agar plates of 3 mm thickness were seeded with the pregrown bacterial pathogens. 4 mg/l of crude extracts was dispensed into agar cups, 6 mm in diameter borne onto the Mueller Hinton agar plates. The plates were kept standing at low temperature (4°C) for 15 minutes, incubated at 37°C and monitored for growth over a period of 24 h. Assay was carried out in triplicates and mean was recorded. Controls were maintained for each test pathogen. Each experimental data set was carried out aseptically. Zones of inhibition were measured in mms and data was computed using the earlier reported quantification procedure of Velho-Perira and Kamat, 2011 to obtain:

Percent area specific differential antibiotic activity score (PASDAAS)=[AWG/TSA]x100.....(1) where AWG is the area on the plate without growth of test pathogen [area of zone of inhibition-area of the plug (28.26 mm^2)], TSA is the total swabbed area of the pathogen on the plate (6358.5 mm^2)

Percent multispecific antibiosis efficiency score (PMSAES), computed using the Eqn. $PMSAES=(\Sigma PASDAASTP_{1-6}/TPS) \times 100$..... (2), where, $\Sigma PASDAASTP_{1-}$

$_6$ is the percent area specific differential antibiotic activity score of test pathogens 1-6 and TPS is total possible score for all test pathogens (i. e. 100 × 6 = 600).

Percent overall inhibition efficiency score (POIES), was calculated using the following Eqn, POIES= (TNIS/TNTS) × 100..... (3), where, TNIS is total number of inhibited species and TNTS is total number of test species. The ideal score for multispecific inhibition would be 100.

Percent overall screening efficiency score (POSES). This is computed by the Eqn, POSES= (TPR/TAS) × 100..... (4), where TPR is total no of positive results of each test pathogen and TAS is total no of bionts.

4.1.6 A. Analysis of Chemical Characterization of antibacterial extracts

The extracellular ethyl acetate extracts of select bioactive extracts were subjected to preliminary chemical characterization using IR Spectroscopy.

4.2 A. Results

4.2.1 A. Screening of bacterial bionts for antibacterial activity

Hundred euryhaline bacterial bionts obtained from marine sponges, bivalve and corals were characterized on the basis of their ability to tolerate a maximum of 3% and 25% NaCl concentrations during growth in Tryptone Yeast Extract medium (TYE) into: Group I: Bionts growing in TYE with 3% NaCl and Group II: Bionts growing in TYE with 25% NaCl

Culture broths of bionts growing in their respective growth medium were screened for the production of extracellular bioactivity by directly exposing individual indicator cultures of bacterial pathogens to specific quantity of culture broth and observed for development of zone of inhibition of growth. The bionts giving a minimum zone of inhibition of 2 mm were considered as bioactive. 20% of Group I bionts were active against Gram positive indicator cultures while 23% were active against the Gram negative indicator cultures. On the other hand, 80% of bionts from Group II were active against Gram positive indicator cultures and 77.27% of bionts were active against Gram negative indicator cultures. Consequently 46 out of 100 bionts were selected for further investigation wherein 2/7 day cell free supernatants of active bionts were extracted in ethyl acetate and used for monitoring of invitro antibacterial activity.

As seen in (Tables 4.1, 4.2 and 4.3) extracts of bionts associated with nine different sponges, five different corals and one bivalve were active, with individual bionts

having indicator culture specificity. The zones of inhibition ranged from 1-30 mm for sponge bionts, 1-20 mm for bivalve bionts and 2-30 mm for coral bionts.

Table 4.1 Antibacterial activity of ethyl acetate extracts of sponge bionts

SPONGES	ISOLATES	ZONE OF INIHIBITION (mm)						GENERA
		S. typhi	*S. marcesans*	*A. aerogens*	*E. coli*	*S. citreus*	*P. vulgaris*	
Petrosia testudinaria (MAM-1)	GUVFPM-1	6	-	6	-	5	-	*Chromohalobacter* sp.
	GUVFPM-2	-	-	2	-	-	-	*Corynebacterium* sp.
	GUVFPM-6	-	-	-	-	-	2	*Pontibacillus* sp.
Cinachyra cavernosa (MAM-2)	GUVFCCM-2	8	-	20	30	12	10	*Haloarchaea*
Haliclona sp. (MAM-4)	GUVFHM-2	7	-	8	-	7	-	*Chromohalobacter* sp.
Unidentified (MAM-5)	GUVFUM-1	10	-	8	-	5	11	*Corynebacterium* sp.
Callyspongia fibrosa (MAM-6)	GUVFCFM-3	1	-	-	-	-	-	*Corynebacterium* sp.
Heteronema erecta (MAM-10)	GUVFHEM-4	-	-	-	10	20	18	*Pseudomonas* sp.
Callyspongia reticutis var solomonensis (NIO1)	GUVFCM-1	2	4	-	-	3	6	*Haloarchaea*
Fasciospongia cavernosa (NIO2)	GUVFFM-1	-	10	1	1	-	17	*Haloarchaea*
	GUVFFM-2	7	6	5	-	-	-	*Haloarchaea*
	GUVFFM-3	12	-	9	11	10	-	*Chromohalobacter* sp.
Unidentified (NIO3)	GUVFSM-1	7	6	5	3	-	14	*Chromohalobacter* sp.

Table 4.2 Antibacterial activity of ethyl acetate extracts of coral bionts

CORALS	ISOLATES	ZONE OF INIHIBITION (mm)						GENERA
		S. typhi	*S. marcesans*	*A. aerogens*	*E. coli*	*S. citreus*	*P. vulgaris*	
Telesto sp. (MAM-3)	GUVFTM-1	2		2	-	-	-	*Bacillus* sp.
	GUVFTM-2	6	-	15	12	12	12	*Chromohalobacter* sp.
	GUVFTM-3	2	-	-	-	-	-	*Bacillus* sp.
	GUVFTM-4	-	2	3	-	-	-	*Bacillus* sp.
	GUVFTM-5	2	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFTM-6	-	-	2	-	-	-	*Chromohalobacter* sp.
Echinogorgia reticulata (MAM-7)	GUVFERM-1	-	-	2	-	-	-	*Corynebacterium* sp.
	GUVFERM-2	-	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFERM-7	-	-	8	-	-	-	*Chromohalobacter* sp.
Echinomuricea indica (MAM-8)	GUVFEIM-1	5	-	-	-	-	-	*Chromohalobacter* sp.
	GUVFEIM-3	-	-	30	-	-	-	*Chromohalobacter* sp.
	GUVFEIM-8	-	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFEIM-9	-	-	2	-	-	-	*Chromohlobacter* sp.
	GUVFEIM-14	-	-	2	-	-	-	*Psychrobacter* sp.
Echinogorgia complexa (MAM-9)	GUVFECM-1	-	-	-	10	30	-	*Deinococcus* sp.
	GUVFECM-2	2	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFECM-3	-	-	12	-	-	15	*Chromohalobacter* sp.
	GUVFECM-5	-	6	-	2	5	-	*Chromohalobacter* sp.
	GUVFECM-6	-	5	-	-	-	-	*Chromohalobacter* sp.
	GUVFECM-7	-	-	2	-	-	-	*Bacillus* sp.
	GUVFECM-8	-	-	2	-	-	-	*Virgibacillus* sp.
	GUVFECM-9	-	-	5	-	-	-	*Chromohalobacter* sp.

Table 4.3 Antibacterial activity of ethyl acetate extracts of bivalve bionts.

BIVALVE	ISOLATES	ZONE OF INIHIBITION (mm)						GENERA
		S. typhi	***S. marcesans***	***A. aerogens***	***E. coli***	***S. citreus***	***P. vulgaris***	
Perna virdis (GUVFM)	GUVFPVM-1	7	-	7	2	-	10	*Planococcus* sp.
	GUVFPVM-2	20	-	6	5	15	-	*Bacillus* sp.
	GUVFPVM-3	7	-	-	-	-	-	*Bacillus* sp.
	GUVFPVM-4	2	-	-	4	8	1	*Bacillus* sp.
	GUVFPVM-5	3	-	8	-	-	-	*Psychrobacter* sp.

4.2.2 A. Quantification of antibacterial activity

Differential antibacterial activity of individual bionts towards various indicator pathogens was deduced by comparing zone sizes of antibacterial activity (Figure 4.1) and recorded as Percent area specific differential antibiotic activity score (PASDAAS). Highest PASDAAS of 53.5% was shown by the sponge biont GUVFCCM-2 against *E. coli* and by the coral bionts namely GUVFEIM-3 and GUVFECM-1 against *A.aerogens* and *S. citreus* respectively. Using PASDAAS, Percent multispecific antibiosis efficiency score (PMSAES) was calculated.

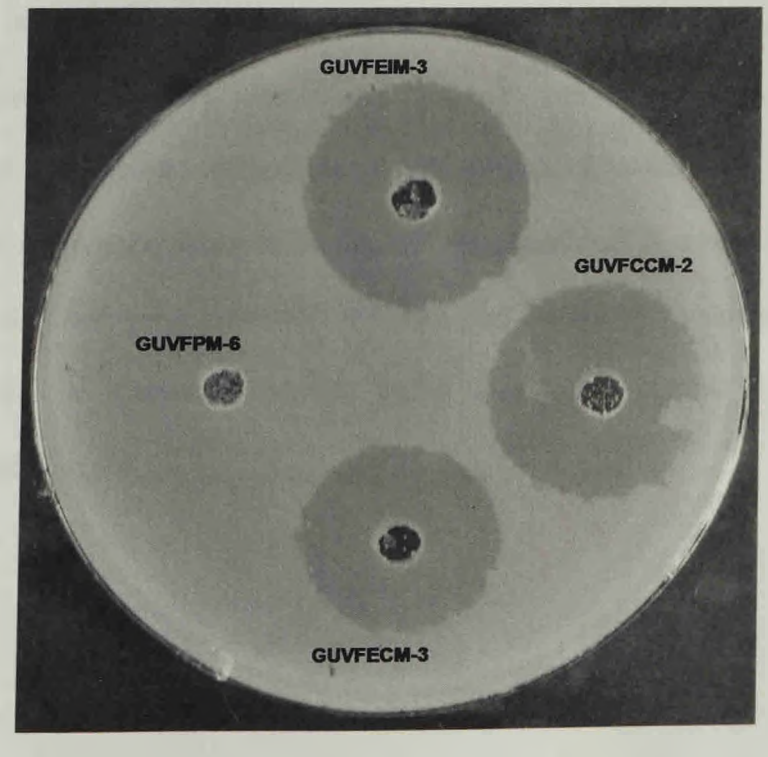


Figure 4.1 Antibacterial activities of bionts GUVFCCM-2, GUVFEIM-3 and GUVFECM-3 against *A. aerogens*

The highest PMSAES (Percent multispecific antibiosis efficiency score) (Figure 4.2) among the sponge bionts was shown by GUVFCCM-2, identified as *Haloarchaea* with a value of 17.2%. It was active against all the tested indicator pathogens except *S. marcesans*. This was followed by GUVFHEM-4 with a value of 9.08%, identified as *Pseudomonas* sp. GUVFHEM-1 was active against *E. coli*, *S. citreus* and *P. vulgaris*. Sponge bionts with PMSAES values in the range 2-6% were GUVFSM-2, with a value of 5.74%, it was identified as *Chromohalobacter* sp. and was active against *S. typhi*, *A. aerogens*, *E. coli* and *S. citreus*. Biont GUVFFM-1 with a value of 4.64% was identified as Haloarchaea and was active against *S. marcesans*, *A. aerogens*, *E. coli* and *P. vulgaris*. Bionts GUVFSM-1 identified as *Chromohaobacter* sp. and biont GUVFUM-1 identified as *Corynebacterium* sp., had near equal PMSAES values of 4.48% and 4.22% respectively. GUVFSM-1 was active against *S. typhi, S. marcesans*, *A. aerogens*, *E. coli* and *P. vulgaris* while biont GUVFUM-1 inhibited *S. typhi*, *S. citreus, P. vulgaris* and *A. aerogens*. Biont GUVFHM-2, had a PMSAES value of 2.38% and was identified as *Chromohalobacter* sp. It was active against *S. citreus*, *A. aerogens* and *S. typhi*.

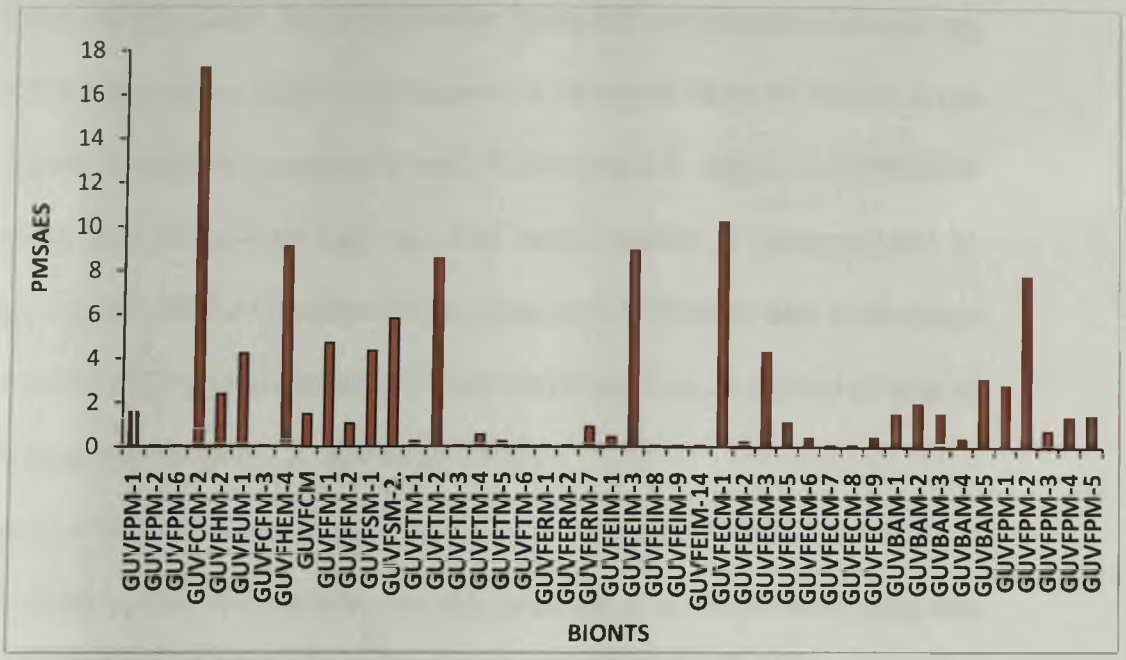


Figure 4.2 Percent Multispecific Antibiosis Efficiency Score (PMSAES) exhibited by bacterial bionts

Bionts having PMSAES values in the range 0.05-2% were GUVFPM-1 identified as *Chromohalobacter* sp., having a PMSAES value of 1.61% and was inhibitory to *S. typhi*, *A. aerogens* and *S. citreus*. Biont GUVFCM with a value of 1.46% was identified as Haloarchaea and was active against *P. vulgaris*, *S. citreus, S. marcesans* and *S. typhi*. GUVFFM-2 identified as Haloarchaea was inhibitory against *E. coli* and *S. marcesans* and had a value of 1.03%. GUVFPM-2 and GUVFPM-6 had similar values of 0.11% and were identified as *Corynebacterium* sp. and *Pontibacillus* sp. respectively. They were active against *A. aerogens* and *P. vulgaris* respectively. GUVFCFM-3 had the lowest value of 0.05%, was identified as *Corynebacterium* sp. and was solely active against *S. typhi*.

Coral biont with highest PMSAES value of 10.23% was GUVFECM-1 identified as *Deinococcus* sp., was active against *S. typhi* and *A. aerogens*. This was followed by GUVFEIM-3 and GUVFTM-2 with near equal PMSAES values of 8.91% and

8.51% respectively. Both the isolates were identified as *Chromohalobacter* sp. GUVFEIM-3 was active solely active against *A. aerogens* while GUVFTM-2 was active against *S. typhi*, *A. aerogens*, *E. coli*, *S. citreus* and *P. vulgaris*. GUVFECM-3 identified as *Chromohalobacter* sp., was active against *A. aerogens* and *P. vulgaris* and had a PMSAES value of 4.36. Biont with PMSAES value in the range 1-2% was GUVFECM-5 identified as *Chromohalobacter* sp., it showed a value of 1.15% against *S. marcesans*, *E. coli* and *S. citreus*.

Bionts with PMSAES values in the range 0.2-1% were GUVFERM-7 at 0.91%, it was identified as *Chromohalobacter* sp. and inhibited *A. aerogens* only. This was followed by biont GUVFTM-4 at 0.53%, identified as *Bacillus* sp. and active against *A. aerogens* and *S. marcesans*. Bionts with equal values of 0.45% were GUVFEIM-1, GUVFECM-6 and GUVFECM-9. They were all identified as *Chromohalobacter* sp. GUVFEIM-1 inhibited *S. typhi*, GUVFECM-6 was active against *S. marcesans* while GUVFECM-9 inhibited *A. aerogens* alone. GUVFTM-5, GUVFTM-1, GUVFECM-2 had similar PMSAES values of 0.23% and were identified as, *Chromohalobacter* sp., *Bacillus* sp. and *Chromohalobacter* sp. and were all active against *S. typhi* and *A. aerogens*. Bionts GUVFTM-3, GUVFTM-6, GUVFERM-1, GUVFERM-2, GUVFEIM-7, GUVFEIM-10, GUVFEIM-12, GUVFECM-8, GUVFECM-7, GUVFECM-8 all showed similar PMSAES value of 0.11%. They were identified as *Bacillus* sp., *Chromohalobacter* sp., *Corynebacterium* sp., *Chromohalobacter* sp., *Chromohalobacter* sp., *Chromohalobacter* sp., *Chromohalobacter* sp., *Bacillus* sp., and *Virgibacillus* sp. respectively. GUVFTM-3 inhibited *S. typhi* and *A. aerogens* while the rest were active solely against *A. aerogens*.

Among the bivalve bionts GUVFPVM-2 identified as *Bacillus* sp. showed the highest PMSAES value at 7.8% and was active against *S. typhi*, *A. aerogens*, *E. coli* and *S. citreus*. This was followed by biont GUVFPVM-1 with a relatively lower value of 2.85%. It was identified as *Bacillus* sp. and was inhibitory against *S. typhi*, *A. aerogens*, *E. coli* and *P. vulgaris*. Bionts GUVFPVM-4 and GUVFPVM-5 identified as *Bacillus*sp.and *Psychrobacter* sp. respectively showed near equal PMSAES values of 1.45% and 1.5% respectively. GUVFPVM-4 inhibited *S. typhi*, *E. coli*, *S. citreus* and *P. vulgaris* while GUVFPVM-5 was active against *S. typhi* and *A. aerogens*. Lowest PMSAES was shown by biont GUVFPVM-3 identified as *Bacillus* sp. with a value of 0.73% against *S. typhi*.

The calculated Percent overall inhibition efficiency score of bionts (POIES) indicated that 33% of the bionts were active against *A. aerogens*, 21% inhibited *S. typhi,* 14% were active against *E. coli,* 13% against *S. citreus* and 12% against *P. vulgaris*. The least inhibited was *S. marcesans* at 6%.

Further the percent overall screening efficiency score of antibacterial activity (POSES) exhibited by bionts was computed by scoring presence or absence of zones by bionts against indicator cultures and depicted in Figure4.3. Highest score of 83.33% was given by sponge bionts GUVFCCM-4 and GUVFSM-1 and coral biont GUVFTM-2.

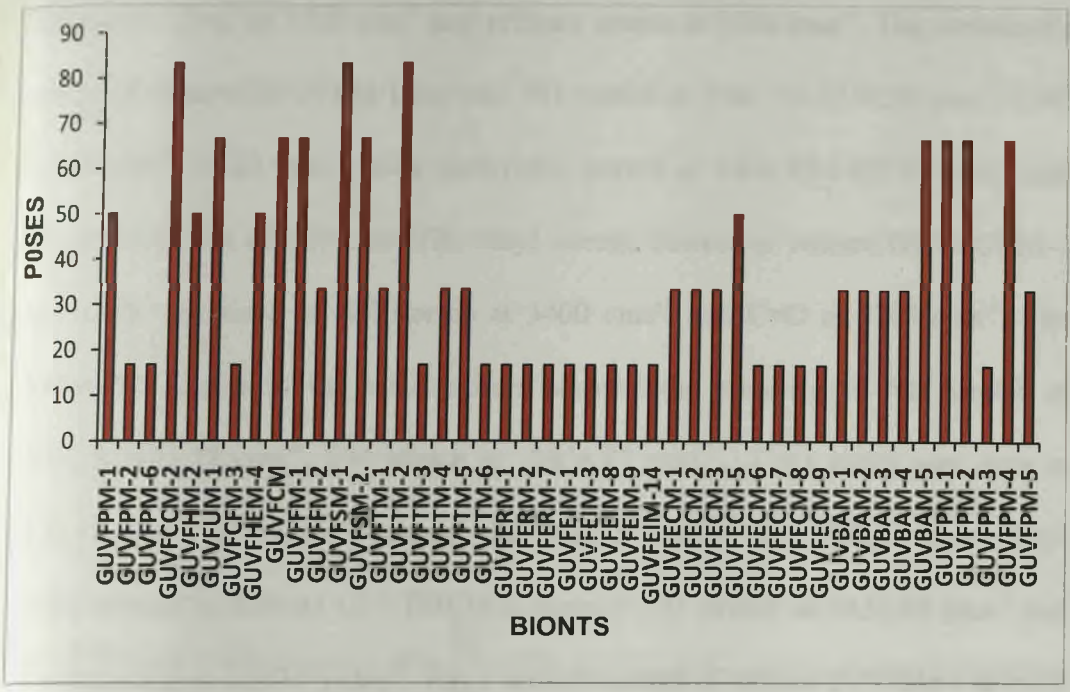


Figure 4.3 Percent Overall Screening Efficiency Score (POSES) of bacterial bionts

4.2.3 A. Analysis and Chemical Characterisation of antibacterial extracts

The ethyl acetate extracts of culture GUVFPM-6 showed the presence of OH bending at 3350 cms^{-1},C-H methylene stretch 2852 and 2902 cms^{-1}, C=C stretch at 1650 cms^{-1}, N-H bending of primary amine 680 cms^{-1} ,1150 cms^{-1} tertiary alcohol 1400 and 1460 cms^{-1} Nitro group of nitro alkane. The methanolic extract of culture GUVFPM-6 showed OH bending at (3400 cms^{-1}), (CH stretch at 2927.94-2958.8 cms^{-1}),ester group was detected at 1730.15 cms^{-1},C=C was detected at 1647.21 cms^{-1},CH methylene bend at 1456.26 cms^{-1},aryl vinyl groups at 1286.52-1271.09 cms^{-1},C-O-C ether group at 1122.57 cms^{-1} and primary amine at 742.59 cms^{-1}.

The methanolic extract of culture GUVFUM-1 showed OH bending (3334.92-3598.16 cms^{-1}), C-H methylene stretch (2852.72 cms^{-1}), C-N aliphatic stretch (C-N aromatic stretch (1338.6 cms^{-1}) and primary amine (1624-1633.71 cms^{-1}). The ethyl acetate extract of culture GUVFFM-1 showed the presence of NH stretch at

3400 cms^{-1}, C=O at 1700 cms^{-1} and primary amine at 1050 cms^{-1}. The methanolic extract of culture GUVFFM-1 showed NH stretch at 3361.93-3518.16 cms^{-1}, C=C at 1624.06-1647.21 cms^{-1}, C-H methylene stretch at 1436.97-1489.05 cms^{-1} and primary amine at 615.29 cms^{-1}The ethyl acetate extract of culture GUVFCCM-2 showed the presence of NH stretch at 3400 cms^{-1} and C=O at 1700 cms^{-1}. The methanolic extract of the same culture showed the presence of NH stretch at 3221.12-3435.22 cms^{-1}, CH stretch at 2956.87 cms^{-1} , C=O group was seen at 1730.15 cms^{-1} and primary amine was seen at 742.59 and 702.09 cms^{-1}.Ethyl acetate extract of culture GUVFHEM-1 showed CH stretch at 2924.09 cms^{-1} and primary amine at 1658.78 cms^{-1}. Ethyl acetate extract of culture GUVFTM-5 showed the presence of NH stretch at 3400.5 cms^{-1}, CH stretch at 2945.3 cms^{-1}, primary amine at1658.78 cms^{-1} and primary alcohol at 1026.13 cms^{-1}. GUVFPM-9 had NH stretch at 3172.9-3278.99 cms^{-1},CH stretch at 2927.94 and 2956.87 cms^{-1},primaryamine at 1643.35-1658.78 cms^{-1} and CH methylene at 1444.68 cms^{-1}. GUVFPM-4 showed the presence of NH stretch at 3400 cms^{-1},CH stretch at 28450-2980 cms^{-1}, CH methylene stretch at 1400-1550 cms^{-1} and primary amine at 1560-1650 cms^{-1}. GUVFPM-2 has NH stretch at 3373.5 and 3358.07 cms^{-1},CH stretch at 2993.73 and 2958.8 cms^{-1}, C=C at 1641.42 and 1654.92 cms^{-1},CH methylene stretch at 1400-1550 cms^{-1}, primary alcohol at 1029.99 cms^{-1} and primary amine at 736.81 and 846.76 cms^{-1}.

Culture cells/biomass of culture GUVFSM was extracted in three solvents namely acetone showing the groups C=O at 1739.79 cms^{-1} and primary amine at signals 671.23 and 648.08 cms^{-1}, chloroform showed signal at 3400 cms^{-1} representing the NH stretch, signal at 1689.64 and 1639.49 cms^{-1} represents C=C, primary alcohol showed a signal at 1093.64 cms^{-1} and signals at 671.23 and 648.08 cms^{-1} represents

primary amine, methanol extract showed a signal at 3361.93 cms^{-1} corresponding to the NH stretch,signal at 2945.3 cms^{-1} corresponding to the CH stretch and signal at 1028.06 cms^{-1} corresponding to primary alcohol. The cell free supernatant extracted in ethyl acetate showed signals at 1745.58 cms^{-1} corresponding to the C=O group,signal at 1045.42 cms^{-1} corresponding to the primary alcohol group and signals 779.24 and 792.74 cms^{-1} representing primary amine (Figure 2.31).

4.3 A. Discussions

The present study was an attempt to investigate the antibacterial activity of bacterial bionts from sponges, corals and bivalve thought to be involved in the epibacterial chemical defense of the host (Thakur and Anil, 2000). In this regard it was a noteworthy observation that 46 isolates out of the 100 screened, showed promising antibacterial activity. These bionts having activity against multidrug resistant clinical pathogens, isolated from hospital patients, have potential to serve as drug candidates. The inhibition of the pathogens by the extracts obtained from the invertebrate associated bacteria strongly supports the hypothesis of the microbial origin of the compounds formerly ascribed to these macro invertebrates as there are several reports on the antibacterial potential of the marine invertebrates used in this study (Rodrigues *et al.*, 2004; Jeyasekaran *et al.*, 2002; Almeida *et al.*, 2011)

Halophilic bacterial strains exhibited a higher antimicrobial activity against the Gram negative bacteria than against the Gram-positive bacteria. These results are not consistent with previous studies wherein Gram-positive bacteria were more susceptible to antibiotics than Gram negative bacteria (Pelaez *et al.*, 1998) The

probable reason for this finding is that only a single Gram positive indicator test was included in the test panel.

Our study corroborates with the findings of Whang *et al.,* (2010), in that approximately 50% of the culturable bionts exhibited antibacterial activity. The results thus confirm that invertebrate-associated microorganisms are highly potential resources of bioactive natural products (Burgess *et al.*, 1999).

The absence of antimicrobial activity in the remaining 50% bionts in the bioassays conducted does not necessarily indicate a lack of antimicrobial chemical defence, as the diffusion assay only measures cell death however there are reports of inhibition of other phenotypic characteristics such as chemotaxis, swarming attachment, swimming etc. which is also a means of counteracting bacterial invasion. Another proposed hypothesis by Geffen and Rosenberg, 2005 for no bioactivity of some of the isolates could be that the release of the bioactive factor is only seen following induction by deleterious micro-organisms and mechanical stress which was not done in our present study. It could also be possible that the bioactive component was not extractable in the ethyl acetate solvent or that it diffuses poorly in the agar medium employed. The demonstration of poor bioactivity by some bacterial isolates associated with the invertebrates is suggestive that the invertebrates resort to some other means of defence rather then production of chemical compounds as reported by Rublee *et al.*, 1980

The isolates having the greatest antimicrobial activity belonged to the genus *Chromohalobacter*, followed by *Bacillus* and *Corynebacterium.* The genus *Bacillus* have been well known to produce lipoproteins, phenolic derivatives, aromatic acids, acetylamino acids (amino acid analogs), peptides (Gebhardt *et al.*, 2002) isocoumarin antibiotics(Pinchunk *et al.*, 2002) bacteriocin like substances

(Bizani and Brandelli, 2002) having a broad antibiotic spectrum, the genus *Corynebacterium* is also increasingly reported as a source of bioactive agents capable of displaying competitive biosynthetic capabilities (Zheng *et al.*, 2000), however the potential of the genus *Chromohalobacter* as a promising resource for antimicrobial compounds is fairly recent with no reports on the structure elucidation of the antimicrobial compound and scarce reports on its antimicrobial activity (Wang *et al.*, 2010) Thus our first report on isolation of halophilic bacterial strains from marine invertebrates, as promising sources for the discovery of novel bioactive compounds andis of immense importance.

The high proportion of antimicrobial producing strains may be associated with an ecological role, i.e. a defensive action to maintain their niche, preventing the invasion of microbial competitors into an established microbial community. Thus marine invertebrates represent an ecological niche harbouring a largely uncharacterized microbial community with unexploited potential sources of new secondary metabolites. Chemical characterization of active extracts by IR Spectroscopy revealed the presence of varied functional groups. It was interesting to note that almost all the extacts showed a lipoproteinaceous nature due to the presence of of NH stretch, primary amine ,carbonyl and aliphatic stretch. As yet, there have been no published reports on the antibacterial activity of all the marine organisms discussed so far from the GoM. Thus this present first report proves that the ethyl acetate extracts of marine bacteria associated with sponges, bivalve and corals are a promising resource having profound antibacterial activity and thus may have potential use in medicine.

Section B: Screening of bionts from Sponges, Corals and Bivalve for antioxidant activity

Shallow-water invertebrates appear to be acclimatized to oxidative stress due to high levels of reactive oxygen species (ROS) (Lesser *et al.,* 1990; Shick, 1991; Tyrrell, 1991) generated through a combination of photosynthesis, symbiont oxygen production, and exposure to intense sunlight (Cunningham *et al.,* 1985). ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•−) and superoxide radicals (O2•−) results from uncoupling at electron transfer sites or via auto-oxidation reactions (Cadenas, 1989), produced during normal aerobic cellular metabolism. As a recognized biological hazard affecting survival, physiology and growth of marine organisms, defense against free-radicals is an important evolutionary step among invertebrates living in shallow-water environments (Jokiel, 1980; Jokiel and York, 1982; Hader and Worrest, 1991). The antioxidant defense system in marine invertebrates is influenced by environmental factors such as water, temperature and oxygen availability, which play a crucial role in the oxidativestress capacity in the different tissues (Verlecar *et al.*, 2008). As temperatures increases, oxygen supplies and feeding-activity are altered and more ROS are produced by the coastal marine invertebrates (Dykens and Shick, 1982; Lesser, 1996, 1997; Nii and Muscatine, 1997; Downs *et al.*, 2000, 2002)

The marine invertebrates reduce ROS by boosting up the concentrations of antioxidants such as catalase, glutathione, α-tocopherol, ascorbic acid, superoxide dismutase (SOD), peroxidase etc. which protect cells and attenuates the damages related to their high reactivity (Halliwell and Gutteridge, 1989).

In recent years, the antioxidant defense of marine invertebrates is also being ascribed to the microbial bionts associated with it (Takamatsu *et al.*, 2003), an aspect which has received little attention so far. Microbial bionts gain access in the host interiors by virtue of its filter feeding mode of nutrition and can play an important role in the invertebrate-microbe metabolic relationships. The conjecture that organisms highly exposed to ROS will have effective antioxidant mechanisms has certainly not disappointed natural product chemists and hence marine organisms and microorganisms associated with it have been mined for antioxidant compounds (Griffin and Chan, 2006; Dunlap *et al.*, 1995). The diverse microbial assemblage occurring in shallow-water coastal environments with extreme variations in pressure, salinity, and temperature have evolved unique metabolic and physiological capabilities to be able to survive in such extreme habitats leading them to produce novel metabolites (Penesyan *et al.*, 2009; Armstrong *et al.*, 2001; Egan *et al.*, 2008)

The present study therefore examines antioxidant producing abilities and their chemical characteristics from a large repository of microbial-bionts retrieved earlier from several invertebrates, native to a coastal embayment of Mandapam in the South-eastern peninsular India.

The present work also facilitates the availability of large number of such cultures for the depository and is therefore of immense significance. In this study we examine the hypothesis that certain peptides may also serve in a complementary fashion as antioxidants for protection against photooxidative stress induced by oxyradicals

4.1 B. Methodology

4.1.1 B. Bacterial bionts

Bacterial bionts isolated from marine sponges, corals and bivalve was screened for antioxidant activity.Identification was carried using Bergeys keys of Systematic Bacteriology.

4.1.2 B. Screening of bionts for antioxidant activity

Hundred halophilic eubacterial bionts isolated from nine sponges, five corals and a bivalve were maintained on 3% TYE and NTYE agar slopes and were inoculated on 3% TYE and NTYE agar plates respectively, using a template of 15 squares. Plates were incubated for growth at ambient temperature (27^0C-30^0C) for a period of 7 days, for cultures growing in NTYE and for 24 hours, for cultures growing on 3% TYE. Thereafter a sterile filter paper (Whatman no. 1) was placed on the growth on agar plates and further incubated for a day at room temperature. The filter paper was then taken out, sprayed with DPPH solution (80μg/ml in ethanol), allowed to dry at ambient temperature (27^0C-30^0C) till white spots were formed around colonies against a purple background. Bacterial bionts showing a zone greater than 3 mms in the initial screening experiment were selected for sequencing of 16S rDNA gene.

4.1.3 B. Demonstration of extracellular antioxidant factor

Individual bacterial bionts were cultured in 3% TYE and NTYE at R.T. (22-28^0C) for a minimum of 2 days for cultures growing in 3% TYE and a maximum of 7 days for cultures growing on NTYE at 150 rpm (Remi-India). The cell free supernatant obtained on separation of cells by centrifuging at 12000 × g for 20

minutes at 4^0C (Remi-India) was extracted three times in ethyl acetate (EA), concentrated to dryness under vacuum using rotary evaporator (Buchi-Germany). Individual crude extracts were weighed and dissolved in methanol to get a 1000µg/ml test solution of crude extract.

4.1.4 B. Free radical scavenging ability of crude extracts of bionts by 1,1-Diphenyl-2-picryl hydrazyl (DPPH) assay.

The free-radicals scavenging capacity of various bacterial extracts of bionts were tested by its ability to bleach the stable 2,2 di-phenyl 2-picryl hydrazyl (DPPH) radical according to the method of Zhou and Elledge, (2000). To 1.5 ml of 0.1 mM DPPH solution in ethanol, 1.5 ml of 1mg/ml concentration of individual extracts were added to make the working volume of 3 ml. The mixture was shaken vigorously and allowed to reach a steady state for 30 minutes at R.T. Decolourization of DPPH was determined by measuring the decrease in absorbance at 517 nms, and the free radical scavenging activity was calculated using the generalisation.

% DPPH scavenging activity = $(A_{control}) - (A_{test}) / (A_{control}) \times 100$

Absorbance of DPPH solution ($A_{Control}$) was measured against 95% ethanol blank. Absorbance of test agent (A_{Test}) was read immediately after 30 minutes. 0.1mg/ml of L-Ascorbic acid was used as the standard for comparison.E xperiments were performed in triplicates and the results averaged. Biont extracts of GUVCCM-4, GUVFECM-7 and GUFPVM-5 showing maximum antioxidant activity was prepared from 1mg/ml stock solution to find the "efficient concentration" or

EC_{50}/IC_{50} value, which is the concentration of substrate that causes 50% loss of the DPPH activity.

4.1.5 B. Superoxide anion radical scavenging ability of crude extracts of bionts

Superoxide anion scavenging activity of crude extracts of bionts was carried out according to Nishimiki, *et al.* 1972. Superoxide radicals were generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the superoxide radicals were generated using 1.0ml of (50 μM) NBT solution, 1 ml (78μM) NADH solution each prepared in 16 mM of Tris HCl buffer pH 6. To this system crude methanolic extracts were added (1mg/ml). The reaction was initiated by adding 1.0ml of (10 μM) phenazine methosulphate (PMS) solution to the mixture, incubated at 25°C for 5 minutes, and monitored for absorbance at 560 nm against blank samples. 0.1% L-Ascorbic acid was used as a reference compound. Decreased absorbance of the reaction mixture was indicated as an increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated by considering the absorbance without the extract as 100%.

Scavenging capacity of crude extracts was calculated using the generalization,

SOD scavenged (%) = $(A_{cont}-A_{test})/ A_{cont}$ x100. Where, A_{cont} is the absorbance of the control, A_{test} is the absorbance of crude extracts.

Biont extracts of GUVCCM-4, GUVFECM-7 and GUFPVM-5 showing maximum antioxidant activity were prepared from 0.1% stock solution to find the "efficient concentration" or EC_{50}/IC_{50} value, which is the concentration required for a 50% inhibition (IC_{50}) of formation of superoxide radicals.

4.1.6 B. Total reducing capacity of crude extracts of bionts

According to the methology of Ulc *et al.*, 2006, crude extracts of culture supernatant of bacterial bionts 100-1000µg/ml were mixed with 2.0 ml of 0.2 M phosphate buffer pH 6.6 and 2.0 ml of 1% potassium ferricyanide $[K_3Fe(CN)_6]$, incubated at 50°C. After 20 minutes, a 2.0 ml aliquot of 10% trichloroacetic acid was added to the mixture and centrifuged at 1036 x g for 10 minutes. 2.5 ml of the upper layer of solution was mixed with 0.5 ml of freshly prepared 1% $FeCl_3$ and the absorbance was measured at 700 nm against reagent blank. Measurements were performed in triplicate.

4.1.7 B. Rapid qualitative assay for antioxidant activity using TLC

Bacterial extracts of only 3 bacteria namely GUVFCFM-3, GUFPVM-5, and GUFPVM-5 showing highest scavenging of DPPH and superoxide free radicles, were spotted onto TLC plates pre-coated with silica gel F_{254}, allowed to dry and were developed in a mixture of solvents namely toluene: cyclohexane in the ratio (8:2) (v/v). The plates were dried at R.T. and were sprayed with 0.02% DPPH in ethanol, allowed to stand at R.T. After 10 minutes white bands appearing against purple background were noted and Rf determined (Sadhu *et al.*, 2003).

4.1.8 B. Characterization of antioxidant factor

Crude ethyl acetate extracts were spotted onto thin layer aluminum sheets pre-coated with silica (SD fine-chem limited Aluchrosep silica gel 60/UV 254). The solvent systems consisted of toluene: cyclohexane (8:2) (v/v). Presence of amino acids were determined by spraying with 0.25% ninhydrin prepared in acetone, sugars were detected using the phenol–sulphuric acid reagent and lipids were visualized using an iodine-saturated chamber. The infra-red spectrum of the antioxidant factor obtained by preparative TLC was recorded on a Fourier Transform Infra-Red spectrometer (Shimadzu IR Prestige – 21) in the 4000 to 400 cm^{-1} spectral region at a resolution of 1902 cm^{-1} after grinding with a 0.23-mm KBr pellet.

4.1.9 B. Statistical analysis

All determinations were conducted in triplicate. The values are expressed as mean ± SD.

4.2 B. Results

4.2.1 B. Screening of bionts for antioxidant activity

Out of the 100 colonies of bionts screened as filter paper replicates for antioxidant activity 55 were positive. 28 bionts exhibiting a white zone greater then 3 mms (Figure 4.4) on a purple background were selected for analysis. Of these eleven were sponge bionts, thirteen were coral bionts and the remaining four were bivalve bionts. *Chromohalobacter israelensis* dominated the cultural assemblage exhibiting antioxidant activity with 46.42% which was followed by *Psychrobacter celer* with 14.28%. 7.14% of the bionts belonged to the genera *Chromohalobacter*

canadensis. *Burkholderia* sp., *Salinicoccus luteus*, *Staphylococcus equorum*, *Lysinibacillus boronitolerans*, *Virgibacillus salexigens*, *Chromohalobacter saresensis*, *Psychrobacter okhotskensis*, *Bacillus lehensis* and *Chromohalobacter nigrandesensis* were least represented with a mere 3.57%. Thus it can be clearly inferred that Gram negative bacteria (82.14%) showed greater antioxidant activity as compared to the Gram positive counterparts (17.85%).

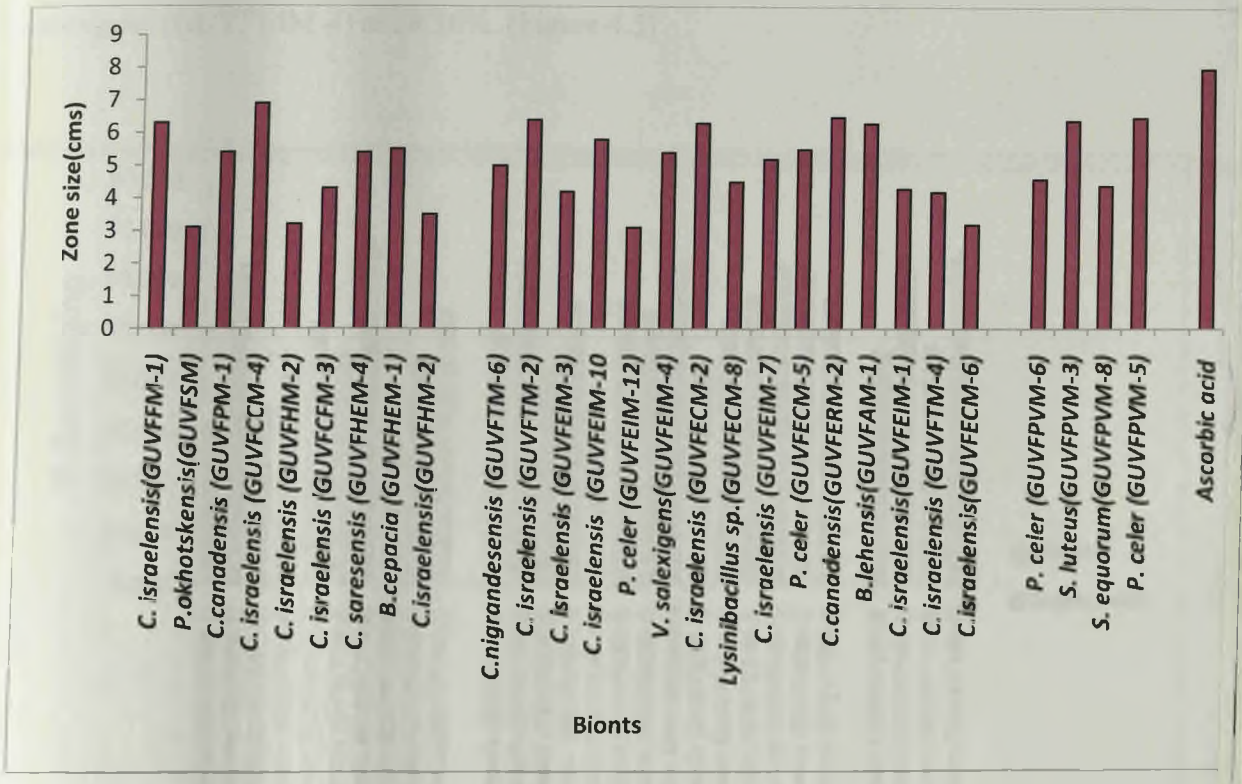


Figure 4.4 Screening of bacterial bionts for antioxidant activity.

4.2.2 B. Free radical scavenging ability of crude extracts of bionts by 1,1-Diphenyl-2-picryl hydrazyl (DPPH) assay.

Among the sponge bionts GUVFCCM-4 showed the highest % scavenging at 67.05% (Figure 1.29). The isolate was identified as *C.israelensis*. Among the coral bionts, GUVFEIM-3 identified as *C. israelensis* showed the highest % scavenging at 63.77%. It was followed closely by biont GUVFEIM-7 identified again as

C.israelensis at 63.52%. Amongst the bivalve bionts isolate GUVFPVM-3 identified as *S.luteus* showed the highest % scavenging at 65.21% followed by GUVFPVM-5 identified as *P.celer* at 64.17%. In our study we found that the extract of sponge biont *C.israelensis* (GUVFCCM-4) showed the maximum DPPH radical scavenging activity 67.05% among all the other extracts tested at a concentration of 1000µg/ml, while the lowest was seen in the coral biont *V. salexigens* (GUVFEIM-4) at 28.56%. (Figure 4.5)

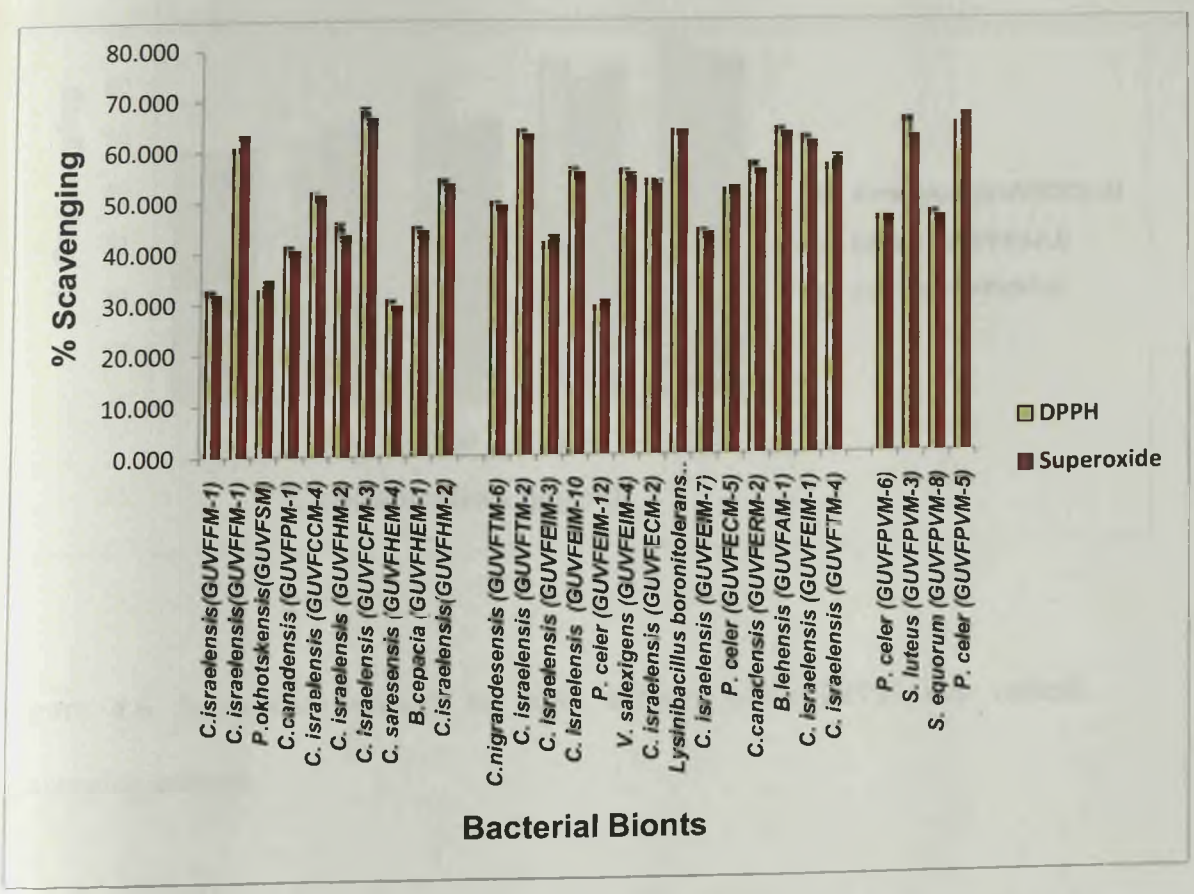


Figure 4.5 DPPH and Superoxide free radical scavenging activities of crude biont extracts.

Extracts of the three cultures *C. israelensis* (GUVFCFM-3), *S. luteus* (GUVFPVM-3), *P. celer* (GUVFPVM-5) showing maximum scavenging activity was further assayed at different concentrations (100, 200, 400, 800, 1000 µg/ml) Figure 4.6.The scavenging of the free radical DPPH occurred in a concentration dependent manner. The IC_{50} values of the extracts for inhibition of free radicals was found to be approximately 169.96, 187.59 and 161.00 µg/ml, respectively.

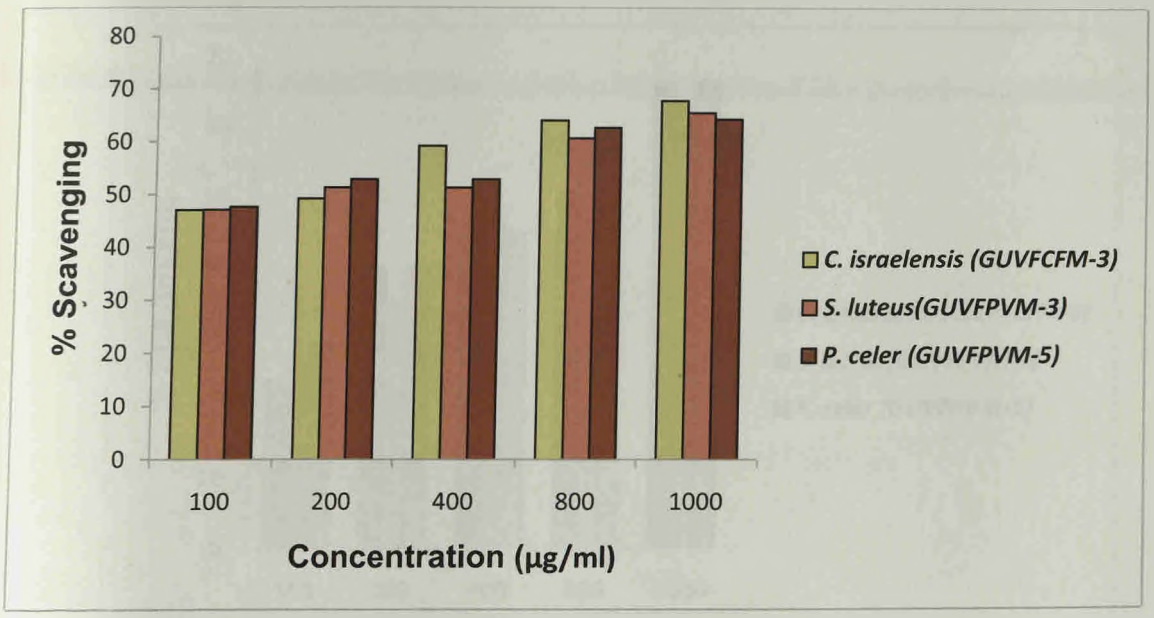


Figure 4.6 Antioxidant activity of biont extracts using DPPH free radical scavenging activity.

4.2.3 B. Superoxide anion radical scavenging ability of crude extracts of bionts

Superoxides were generated endogenously in the PMS-NADH reaction mixture. The superoxide radical formed reduced NBT to a blue coloured complex which can be measured at 560 nm. The highest scavenging ability was shown by culture GUVFCCM-4 with a % scavenging of 65.21% while the lowest was seen in

culture GUVFHM-2 at 29.09% (Figure 4.7). Among the sponge bionts culture GUVFCCM-4 identified as *C.israelensis* showed the highest superoxide scavenging at 67.21%. Amongst the coral bionts culture GUVFECM-2 identified as *C. israelensis* showed the highest scavenging activity at 63.59%. Isolate GUVFPVM-5 showed the highest superoxide scavenging activity among the bivalve bionts at 66.57%

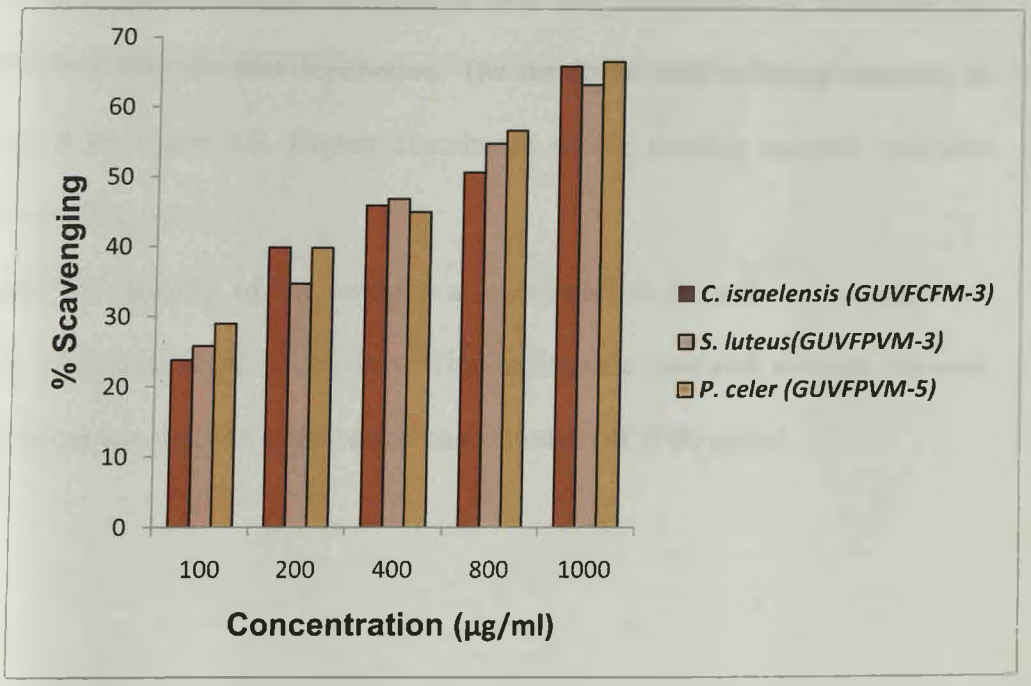


Figure 4.7 Antioxidant activity of biont extracts using Superoxide anion free radical scavenging activity

At different concentrations (10-1000 µg/ml) scavenging of superoxide radical by the three cultures namely, *C. israelensis* (GUVFCFM-3), *S. luteus* (GUVFPVM-3), *P. celer* (GUVFPVM-5) showing maximum scavenging activity, occurred in a concentration dependent manner Figure 4.7. IC_{50} value of *C. israelensis*

(GUVFCFM-3), *S. luteus* (GUVFPVM-3), *P. celer* (GUVFPVM-5 was found to be 629.84, 630.98 and 569.43 μg/ml respectively.

4.2.4 B. Total reducing capacity of crude extracts of bionts

For the measurements of the reductive ability, we investigated the ferric to ferrous transformation in the presence of the extracts of the 28 bacterial extracts in this method. The capacity of extracts to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. The results of total reducing capacity, is represented in Figure 4.8. Higher absorbance of the reaction mixture indicates greater reducing power.

The reducing capacity of the extracts as compared to the reference compound ascorbic acid was found to be less. The methanolic bacterial extracts showed highest/maximum activity at the tested concentration of 1000 μg/ml.

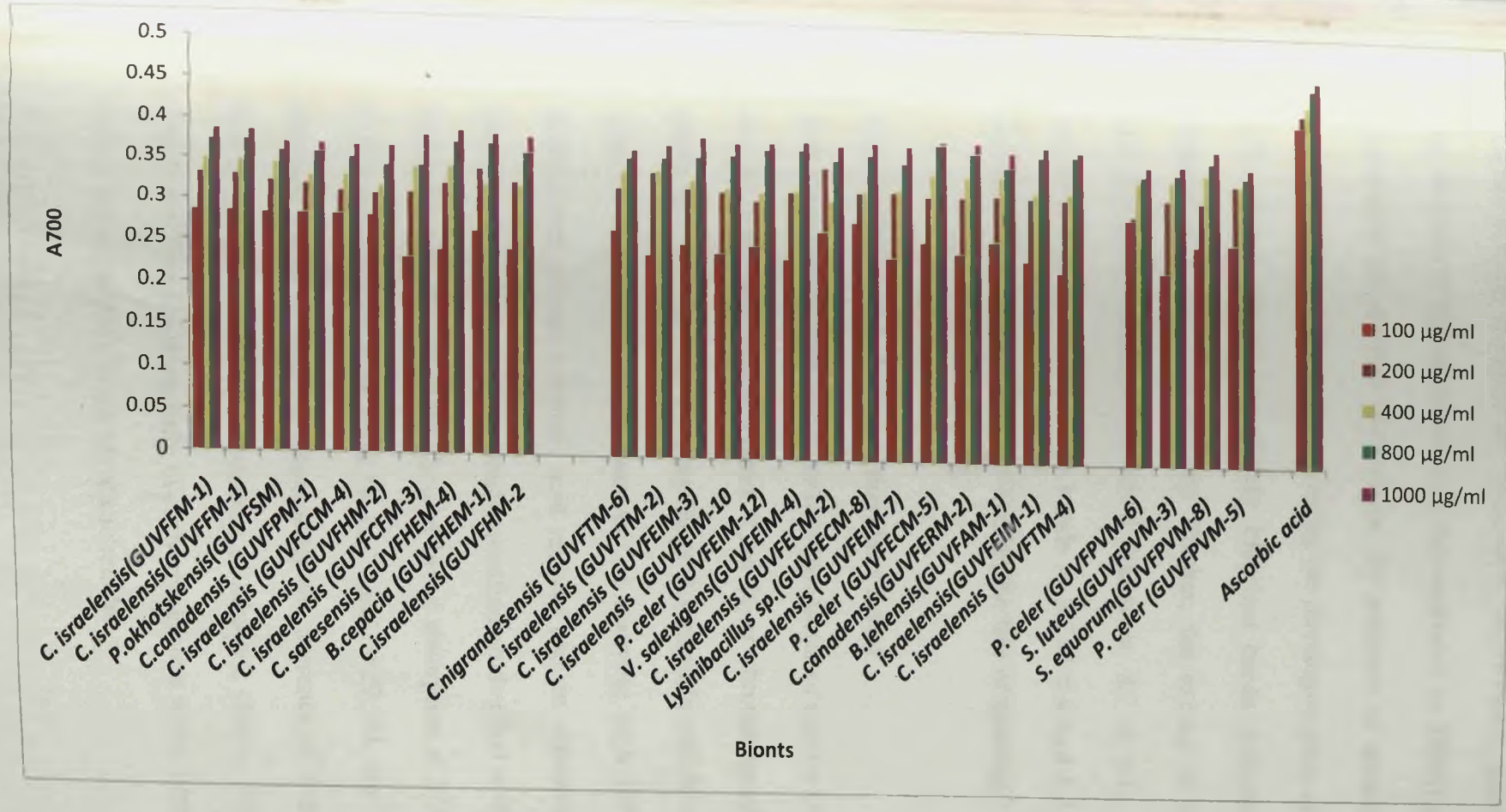


Figure 2.36 Total reducing activities of bacterial bionts

4.2.5 B. Rapid qualitative assay for antioxidant activity using TLC

Culture extracts of bionts GUVFCCM-4, GUFPVM-5, and GUFPVM-5 selected on the basis of highest scavenging demonstrated by DPPH and superoxide anion scavenging ability were checked for the presence of antioxidant principles. The chromatogram obtained after spraying the developed plate of with 0.2% DPPH in 95% ethanol showed immediately bleached bands, referring to the antioxidant capacity of all the three extracts. Further, the extract of culture *C. israelensis* (GUVFCFM-3) displayed bleached bands at Rf of 0.6, extract of *P. celer* (GUFPVM-5) showed bleached bands at Rf of 0.4 and 0.6 Extract of *S. luteus* showed a bleached band at Rf 0.9 and on the line of spotting.

4.2.6 B. Characterisation of antioxidant factor

Preliminary characterization of antioxidant factor of culture GUVFCCM-3 showed that the compound was a protein. Thin layer chromatography showed a single purple spot with Rf = 0.6 on spraying with ninhydrin which coincided with the Rf value of the bleached band observed on spraying with DPPH. No spots were detected with phenol–sulphuric acid reagent and on exposure to iodine vapours. The infrared spectrum of the partially purified crude ethyl acetate extract, showing the bleached band with DPPH showed broad absorption at 3535–3000 cm^{-1} due to N–H stretching, bands at 1658.78 cm^{-1} and 1529.551 cm^{-1} were due to C–O stretching and N–H bending, implicating the presence of peptide bonds. Band at 2954.95 cm^{-1} indicated C–H stretching of aliphatic chains. Vibrations observed at 1091.71 cm^{-1} were characteristic of carbonyl groups while vibration at 1408.4 cm^{-1} is characteristic of the CN stretch of the amide.

4.3 B. Discussions

Marine bacteria constitute a diverse group, which has received much attention in the recent years as potential natural antioxidant producers in terms of their ability to act as efficient radical scavengers and it is believed to be mainly due to their redox properties. Hence 9 marine sponges, 5 corals and 1 bivalve sample, all collected from Mandapam in the South east region of the Indian subcontinent were selected for the isolation of halophilic bacteria. DPPH is a stable nitrogen centered free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of extracts. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine and the absorption decreases. The decrease in absorption is taken as a measure of the extent of radical scavenging (Koleva *et al.*, 2001). The solution therefore looses colour stoichiometrically depending on the number of electrons taken up.

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Mariod *et al.*, 2009). The ability of the extracts to scavenge superoxide radicles suggests its ability to safeguard cellular systems from oxidative damage.

The presence of reductants such as antioxidant substances in the samples caused the reduction of the Ferric/ferricyanide complex to the ferrous form. The reducing power of the extracts increased as concentration increased indicating the presence of electron donors that can react with free radicals to convert them to stable products and terminate the radical chain reactions.The reducing power of the extracts increased as concentration increased indicating the presence of electron

donors that can react with free radicals to convert them to stable products and terminate the radical chain reactions. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity has been attributed to various chain mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging (Diplock, 1997).

The finding that the antioxidant factor is a proteinaceous compound is reported by previous researchers. Proteinaceous extracellular peptides such as diketopiperazines have been reported for their antioxidant activity by virtue of scavenging of the DPPH free radicals from an Antarctic psychrophile *Psedoalteromonas haloplanktis.* Their free radical scavenging properties could be ascribed to the presence of a phenyl group in their structure (Mitova *et al.*, 2005). Previously DKPs were reported as antioxidants. Previous reports (Boldyrev *et al.*, 1989) indicated that antioxidant activity of DKPs demonstrated and their free radical scavenging properties were not correlated.

Chromohalobacter sp. dominated the cultural assemblage bacteria having antioxidant bacteria. This euryhaline bacterium best known for its unique salt tolerating abilities by virtue of the production of osmolytes is not much known for its antioxidant capabilities. Though there are no published reports of antioxidant bacteria of this genera Proteonomic studies have inferred from electronic annotation that there exists a putative sequence of 182 AA having antioxidant activity.

The antioxidant potential of the symbionts associated with these marine organisms adapted to shallower environments and greater fluctuations in temperature and

irradiance can be understood from the fact that ROS increases with elevated temperature. Pigments such as fluorescent proteins (Salil *et al.*, 2000), UV-absorbing compounds (mycosporine-like amino acids, MAAs) and xanthophylls protect the symbiont from UV and/or solar damage that can occur during increased irradiance through dissipation of excess absorbed excitation energy in the symbionts and enzymes with antioxidant functions. Enzymes such as superoxide dismutase (SOD) and catalase react to short term fluctuations in light and temperature over a period of hours (Ambarsari *et al.*, 1997; Brown *et al.*, 1999) as accumulation of these ROS are toxic to the cell if not scavenged from time to time.

Due to sand quarrying, fly ash discharge from thermal power stations and discharge of effluents from chemical industries in the GOM area, the waters of Mandapam are under relenting stress. These discharges have resulted in elevated temperatures of surface waters and also an acidic pH both of which enhance ROS production. To combat this menace the marine invertebrates thriving in these waters and its associated microflora produce antioxidant compounds.

The marine invertebrates have acclimatized to heated and acidic effluents from electric and chemical plants, have also adapted to shallower environments with greater fluctuations in temperature, irradiance and global warming by production of antioxidant enzymes and compounds. Acid stress has been shown to be associated with the induction of Mn superoxide dismutase (MnSOD) in *Lactococcus lactis* and *Staphylococcus aureus* (Hassan *et al.*, 1997). To offset the harmful effects of ROS, most organisms have evolved protective mechanisms that utilize antioxidant enzymes, such as superoxide dismutases and hydroperoxidases (i.e., catalases and peroxidases or KatE and KatG), which scavenge superoxide

radicals and hydrogen peroxide, respectively, and thus prevent the formation of HO (Fenton, 1984).

4.4 B. Conclusions

In conclusion we report the presence of halophilic bionts from sponges, bivalves and corals with antioxidant activity. Culture *C. israelensis* (GUVFCCM-4) a sponge biont from sponge *Cinachyra cavernosa* was the most promising antioxidant producer capable of scavenging both DPPH and superoxide free radicles. The % scavenging of DPPH free radicals was 67.05% and of superoxide radicals was 67.21% respectively. Preliminary characterization of the extracellular antioxidant factor revealed that it was peptide. Thus strain *C. israelensis* (GUVFCCM-4) holds potential for effectively treating neurodegenerative and other disorders caused due to the activity of free radicals. The results obtained from this study show that multiple *in vitro* methods targeting different radical species are important for testing antioxidant potential of bacterial bionts associated with the sponges, corals and bivalve. Employment of more than one test method specific to a radical species gives a better estimate of comparative antioxidant potential of a test compound.

Section C: Screening of bionts from Sponges, Corals, Bivalve for hemolytic activity

Hemolysis is the breakdown of red blood cells. *In vivo* hemolysis can be caused by a large number of medical conditions, including many Gram positive bacteria (*e.g.*, *Streptococcus*, *Enterococcus* and *Staphylococcus*, some parasites (*e.g.*,

Plasmodium), some autoimmune disorders (*e.g.* Hemolytic disease of the newborn), and some genetic disorders (*e.g.*, Sicklecell disease or G6PD deficiency). A hemolytic crisis, or hyperhemolytic crisis, characterized by an accelerated rate of red blood cell destruction leading to anemia, jaundice and reticuculocytosis are major concerns with sickle-cell disease and G6PD deficiency.

Erythrocytes, which are the most abundant cells in the human body, possessing desirable physiological and morphological characteristics, are exploited extensively in drug delivery (Hamidi and Tajerzadeh, 2003). Oxidative damage to the erythrocyte membrane (lipid/protein) may be implicated in hemolysis associated with some hemoglobinopathies, oxidative drugs, transition metals, radiation, and deficiencies in some erythrocyte antioxidant systems (Koh *et al.*, 1997). The hemolytic assay is useful for screening studies on various molecules and their metabolites, on one hand by molecules having an oxidizing or antioxidizing activity and on the other hand, molecules having a long term action (Djeridane *et al.*, 2007). RBC has been used as an *in vitro* model to study the oxidant/antioxidant interaction since its membrane is rich in polyunsaturated fatty acids, which are extremely susceptible toperoxidation (Chaudhuri *et al.*, 2007). In recent years, a few interesting studies indicating the protective effect of some plants extracts against oxidative damage in intact RBC membranes have been reported (Magalhaes *et al.*, 2009; Ebrahimzadeh *et al.*, 2010). In view of this the aim of this study was to screen for the presence of hemolytic factor present in bionts associated with marine invertebrates from Mandapam.

4.1 C. Methodology

4.1.1 C. Screening bionts for hemolytic activity

All the bionts asscociated with sponges, corals and bivalve were checked for hemolytic activity by streaking onto blood agar plates.

4.1.2 C. Measurement of Hemolytic Activity in extracts of bionts

Fresh human red blood cells (RBCs, 30 ml) were suspended in 10 ml of Tris saline, washed 3 times by centrifugation (5 min at 1500 rpm) and resuspended in Tris saline. Ethyl acetate extracts of cell free supernatents of bacterial bionts of sponges, corals and bivalve were subjected to this assay. Freshly prepared extracts dissolved in normal saline of different concentrations were added to 80 ml of the above prepared RBC suspension to reach a final volume of 100 ml in a 96 well plate. The plate was gently shaken and allowed to stand for 2 hrs at room temperature and the results were recorded. Uniform red coloured suspension in the wells was considered as positive hemolysis and a button formation at the bottom of the wells was considered as absence of hemolysis. All the experiments were performed in triplicate.

4.2 C. Results

4.2.1 C. Screening bionts for hemolytic activity

Among all the extracts tested only the isolates from coral *E. complexa* namely GUVFECM-5 and GUVFECM-13 showed α- hemolysis due to partial decomposition of haemoglobin. The agar under the colony was dark greenish. The

other isolates showed γ -hemolysis as the region around them was unchanged There were no isolates showing ß -hemolysis.

4.2.2 C. Measurement of Hemolytic Activity in extracts of bionts

Among all the extracts tested for hemolytic activity the extracts of the sponge isolates GUVFCFM-1,GUVFCFM-3(from *Callyspongia fibrosa*), GUVFHM-2 (from *Haliclona* sp.), GUVFHEM-1(from *Heteromema erecta*), extracts of coral bionts GUVFECM-1(from *E. complexa*), GUVFEIM-1(from *E.indica)*,GUVFTM-5 (from *Telesto* sp.) extracts from the bivalve biont GUVFPVM-9 showed a uniform red suspension indicating effective hemolysis.The extracts of the other cultures were non hemolytic as they showed the formation of a clump of cells at the base of the microtitre well. The extracts of all the other bionts tested thus have potent hemolytic activity.

4.3 C. Discussions

Streptococcus pneumonia and a group of oral streptococci (*Streptococccus viridans* or *viridans Streptococci*) are known to display α- hemolysis. This is sometimes called *green hemolysis* because of the color change in the agar. Other synonymous terms are *incomplete hemolysis* and *partial hemolysis*. α- hemolysis is caused by hydrogen peroxide produced by the bacterium, oxidizing hemoglobinto green methemoglobin.

Beta hemolysis (β-hemolysis), sometimes called *complete hemolysis*, is a complete lysis of red cells in the media around and under the colonies: the area appears lightened (yellow) and transparent. Streptolysin, an exotoxin, is the enzyme

produced by the bacteria which causes the complete lysis of red blood cells. There are two types of streptolysins: Streptolysin O (SLO) and streptolysin S (SLS). Streptolysin O is an oxygen-sensitive cytotoxin, secreted by most Group A streptococcus (GAS), and interacts with cholesterol in the membrane of eukaryotic cells (mainly red and white blood cells, macrophages, and platelets), and usually results in β-hemolysis under the surface of blood agar. Streptolysin S is an oxygen-stable cytotoxin also produced by most GAS strains which results in clearing on the surface of blood agar. SLS affects immune cells, including polymorphonuclear leukocytes and lymphocytes, and is thought to prevent the host immune system from clearing infection. *Streptococcus pyogenes*, or Group A beta-hemolytic Strep (GAS), displays beta hemolysis. Some weakly beta-hemolytic species cause intense beta hemolysis when grown together with a strain of *Staphylococcus* sp.

If an organism does not induce hemolysis, it is said to display gamma hemolysis (γ-hemolysis): the agar under and around the colony is unchanged (this is also called *non-hemolytic*). *Enterococcus faecalis* (formerly called Group D Strep) displays gamma hemolysis.

The probable reasoning to the hemolytic activity could be attributed to the presence of surfactants which eventually cause complete or partial solubilization of membrane lipids and proteins of RBC's by formation of mixed micelles (Maher and Singer, 1984, Jones, 1999). At low concentrations, the main result of the intercalation of the surfactant into the membrane is a change in the membrane permeability. In the case of an ionic suspending solution, this change induces a Donnan distribution across the cell membrane, which is followed by water

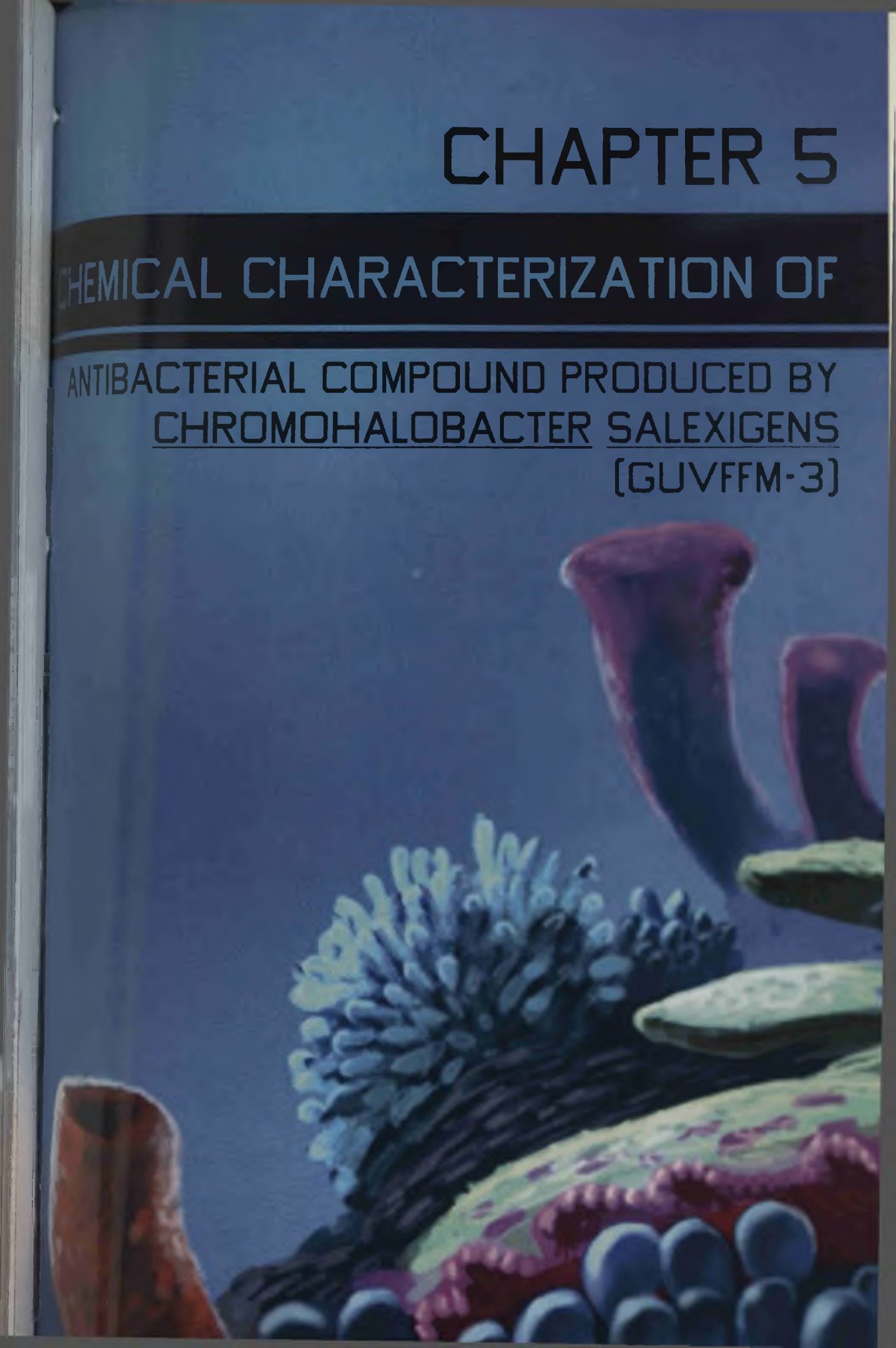
penetration, cell swelling, and membrane rupture (Bielawski, 1990, Isomaa, 1979; Isomaa *et al.*, 1989, 1986).

4.4 C. Conclusions

Extracts of bionts exhibited hemolysis of erythrocytes attributed to the bioactive constituents present in the extracts. These extracts hold promise for further pharmacological studies, which should be focused on evaluating other biological effects or improving mechanism of these effects.

CHAPTER 5

CHEMICAL CHARACTERIZATION OF ANTIBACTERIAL COMPOUND PRODUCED BY CHROMOHALOBACTER SALEXIGENS (GUVFFM-3)



Marine surface associated microorganisms are under persistent threat of infection by resident pathogenic bacteria and thus have proven to be a rich source for novel bioactives because of the necessity to evolve allelochemicals from a diverse set of biological precursors (Parkes *et al.*, 1994). Chemically driven interactions are also important for the establishment of cross-relationships between microbes and their eukaryotic hosts, in which organisms producing antimicrobial compounds ("antimicrobials") may protect the host surface against over colonisation in return for a nutrient rich environment. Since sponges are simple and sessile organisms; during evolution they have developed potent chemical defensive mechanism to protect themselves from competitors and predators as well as infectious microorganisms (Villa and Gernwick, 2010; Mayer *et al.*, 2011). Studies show that secondary metabolites in sponges play a crucial role in their survival in the marine ecosystem (Slattery *et al.*, 2001). These natural products have interesting biomedical potential, pharmaceutical relevance and diverse biotechnological applications (Blunt *et al.*, 2011, Thakur and Muller, 2005; Jensen and Fenical, 1994; Bernan *et al.*, 1997; Haygood *et al.*, 1999). Interestingly, out of the 13 marine natural products that are currently under clinical trials as new drug candidates, 12 are derived from invertebrates. Among them, Porifera remains the most important phylum, as it provides a greater number of natural products, especially novel pharmacologically active compounds (Osinga *et al.*, 2001; Thakur *et al.*, 2005).

Occurrence or emergence of multidrug resistant (MDR) strains of human bacterial pathogens necessitates discovery of newer antibiotics with a promise for combating MDR strains. Harnessing of large quantities of bioactive products from producer bacteria is both energy and cost intensive. Unveiling of the chemical

structure is therefore important as this opens possibilities of chemically synthesizing the potent compound. The culture GUVFFM-3 identified as *Chromohalobacter salexigens* (GenBank accession number AJ295146) in Chapter 2 an euryhaline bacterial biont of *Fasciospongia cavernosa*, an inhabitant of Mandapam-India was seen to have antibacterial activity against *S. marcesans*, *A.aerogens*, *S.citreus*, *S.typhi* and *Sh. flexneri* as recorded in Chapter 4. The present study undertook the production of the extracellular bioactive antibacterial factor, its isolation, chemical characterization and elucidation of its chemical structure.

5.1 Methodology

5.1.1 Production of extracellular antibacterial compound

GUVFFM-3 was mass cultured in 3% TYE medium using a fermentor (B Braun Biotech international Biostat B) set to (37^0C) and 150 rpm. The cell free supernatant obtained by centrifuging at 12000 g for 20 minutes at 4^0C was extracted three times in ethyl acetate. The pooled fraction was subjected to concentration under vacuum using a rota evapotator (Buchi Germany) at 35^0C. The concentrate dissolved in water, was used as crude samples for its bioactivity determination using the agar cup diffusion assay.

5.1.2 Assay of Bioactivity

Ethyl acetate of crude extracts of *Chromohalobacter salexigens* GUVFFM-3 redissolved in water and purified ethyl acetate fractionated compounds were tested against ten clinical bacterial strains viz. *Escherichia coli, Pseudomonas* sp., *Staphylococcus citreus, A. aerogens, S.typhi, Bacillus subtilis, Candida albicans,*

Shigella flexneri, Proteus vulgaris, Serratia marcesans using the agar cup diffusion assay. 4 mg ml^{-1} of crude ethyl acetate extract dissolved in water was dispensed into agar cups (6 mm in diameter) borne into Mueller Hinton Agar plates seeded with 200µl of 24 hr old clinical pathogen at a concentration of 1.6 x 10^8 – 2.5x 10^8cfu/ml. Solvent controls were maintained on the same plates. After 24 hrs of incubation of plates at 37^0C, the diameter of each zone of growth inhibition was measured in mms to obtain a semi quantitative determination of the antibiotic nature of the extracts. The assay was carried out in triplicates.

Minimum Inhibitory Concentration (MIC) of crude ethyl acetate extract was determined for serially diluted concentrations (0.2-4 mg/ml) against each individual human pathogenic bacterial strain. Previously described well diffusion assay was used to calculate the MIC and to determine the bioactivity of fractionated ethyl acetae extracts.

5.1.3 Purification of antibacterial compound

The crude ethyl acetate extract of cell free culture broth was lyophilized (138 mg) and fractionated using flash chromatography equipped with a column of silica gel (60–120 mesh) column and equilibrated with petroleum ether and eluted with solvents of increasingpolarities. Eluates collected in 10 ml fractions were pooled together based on their TLC profile using ethyl acetate-petroleum ether (15:85, v/v) as developing solvent system. Spots were visualized in long and short UV and by spraying with 5% (v/v) sulphuric acid in methanol followed by heating of the TLC plate in an oven for 2 minutes at 110 °C.

5.1.4 Analytical Methods/Instrumentation

Lyophilization of sample was carried out in a Christ, A- 2–4 LD plus instrument. NMR spectra were recorded at 23 °C on a Bruker Avance AC-300 spectrometer, operating at 300 and 75 MHz for ^{1}H and ^{13}C, respectively, using tetramethylsilane (TMS) as an internal standard. Mass data was recorded on an Electrospray Ionization Tandem Mass Spectrometer (ESI/MS-MSa QTOF-XL MS/MS Applied Biosystem instrument equipped with MDS Sciex Analyst software (Concord, Ontario, Canada). The sample dissolved in 1:1 MeOH: 0.1% TFA and was directly infused at a constant flow rate of 10μL/min into the ion spray source using an integrated syringe pump. The instrument was operated in positive ionization mode with the following settings: Nebulizer gas (N2) 28(arbitrary units); curtain gas (N2) 18 (arbitrary units); Ion spray voltage-5700 V; Declustering potential (DP)-120V; Focusing potential (FP)-365V; Declustering potential (DP2)-14V andCollision gas (CAD) 3 (arbitrary units). Full-scan data acquisition was performed; scanning from m/z 100 to m/z 2000 in profile mode and using a cycle time of 1s. TLC was performed on aluminium sheets pre-coated with silica gel 60 F_{254} (Merk KgaA, Damstadt, Germany, Cat No. 1.05554). All the solvents used were AR grade and distilled.

5.2 Results

5.2.1 Production of extracellular antibacterial compound

Ethyl acetate extracts of tissue samples of *Fasciospongia cavernosa* inhabiting Mandapam in India, showed remarkable activity against 5 different emerging pathogenic bacteria isolated in a state hospital and resistant to antibiotics such as chloramphenicol, ampicillin, trimethoprim and beta lactam antibiotics. In recent

years microbes associated with sponges are reported as the actual producers of bioactivity. Attempts were made to isolate such microbes from the sponge under study. Bacterial colony giving an activity similar to that given by the sponge tissue was designated as GUVFFM-3 and its 16S rRNA sequence is deposited at the Genebank with an accession number JF 330126 and was used for the production of bioactive compound. The bacterium was identified as *Chromohalobacter salexigens.*

5.2.2 Assay of Bioactivity

For identification of this antibacterial activity, *Chromohalobacter salexigens* was mass cultured. It grew as a creamish white suspension possessing an unpleasant odour. Ethyl acetate extract of the cell free supernatant yielded a caramel brown colour concentrate of 27.6 mg/L bearing an unpleasant odor.

5.2.3 Purification of antibacterial compound

Since the ethyl acetate extract showed remarkable activity against 5 out of the 10 hospital strains tested, it was selected for further fractionation of the individual compounds using flash chromatography. The crude ethyl acetate extract when spotted onto TLC plates pre-coated with silica gel 60 F_{254} showed the presence of 6 spots of Rf values 1) 0.89, 2) 0.7346, 3) 0.421, 4) 0.1632, 5)0.1224, 6) 0.061 (Figure 5.1). Spots 1, 2, 4, 5 and 6 were seen in iodine. Spot 3 on the other hand was not seen in iodine but was only seen under long UV. All the compounds could be visualized in long UV.

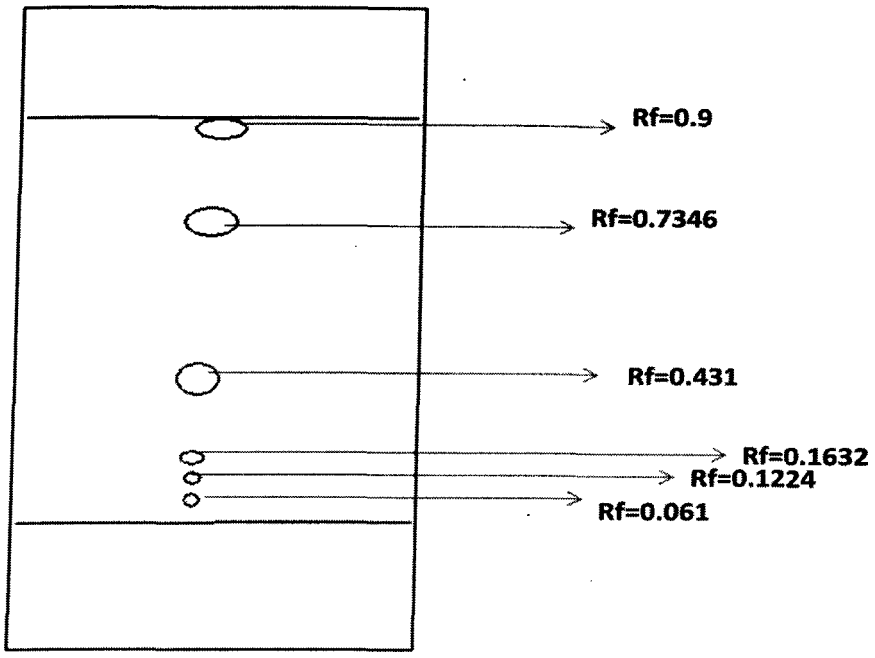


Figure 5.1: TLC of crude ethyl acetate extract developed in Ethyl acetate.

Concentrates of crude extract separated on flash chromatography as distinct peaks. Compounds 1 and 2 were eluted in pet ether, compounds 3 and 4 in 100% ethyl acetate while compounds 5 and 6 were eluted in 5% ethanol and 95% ethyl acetate.

All the extracts were checked for antimicrobial activity and the key antibacterial compound (C_5) which eluted in 5% (w/v) methanol in ethyl acetate (Figure 5.2) was selected for further purification.

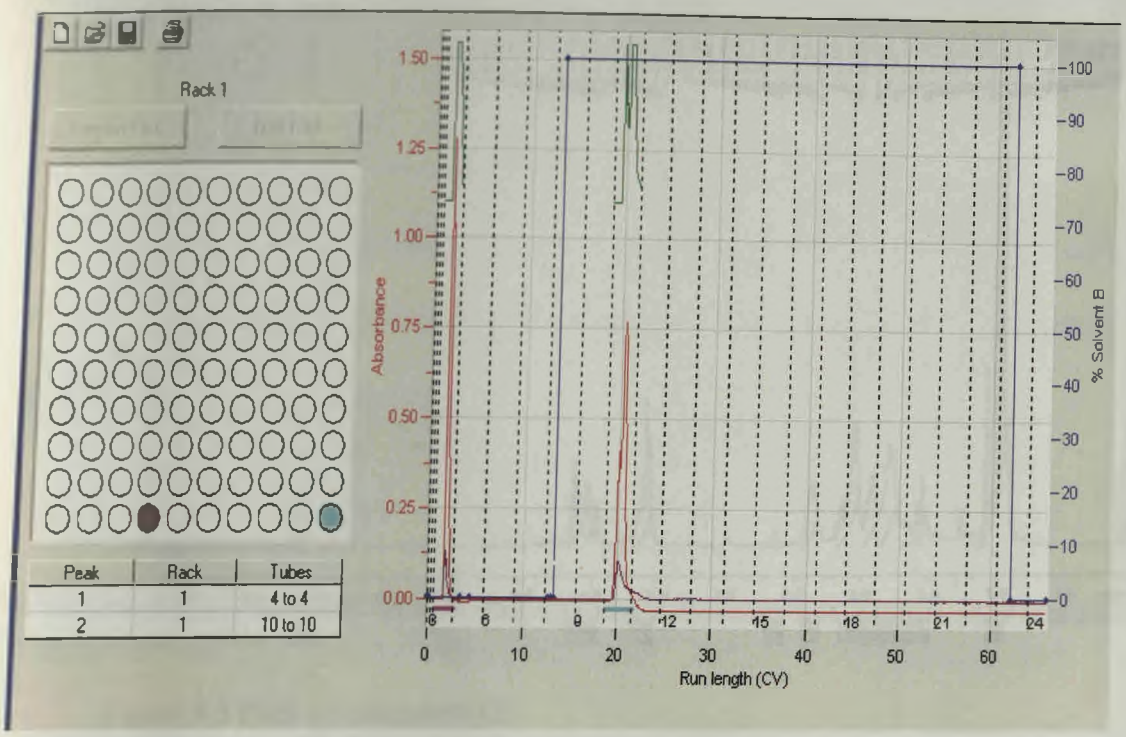


Figure 5.2 Flash chromatogram showing the separation of C_5

5.2.4 Identification of key antibacterial compound

The electrospray ionization mass spectrum (ESI-MS) of compound C_5 showed the presence of a signal at 211.04 due to $[M+H]^+$ indicating its molecular formula to be $C_{11}H_{18}N_2O_2$. The ^1H NMR data (Figure 5.3) showed a single broad singlet at δ 6.169 for the hydrogen attached to the nitrogen group (–NH proton) and hence appeared downfield. The 2 other signals at δ 3.62 and δ 3.647 correspond to the two CH groups that also appeared downfield as these aromatic H are bonded to a single carbon atom which in turn is bonded to a nitrogen atom. The corresponding carbons showed signals at δ 53.41 and δ 58.78 respectively (Figure 5.4).

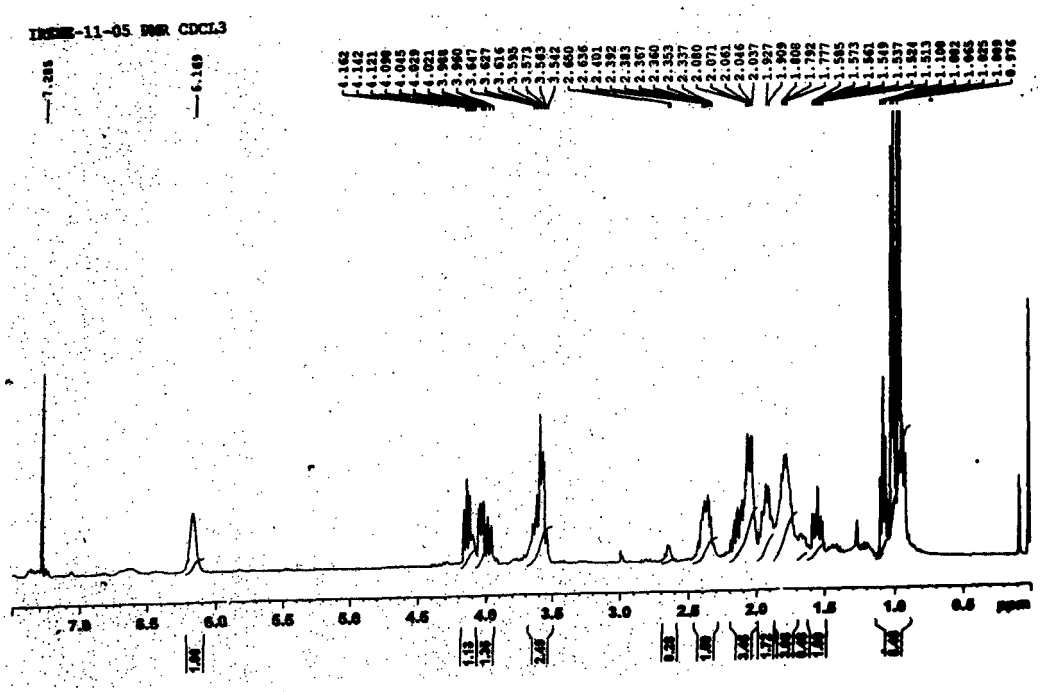


Figure 5.3 PMR of compound C_5

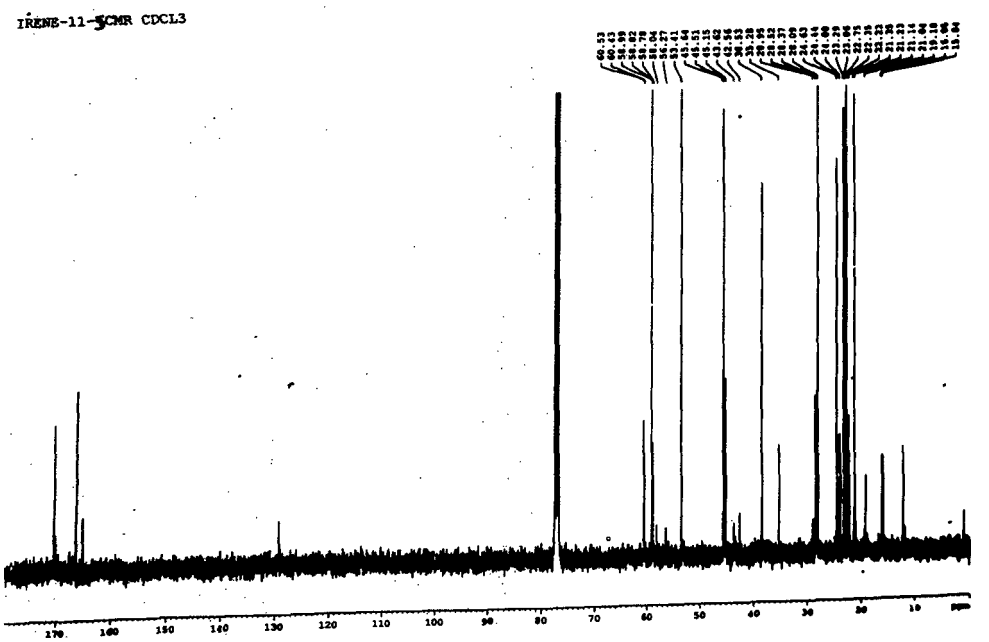


Figure 5.4 CMR of compound C_5

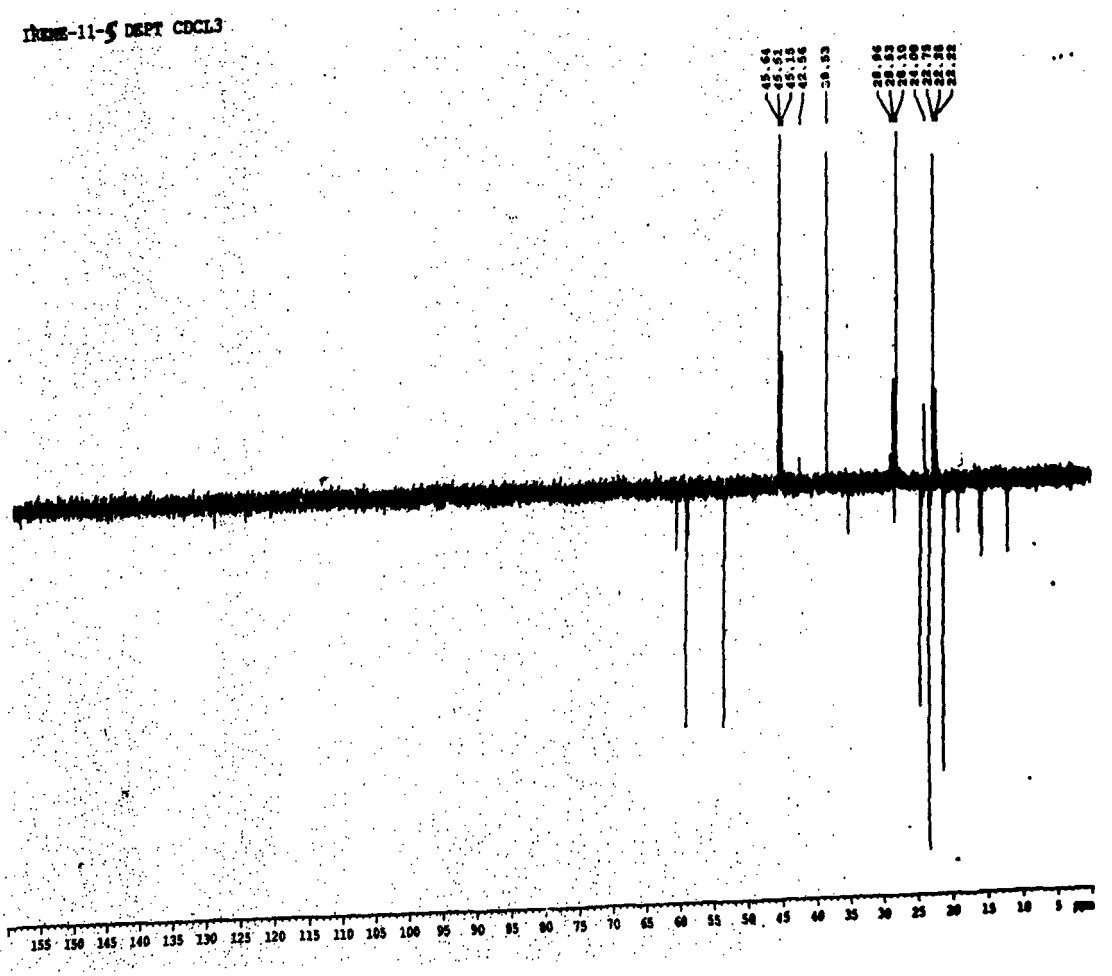


Figure 5.5 DEPT of compound C5

The signal due to methylene was evident as a single triplet in the 1H-NMR spectra centered at δ 3.542. This signal occurred downfield as it is attached to a carbon atom which in turn is attached to a nitrogen atom. The corresponding carbon occurred at a signal of δ 45.51. The 6 aromatic protons of the 3 methylene groups were seen as triplet signals between δ 1.909- δ 2.080 with the corresponding carbons showing doublet signals at δ 22.35, 28.10 and δ 38.53. The protons of the 2 methyl groups had signals at δ 1.549 with the corresponding carbons at δ 21.04 and δ 23.06. The single CH bonded to the two methyl groups were seen as a signals at δ 1.537 with corresponding carbon at δ 23.29. The two carbonyl carbons were seen as signals at δ 170.5 and δ 167 respectively. The carbon data was revealed by DEPT (Figure 5.5) and CMR. On the basis of the above spectral data the compound has been identified as 3-isopropyl-pyrrolopiperazine (Figure 5.6) which was also in good agreement with the spectral data given in Aldrich

catalogue of NMR data. This compound was translucent in colour.The compound exhibited antibacterial activity against multidrug resistant hospital strainsof *Shigella flexneri, S.typhi, A.aerogens, S.citreus* and the observed MIC was 0.2, 0.3 and 0.4 mg/ml.

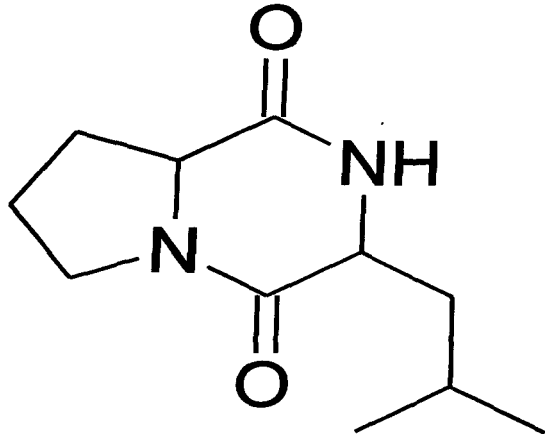


Figure 5.6 Structure of the compound 3-isopropyl-pyrrolopiperazine

5.3 Discussions

Marine sponges are well documented as a source of novel bioactive metabolites such as terpenoids, alkaloids, macrolides, polyethers, nucleoside derivatives and peptides. Both antibacterial and cytoxic activities are reported for peptide compounds such as Discodermins (Matsunaga *et al.*, 1985), Arenastatin A (Kobayashi *et al.*, 1994) Phakellistatins (Pettit *et al.*, 1993), Theonellamides A–F (Matsunaga *et al.*, 1989) , Polydiscamide A, Microsclerodermins C–E (Schmidt *et al.*,1998), Aciculitins A–C (Bewley *et al.*, 1996a), Theonegramide (Bewley *et al.*, 1996a), aligramides A and B, (Rashid *et al.*, 2000). It is suggested that the origin of some of these peptides would be associated with a microbial source or with symbionts within the sponges, because of the similarity of some of these molecules

to marine microbial metabolites. Advanced techniques of molecular biology, such as Polymerase Chain Reaction (PCR), in particular the application of degenerated primers of Non-ribosomal peptide synthetases (NRPS) to amplify gene fragments from peptide producers has allowed screening on the presence of non ribosomal peptides among secondary metabolite-producing microorganisms (Radjasa *et al.*, 2007). The potential for the sponge microbiota of *Haliclona simulans* to produce secondary metabolites was also analysed by polymerase chain reaction amplification of polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) gene, also further confirms the microbial origin of peptides.

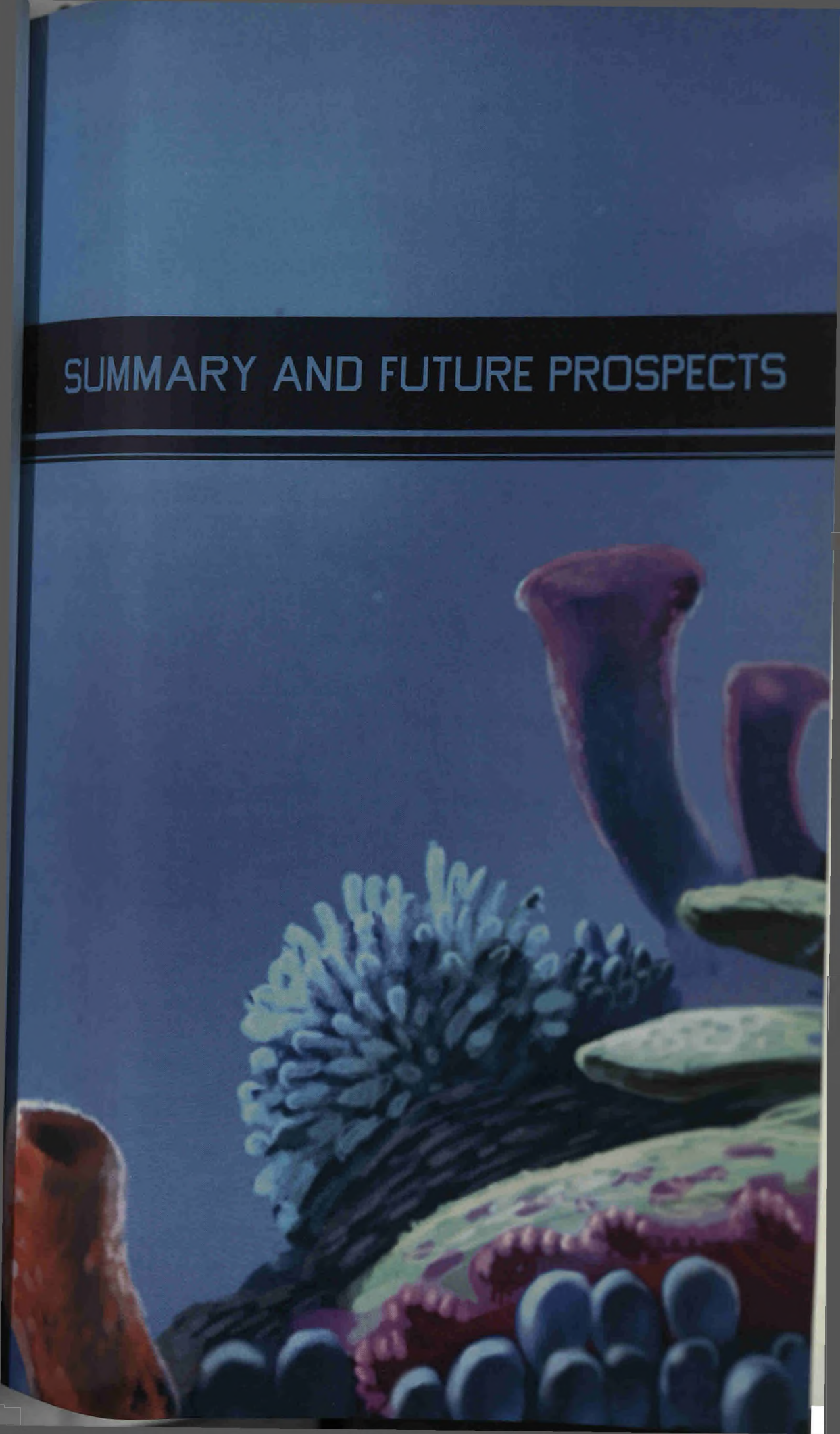
There is no literature available to show that the compound isolated in this study was ever reported earlier from any marine sponge biont *Chromohalobacter salexigens*. Hence, the present study becomes the first report of this compound from *Chromohalobacter salexigens*. There have been other reports of these compounds from marine bacterium *Bacillus* sp., Fungus *Fusarium* sp., *Rhizopus* sp., *Trichothecium* sp., *Penicillium* sp., *Gliocladium* sp., *Monoscus purpureus*, *Micromonospora* sp. and actinomycetes *Streptomyces* sp. There are no reports of the antibacterial activity of this compound and hence this is the first report of antimicrobial activity of this peptide antibiotic.

5.4 Conclusions

The marine bacterium GUVFFM-3 with an accession number JF 330126, is a Gram negative bacterium. It is motile non spore forming aerobe, showing 99% similarity to *Chromohalobacter salexigens* DSM 3043 AJ295146. Cell free supernatant and cell biomass exhibits antibacterial activity. As the ethyl acetate extract showed most promising antimicrobial activity it was selected for further

purification for structure elucidation. Mass culture of *Chromohalobacter salexigens* GUVFFM-3 for chemical characterization led to the isolation and characterization of one compounds out of the six separated using ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) and Electrospray Ionization Mass Spectrometer (ESI-MS)

SUMMARY AND FUTURE PROSPECTS



The present study summarises the research endeavour to isolate halophilic and haloarchaeal bacteria (bionts) associated with marine sponges, corals and the bivalve *P.viridis* isolated from Mandapam the Gulf of Mannar – India and the determination of the bioactive potential of the isolated bacterial bionts. It also collates information available on the title of the thesis.

The culturing strategy for isolation of bacterial bionts, was the plating of samples on a halophilic medium amended with 25% NaCl for the isolation of haloarchaeal and halophilic isolates, plating of samples on media without NaCl for the isolation of non halophiles and plating on media with 3% NaCl for the isolation of marine halophiles. Total viable counts were highest in the Unidentified sponge MAM 5. AODC revealed highest counts of curved rods and cocci in sponge *P.testudinaria.* Short rods were seen in abundance in sponge *Haliclona* sp. and *C. fibrosa* while the counts of coccobacilli were highest in the sponge *Callyspongia reticutis var solomonensis* and NIO3. Sponge *Haliclona* sp. and MAM 5 on the other hand, showed the highest number of long rods. The cultures were isolated, purified, characterized and identified using morphplogical, biochemical, chemotaxonomic and molecular markers/keys.

Among the sponges the most productive in generic diversity were the sponges MAM 1, MAM 2 and MAM 10. Nine genera of bacteria associated with the sponges under study were identified as *Bacillus* sp., *Pseudomonas* sp., *Pontibacillus* sp., *Chromohalobacter* sp., *Corynebacterium* sp.,

Enterococcus sp., *Enterobacter* sp. and *Planococcus* sp., *Loktanella* sp. using Bergeys keys of Systematic Bacteriology.

The total viable counts of retrieved bacteria were highest in coral MAM 6. Among the corals, the most productive in generic diversity was coral MAM 8. The genera identified among the corals using Bergeys keys of Systematic Bacteriology were, *Bacillus* sp., *Corynebacterium* sp., *Chromohalobacter* sp., *Vibrio* sp., *Aeromonas* sp., *Pseudomonas* sp., *Alteromonas* sp., *Alcaligens* sp., *Planococcus* sp., *Psychrobacter* sp., *Pontibacillus* sp. and *Planococcus* sp. The single bivalve *Perna viridis* sampled in our study showed the highest count on the TYE medium. The isolates from the single bivalve were identified as *Planococcus* sp., *Psychrobacter* sp. *Staphylococcus sp.* and *Bacillus* sp. The research resulted in isolation into cultures 42 halophilic isolates belonging to 8 taxa from sponges, 63 isolates from corals belonging to 11 taxa and 9 isolates belonging to 3 taxa in bivalve. The study clearly revealed the presence of cohabitating marine halophiles (3% NaCl), halotolerant or euryhaline halophiles (0-25% NaCl) and extreme halophiles/haloarchaea. It also pointed out the predominance of euryhaline bacteria, capable of coping with osmolal stress in the host interiors, possibly due to the sequestration of DOM from the ambient waters of its habitat, which in turn supports co-existence of bionts of Genera known to produce osmolytes.

The presence of haloarchaea (GUVFCM, GUVFFM-1, GUVFFM-2, GUVFCCM-2) in marine sponges (with a salinity levelof 3%–3.5% NaCl), an econiche in which they are not expected to survive, is a significant finding that reflects the physiological and ecological complexities of

halophilic archaea, and furthermore, raises the question pertaining to the survival mechanism employed in overcoming adverse hyposaline conditions of such an environment. The retrieval of bionts of Genera *Enterococcus* sp., *Corynebacterium* sp., *Enterobacter* sp. and *Pseudomonas* sp. reflects the entry of bacteria, known for pathogenicity to humans, into waters of Mandapam, through sewage and other pollutants. It indicates microbial pollution of Mandapam waters and *Heralds Caution* for taking measures for safeguarding the waters and ensuring sustainability of the biota.

Microbial community analysis of the single hard coral *A. formosa*s sampled from the archipelago of Lakshadweep using DGGE revealed low diversity phylotypes of *Brevundimonas halotolerans MCS 24(T), Luteimonas lutimaris G3(T), Brevundimonas staleyi FWC43(T),Brevundimonas halotolerans MCS 24(T), Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1(T), Pseudomonas toyotomiensis HT-3(T), Lysobacter xinjiangensis RCML-52(T), Candidatus Accumulibacter phosphatis, Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1(T),Pseudomonas toyotomiensis HT-3(T)*

Various bionts showed antibacterial, antioxidant and hemolytic activities. In this regard it was interesting to note that culture GUVFCCM-2 showed maximum antibacterial activity against known clinically implicated pathogens *S.typhi*, *E.coli*, *S.citreus* and *P.vulgaris.* Culture showing maximum antioxidant activity is GUVFCFM-3 while cultures showing promising hemolytic activity were GUVFECM-5 and GUVFECM-13. Cell free supernatant and cell biomass of the marine bacterium GUVFFM-3

identified as *Chromohalobacter salexigens* showed most promising antimicrobial activity. Mass culture of *Chromohalobacter salexigens* GUVFFM-3 for chemical characterization led to the isolation and characterization of the key antibacterial compound using ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) and Electrospray Ionization Mass Spectrometer (ESI-MS) The compound was a peptide antibiotic identified as 3-isopropyl-pyrrolopiperazine.

Ecologically and biologically this work represents the retrievability of some of the eubacterial and haloarchaeal bionts of marine sponges, corals and bivalve sampled from Mandapam coast in Tamil Nadu and their potentiality to produce antibacterial, antioxidant and hemolytic compounds.

CONCLUSIONS

1. The research work presented in this thesis, is a record of halophilic eubacteria and haloarchaeal bionts retrieved into cultures as coinhabitants from marine sponges, corals and bivalve inhabiting Mandapam-India. *Bacillus* sp., *Pseudomonas* sp., *Pontibacillus* sp., *Chromohalobacter* sp., *Corynebacterium* sp., *Enterococcus* sp., *Enterobacter* sp., *Loktanella* sp., *Vibrio* sp., *Aeromonas* sp., *Alteromonas* sp., *Alcaligens* sp., *Psychrobacter* sp. and *Staphylococcus* sp. are the bacterial genera isolated from these invertebrates. Of these genera *Pontibacillus* sp., *Chromohalobacter* sp., *Corynebacterium* sp., *Enterococcus* sp., *Enterobacter* sp., *Loktanella* sp., *Vibrio* sp., *Aeromonas* sp., *Alteromonas* sp., *Alcaligens* sp., *Psychrobacter* sp., are not reported from invertebrates of the region. This is also the first report of culturable haloarchaea, from invertebrates of this region.

2. Reports for the first time a eubacterial community of *Brevundimonas halotolerans*, *Luteimonas lutimaris*, *Brevundimonas staleyi*, *Brevundimonas halotolerans*, *Polynucleobacter necessarius subsp. asymbioticus*, *Pseudomonas toyotomiensis*, *Bacillus thermolactis*, *Lysobacter xinjiangensis* and *Candidatus Accumulibacter phosphatis* associated with *A. formosa* from Kavaratti island in Lakshadweep.

3. This is first report on the antioxidant and antihemolytic potential of the bacterial bionts associated with sponges, corals and bivalve inhabiting the Indian subcontinent.

4. Elucidates that the key antibacterial compound produced by GUVFFM-3 as 3-isopropyl-pyrrolopiperazine

Overall the study confirms the presence of bacterial bionts in three different invertebrates, namely sponges, corals and bivalve producing antibacterial, antioxidant and hemolytic bioactive factors.

FUTURE PROSPECTS

The halophilic and haloarchaeal bionts obtained in this study are a biotechnological resource worth pursuing for the bioactive factors and their potential for commercialization.

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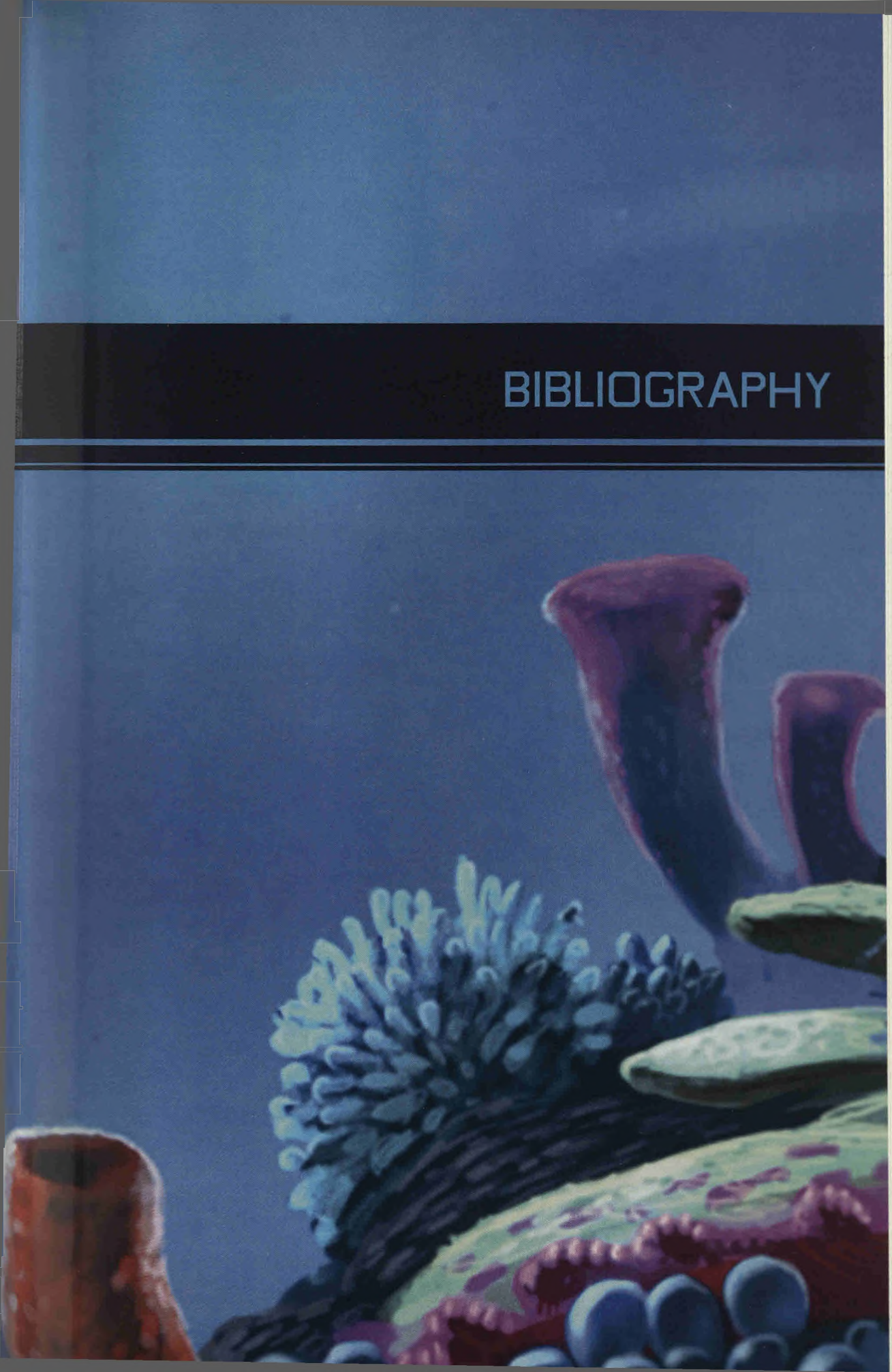

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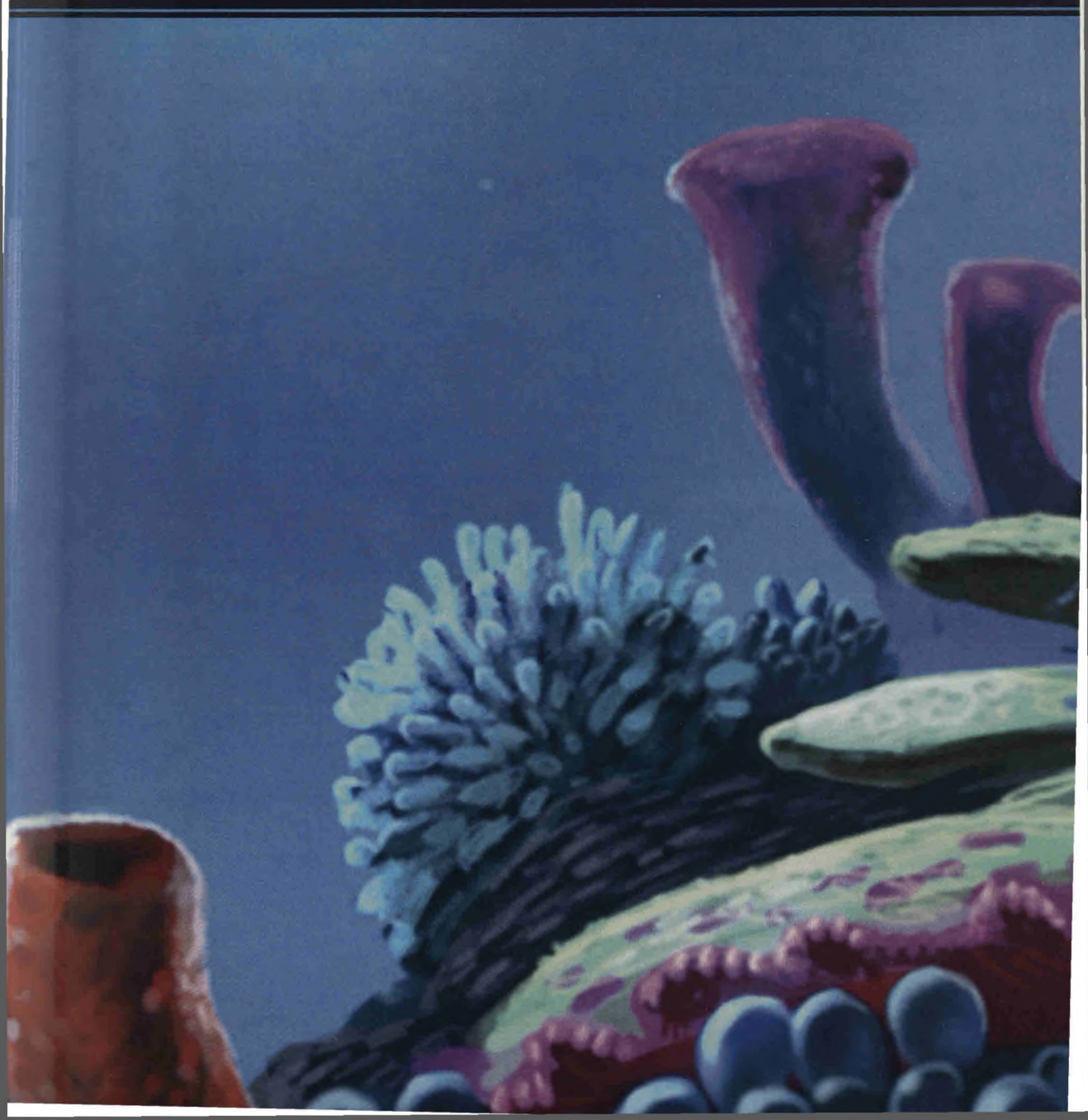
APPENDIX



Reagents for DGGE

40% Acrylamide/Bis (37.5:1)

Reagent	Amount
Acrylamide	38.93 g
Bis-acrylamide	1.07 g
dH_2O	to 100.0 ml

Filter through a 0.45 μ filter and store at 4 °C.

50x TAE Buffer

Reagent	Amount	Final Concentration
Tris base	242.0 g	2 M
Acetic acid, glacial	57.1 ml	1 M
0.5 M EDTA, pH 8.0	100.0 ml	50 mM
dH_2O	to 1,000.0 ml	

Mix. Autoclave for 20–30 minutes. Store at room temperature.

Degas for 10–15 minutes. Filter through a 0.45 μ filter. Store at 4 °C in a brown bottle for approximately 1 month. A 100% denaturant solution requires re-dissolving after storage. Place the bottle in a warm bath and stir for faster results. For denaturing solutions less than 100%, use the volumes for acrylamide, TAE and wate described above in the 100% Denaturing Solution. Use the amounts indicated below for urea and formamide.

Denaturing Solution	10%	20%	30%	40%	50%	60%	70%	80%	90%
Formamide (ml)	4	8	12	16	20	24	28	32	36
Urea (g)	4.2	8.4	12.6	16.8	21	25.2	29.4	33.6	37.8

10% Ammonium Persulfate

Reagent	Amount
Ammonium persulfate	0.1 g
dH_2O	1.0 ml

Store at -20 °C for about a week.

DCode Dye Solution

Reagent	Amount	Final Concentration
Bromophenol blue	0.05 g	0.5%
Xylene cyanol	0.05 g	0.5%
1x TAE buffer	10.0 ml	1x

Store at room temperature. This reagent is supplied in the DCode electrophoresis reagent kit for DGGE, CDGE.

2x Gel Loading Dye

Reagent	Amount	Final Concentration
2% Bromophenol blue	0.25 ml	0.05%
2% Xylene cyanol	0.25 ml	0.05%
100% Glycerol	7.0 ml	70%
dH_2O	2.5 ml	
Total volume	10.0 ml	

Store at room temperature.

1x TAE Running Buffer

Reagent	Amount
50x TAE buffer	140 ml
dH_2O	6,860 ml
Total volume	7,000 ml

RESEARCH PUBLICATIONS

Papers presented

Velho-Pereira S. and Furtado I. Isolation and characterisation of Haloarchaea from the genus *Natrialba* from *Callyspongia reticutis var solomonensis* at the International 7th Asia Pacific Marine Biotechnology Conference, held on 2nd-3rd November 2007 in Kochi-Kerala.

Velho-Pereira S. and Furtado I.. Isolation and characterization of bioactive eubacterial and haloarchaeal epibionts from sponges of Mandapam-India at the National Seminar on Advances in Zoology and Life Processes, held on February 2012 in Goa University.

Publication in Journals

1. Velho-Pereira, S., Furtado, I. (2012) Antibacterial Activity of Halophilic Bacterial Bionts from Marine Invertebrates of Mandapam-India. *Indian J Pharm Sci* 74(4):331-338.

2. Velho-Pereira, S., Furtado, I. (2013) Retrieval of Euryhaline, Eubacterial and Haloarchaeal bionts from nine different benthic Sponges: Reflection of the bacteriological health of waters of Mandapam in India. (*In Press*: *Ind J Mar Sci*).

3. Velho-Pereira S., Naik C.G. and Furtado I. Antimicrobiocity and molecular characterization of the bacterial biont *Chromohalobacter salexigens* the biont of an unidentified sponge from Mandapam- India (communicated).

4. Velho-Pereira S., and Furtado I. Screening of antioxidant producing halophilic eubacterial bionts of marine sponges, corals and bivalve from the Indian subcontinent (communicated).

5. Velho-Pereira S., Parmeshwaran P. and Furtado I. Isolation and Characterisation of antibacterial compound 3-isobutyl-pyrrolopiperazine-1,4-dione produced by halophillic bacteria associated with a rare sponge inhabiting Mandapam-India (communicated).

6. Velho-Pereira S., and Furtado I. Bioactive potential of marine sponge associated haloarchaea *Halorubrum saccharovorum* (communicated).

7. Velho-Pereira S., and Furtado I. Evaluation of halophillic eubacteria and haloarchea from sponges, bivalve corals of Mandapam in GoM-India (under preperation).

8. Velho-Pereira S., Dhakhephalker P., Furtado I. Highly frequent detection of novel 16S rRNA phylotypes associated with *Acropora formosa* (under preparation)

Research Paper

Antibacterial Activity of Halophilic Bacterial Bionts from Marine Invertebrates of Mandapam-India

SHERYANNE VELHO-PEREIRA AND IRENE FURTADO*
Department of Microbiology, Goa University, Taleigao Plateau, Goa-403 206, India

Marine ecosystem and its organisms, particularly the invertebrates are recent targets of bioprospecting and mining for a large group of structurally unique natural products encompassing a wide variety of chemical classes such as terpenes, polyketides, acetogenins, peptides and alkaloids of varying structures, having pronounced pharmacological activities. In view of the limited reports on the antibacterials produced by bacteria, isolated from marine sponges, corals and bivalves of Indian origin, the present study is aimed at investigating the antagonistic activities of 100 heterotrophic, halophilic bacterial bionts isolated from 9 sponges, 5 corals and one bivalve. Culture broths of 46 of these bionts were active against human pathogenic bacteria namely *Staphylococcus citreus, Proteus vulgaris, Serratio marcesans, Salmonella typhi, Aerobacter aerogenes and Escherichia coli.* Further, the ethyl acetate extracts of cell free supernatant confirmed the presence of extracellular bioactive factor, by agar cup diffusion method. Interestingly, highest number of bionts having activity was isolated from corals followed by sponges and bivalve. The study clearly demonstrates that bacterial bionts of marine invertebrates are a rich source of bioactive secondary metabolites against human bacterial pathogens.

Key words: Antibacterial, halophilic-bacterial-bionts, human pathogens

Soil has been widely explored as the source of microorganisms possessing a large number of bioactive molecules. However, the continual and cyclic need of new antibiotics to combat the emerging resistant forms of bacterial pathogens has led to the exploration of newer econiches and biota, thereof. Marine ecosystem and its organisms, particularly the invertebrates, such as sponges, coelenterates (sea whips, sea fans and soft corals), tunicates, molluscs (nudibranchs, sea hares), echinoderms (starfish, sea cucumbers) and bryozoans (moss animals)[1,2] are recent targets of bioprospecting and mining for a large group of structurally unique natural products encompassing a wide variety of chemical classes such as terpenes, polyketides, acetogenins, peptides and alkaloids of varying structures representing and having pronounced pharmacological activities[3]. The diversity of marine organisms and the highly competitive environmental habitats in which access to space and nutrients are limited is responsible for this stunning variety.

One school of thought is that these marine invertebrates combat potential invaders, predators or competitors by producing secondary metabolites as chemical weaponry of their defence mechanisms[4]. Several of these metabolites have been characterised to be enzymes, haemolytic factors and antibiotics. In the recent years, however, investigations reveal that these bioactive factors may not be products of the marine invertebrates but may actually be produced by the microorganisms associated and/or inhabiting the sessile hosts[1,5-8]. Additionally, it is increasingly becoming evident that numerous natural products from marine invertebrates have striking structural similarities to metabolites of microbial origin. This poses a serious question on the role of host-associated microbes: Whether these are the true source of the metabolites used in defence by the host or whether these are intricately involved in biosynthesis of the metabolites used in defence[1]. Studies in these lines have demonstrated that microbes associated with invertebrates far exceed, in their bioactivity, as against that produced by free living planktonic bacteria[9,10]. In spite of this, there are limited reports on studies attempting to retrieve bacteria inhabiting marine invertebrates of Indian origin, into cultures, and scrutinising their antibacterial activity[11-13].

The Gulf of Mannar, is the world's richest marine bioreserve lying between the southern tip of India,

*Address for correspondence
E-mail: ijfurtado@unigoa.ac.in

the south-eastern coast of Tamil Nadu state and the north-west coast of Sri Lanka. It supports a diverse and productive community of marine life. The Gulf of Mannar, is reported to harbour 295 species of sponges, 106 species of corals, 466 species of molluscs including 271 gastropods, 174 bivalves, 5 polyplacophorans, 16 cephalopods, 5 scaphopods, 100 species of echinoderms and 180 species of marine algae and seaweeds[14,15].

In view of the limited reports on the antibacterials produced by bacteria isolated from marine sponges,[11,13,16,17] corals[18,19] and bivalves[20] of Indian origin, the present study is aimed at investigating the antagonistic activities of 100 heterotrophic, halophilic bacterial bionts isolated from nine sponges (*Petrosia testudinaria*, *Cinachyra cavernosa*, *Haliclona* sp., *Callyspongia fibrosa*, *Heteronema erecta*, *Fasciospongia cavernosa*, *Callyspongia reticutis var solomonensis* and two unidentified sponge samples), five corals (*Telesto* sp., *Echinogorgia reticulata*, *Echinomuricea indica*, *Echinogorgia complexa*, *Acropora formosa*) and one bivalve (*Perna viridis*) collected from the Mandapam in the Gulf of Mannar. Their activity was tested against multidrug-resistant bacterial strains namely *S. typhi*, *E. coli*, *P. vulgaris*, *A. aerogenes*, *S. marcesans* and *S. citreus*.

MATERIALS AND METHODS

Bacterial bionts were isolated by plating aliquots of saline macerates of tissues of sponges/corals/bivalve collected from Tamil Nadu on the South-Eastern coast of the Indian peninsula situated at (Lat. 09°19′ 37.3″N and Long. 79°10′ 20.5″E), onto nutrient-rich sterile, tryptone yeast extract agar (TYE)[21] having (g/l) $MgSO_4$-20; $CaCl_2$-0.2; Tryptone-5; Yeast extract-3, adjusted to pH 7, using 1 N NaOH and solidified with Agar 1.5. TYE supplemented with 3% NaCl and 25% NaCl, refered, hereafter as, (3%TYE) and NTYE)[22-24], respectively. Colonies were purified and identified following schemes given by Smibert and Krieg, and Holt *et al.*[23,24] and maintained on TYE agar slopes having 3/25% NaCl. All the chemicals used were from Himedia, Mumbai, India.

Human bacterial pathogens:

Salmonella typhi, *E. coli*, *P. vulgaris*, *A. aerogenes*, *S. marcesans* and *S. citreus* obtained from Goa Medical College, Bambolim were pregrown separately in 5 ml nutrient broth medium to an absorbance of 1 at 600 nm for 24 h at R.T.

Screening of bacterial bionts for antagonistic activity:

Individual bacterial bionts were inoculated into 5 ml of 3% TYE and NTYE. After 2/7 days, aliqouts were dispensed into agar cups, borne onto Mueller Hinton agar spread plated with the individual pregrown human bacterial pathogens.

Demonstration of extracellular bioactive factor:

Individual bacterial bionts were cultured in 3% TYE and NTYE at R.T. (22-28°) for a minimum of 2 days and a maximum of 7 days at 150 rpm. The cell-free supernatant obtained on separation of cells by centrifuging at 12,000×*g* for 20 min at 4° was extracted three times in ethyl acetate (EA), concentrated to dryness under vacuum using rotary evaporator (Buchi, Essen Germany). Crude extract was dissolved in methanol and used for bioactivity studies.

Quantification of antibacterial activity:

Mueller Hinton agar plates of 3 mm thickness were seeded with the pregrown bacterial pathogens. Four milligram per litre of crude extracts was dispensed into agar cups, 6 mm in diameter borne onto the Mueller Hinton agar plates. The plates were kept standing at low temperature (4°) for 15 min, incubated at 37° and monitored for growth over a period of 24 h. Assay was carried out in triplicates and mean was recorded. Controls were maintained for each test pathogen. Each experimental data set was carried out aseptically. Zones of inhibition were measured in mms and data was computed using the earlier reported quantification procedure[25] to obtain:

Percent area specific differential antibiotic activity score (PASDAAS)=[AWG/TSA]×100 (1)

where AWG is the area on the plate without growth of test pathogen [area of zone of inhibition-area of the plug (28.26 mm^2)], TSA is the total swabbed area of the pathogen on the plate (6358.5 mm^2).

Percent multispecific antibiosis efficiency score (PMSAES), computed using the following equation:

$$PMSAES=(\Sigma PASDAASTP_{1\text{-}6}/TPS)\times 100 \quad (2)$$

where $\Sigma PASDAASTP_{1\text{-}6}$ is the percent area specific differential antibiotic activity score of test pathogens 1-6 and TPS is total possible score for all test pathogens (i.e., 100×6=600).

Percent overall inhibition efficiency score (POIES), was calculated using the following equation:

$$POIES=(TNIS/TNTS)\times100 \quad (3)$$

where TNIS is total number of inhibited species and TNTS is total number of test species. The ideal score for multispecific inhibition would be 100.

Percent overall screening efficiency score (POSES). This is computed by,

$$POSES=(TPR/TAS)\times100 \quad (4)$$

where, TPR is the total number of positive results for each test pathogen and TAS is the total number of bionts.

RESULTS

Hundred euryhaline bacterial bionts obtained from marine sponges, bivalve and corals were characterised on the basis of their ability to tolerate a maximum of 3 and 25% NaCl concentrations during growth in TYE medium into: Group I: Bionts growing in TYE with 3% NaCl and Group II: Bionts growing in TYE with 25% NaCl.

Culture broths of bionts growing in their respective growth media were screened for the production of extracellular bioactivity by directly exposing individual indicator cultures of bacterial pathogens to specific quantity of culture broth and observed for development of zone of inhibition of growth. The bionts giving a minimum zone of inhibition of 2 mm were considered as bioactive. Twenty percentage of Group I bionts were active against Gram-positive indicator cultures whereas 23% were active against the Gram-negative indicator cultures. On the other hand, 80% of bionts from Group II were active against Gram-positive indicator cultures and 77.27% of bionts were active against Gram-negative indicator cultures. Consequently, 46 out of 100 bionts were selected for further investigation wherein 2/7 day cell-free supernatants of active bionts were extracted in EA and used for monitoring of *in vitro* antibacterial activity. As seen in (Tables 1-3) extracts of bionts associated with nine different sponges, five different corals and one bivalve were active, with individual bionts having indicator culture specificity. The zones of inhibition ranged from 1-30 mm for sponge bionts, 1-20 mm for bivalve bionts and 2-30 mm for coral bionts.

Differential antibacterial activity of individual bionts towards various indicator pathogens was deduced by comparing zone sizes of antibacterial activity and recorded as percent area specific differential antibiotic activity score (PASDAAS). Highest PASDAAS of 53.5% was shown by the sponge biont GUVFCCM-2 against *E. coli* and by the coral bionts namely GUVFEIM-3 and GUVFECM-1 against *A. aerogenes* and *S. citreus*, respectively. Using PASDAAS, percent multispecific antibiosis efficiency score (PMSAES) was calculated. The highest PMSAES (fig. 1) amongst the sponge bionts was shown by GUVFCCM-2, identified as *Chromohalobacter* sp. with a value of 17.2% (fig. 2). It was active against all the tested indicator pathogens except *S. marcesans*. This was

TABLE 1: ANTIBACTERIAL ACTIVITY OF ETHYL ACETATE EXTRACTS OF SPONGE BIONTS

Sponges	Isolates	Zone of inhibition (mm)						Generic identity
		S. typhi	*S. marcesans*	*A. aerogens*	*E. coli*	*S. citreus*	*P. vulgaris*	
Petrosia testudinaria (MAM-1)	GUVFPM-1	6	-	6	-	5	-	*Chromohalobacter* sp.
	GUVFPM-2	-	-	2	-	-	-	*Corynebacterium* sp.
	GUVFPM-6	-	-	-	-	-	2	*Pontibacillus* sp.
Cinachyra cavernosa (MAM-2)	GUVFCCM-2	8	-	20	30	12	10	*Chromohalobacter* sp.
Haliclona sp. (MAM-4)	GUVFHM-2	7	-	8	-	7	-	*Chromohalobacter* sp.
Unidentified (MAM-5)	GUVFUM-1	10	-	8	-	5	11	*Corynebacterium* sp.
Callyspongia fibrosa (MAM-6)	GUVFCFM-3	1	-	-	-	-	-	*Corynebacterium* sp.
Heteronema erecta (MAM-6)	GUVFHEM-4	-	-	-	10	20	18	*Pseudomonas* sp.
Callyspongia reticutis var solomonensis (NIO1)	GUVFCM	2	4	-	-	3	6	*Halobacteria* sp.
Fasciospongia cavernosa (NIO2)	GUVFFM-1	-	10	1	1	-	17	*Halobacteria* sp.
	GUVFFM-2							*Halobacteria* sp.
Unidentified (NIO3)	GUVFSM-1	7	6	5	3	-	14	*Halobacteria* sp.
	GUVFSM-2	12	-	9	11	10	-	*Chromohalobacter* sp.

TABLE 2: ANTIBACTERIAL ACTIVITY OF ETHYL ACETATE EXTRACTS OF CORAL BIONTS

Corals	Isolates	Zone of inhibition (mm)						Generic identity
		S. typhi	*S. marcesans*	*A. aerogens*	*E. coli*	*S. citreus*	*P. vulgaris*	
Telesto sp. (MAM-3)	GUVFTM-1	2		2	-	-	-	*Bacillus* sp.
	GUVFTM-2	6	-	15	12	12	12	*Chromohalobacter* sp.
	GUVFTM-3	2	-	-	-	-	-	*Bacillus* sp.
	GUVFTM-4	-	2	3	-	-	-	*Bacillus* sp.
	GUVFTM-5	2	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFTM-6	-	-	2	-	-	-	*Chromohalobacter* sp.
Echinogorgia reticulata (MAM-7)	GUVFERM-1	-	-	2	-	-	-	*Corynebacterium* sp.
	GUVFERM-2	-	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFERM-7	-	-	8	-	-	-	*Chromohalobacter* sp.
Echinomuricea indica (MAM-8)	GUVFEIM-1	5	-	-	-	-	-	*Chromohalobacter* sp.
	GUVFEIM-3	-	-	30	-	-	-	*Chromohalobacter* sp.
	GUVFEIM-8	-	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFEIM-9	-	-	2	-	-	-	*Chromohlobacter* sp.
	GUVFEIM-14	-	-	2	-	-	-	*Psychrobacter* sp.
Echinogorgia complexa (MAM-9)	GUVFECM-1	-	-	-	10	30	-	*Deinococcus* sp.
	GUVFECM-2	2	-	2	-	-	-	*Chromohalobacter* sp.
	GUVFECM-3	-	-	12	-	-	15	*Chromohalobacter* sp.
	GUVFECM-5	-	6	-	2	5	-	*Chromohalobacter* sp.
	GUVFECM-6	-	5	-	-	-	-	*Chromohalobacter* sp.
	GUVFECM-7	-	-	2	-	-	-	*Bacillus* sp.
	GUVFECM-8	-	-	2	-	-	-	*Virgibacillus* sp.
	GUVFECM-9	-	-	5	-	-	-	*Chromohalobacter* sp.
Acrospora formosa (GUBFM)	GUVBAM-1	8	-	3	-	-	-	*Bacillus* sp.
	GUVBAM-2	-	-	2	13	-	-	*Pontibacillus* sp.
	GUVBAM-3	-	-	9	-	5	-	*Corynebacterium* sp.
	GUVBAM-4	-	-	1	4	-	-	*Bacillus* sp.
	GUVBAM-5	7	-	3	8	-	8	Unidentified

TABLE 3: ANTIBACTERIAL ACTIVITY OF ETHYL ACETATE EXTRACTS OF BIVALVE BIONTS

Bivalve	Isolates	Zone of inhibition (mm)						Generic Identity
		S. typhi	*S. marcesans*	*A. aerogenes*	*E. coli*	*S. citreus*	*P. vulgaris*	
Perna virdis (GUFM)	GUVFPM-1	7	-	7	2	-	10	*Planococcus* sp.
	GUVFPM-2	20	-	6	5	15	-	*Bacillus* sp.
	GUVFPM-3	7	-	-	-	-	-	*Bacillus* sp.
	GUVFPM-4	2	-	-	4	8	1	*Bacillus* sp.
	GUVFPM-5	3	-	8	-	-	-	*Psychrobacter* sp.

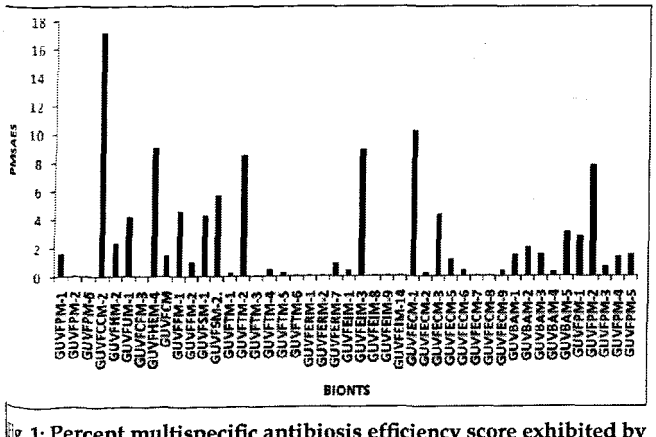
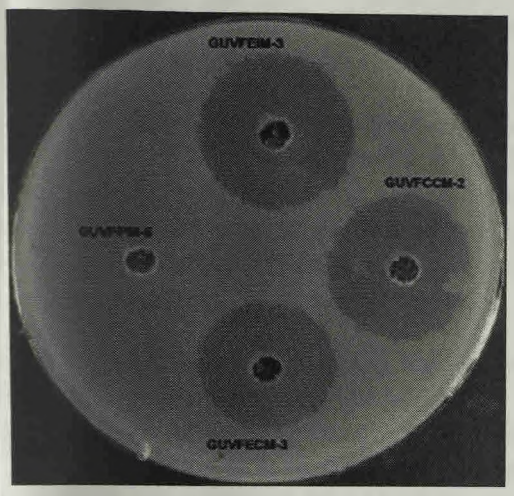


Fig. 1: Percent multispecific antibiosis efficiency score exhibited by bacterial bionts

followed by GUVFHEM-4 with a value of 9.08%, identified as *Pseudomonas* sp. GUVFHEM-1 was active against *E. coli*, *S. citreus* and *P. vulgaris*. Sponge bionts with PMSAES values in the range 2-6% were GUVFSM-2, with a value of 5.74%, it was identified as *Chromohalobacter* sp. and was active against *S. typhi*, *A. aerogenes*, *E. coli* and *S. citreus*. Biont GUVFFM-1 with a value of 4.64% was identified as Haloarchaea and was active against *S. marcesans*, *A. aerogenes*, *E. coli* and *P. vulgaris*. Bionts GUVFSM-1 identified as Haloarchaea and biont GUVFUM-1 identified as *Corynebacterium* sp., had near equal PMSAES values of 4.48 and 4.22%, respectively. GUVFSM-1 was active against *S. typhi*, *S. marcesans*, *A. aerogenes*, *E. coli* and *P. vulgaris* whereas biont GUVFUM-1 inhibited *S. typhi*, *S. citreus*, *P. vulgaris* and *A. aerogenes*. Biont GUVFHM-2, had a PMSAES value of 2.38% and was identified as *Chromohalobacter* sp. It was active against *S. citreus*, *A. aerogenes* and *S. typhi*.



ig. 2: Antibacterial activity of bacterial bionts GUVFCCM-2, GUVFEIM-3 and GUVFECM-3 against *A. aerogens*

Bionts having PMSAES values in the range 0.05-2% were GUVFPM-1 identified as *Chromohalobacter* sp., having a PMSAES value of 1.61% and was inhibitory to *S. typhi*, *A. aerogenes* and *S. citreus*. Biont GUVFCM with a value of 1.46% was identified as Haloarchaea and was active against *P. vulgaris*, *S. citreus*, *S. marcesans* and *S. typhi*. GUVFFM-2 identified as Haloarchaea was inhibitory against *E. coli* and *S. marcesans* and had a value of 1.03%. GUVFPM-2 and GUVFPM-6 had similar values of 0.11% and were identified as *Corynebacterium* sp. and *Pontibacillus* sp., respectively. They were active against *A. aerogenes* and *P. vulgaris*, respectively. GUVFCFM-3 had the lowest value of 0.05%, and was identified as *Corynebacterium* sp. and was solely active against *S. typhi*.

Coral biont with highest PMSAES value of 10.23% was GUVFECM-1 identified as *Deinococcus* sp., was active against *S. typhi* and *A. aerogenes*. This was followed by GUVFEIM-3 and GUVFTM-2 with near-equal PMSAES values of 8.91 and 8.51%, respectively. Both the isolates were identified as *Chromohalobacter* sp. GUVFEIM-3 was active solely active against *A. aerogenes* whereas GUVFTM-2 was active against *S. typhi*, *A. aerogenes*, *E. coli*, *S. citreus* and *P. vulgaris*. GUVFECM-3 identified as *Chromohalobacter* sp., was active against *A. aerogenes* and *P. vulgaris* and had a PMSAES value of 4.36%. It was followed by GUVFAM-5 with a value of 3.15%. This isolate remained unidentified as it failed to grow on repeated subculture and was active against *S. typhi*, *A. aerogens*, *E. coli* and *P. vulgaris*. Isolate with PMSAES values ranging from 1 to 2% were GUVFAM-2 identified as *Pontibacillus* sp., active against *A. aerogenes* and *E. coli* and had a PMSAES value of 2.03%. Near-equal PMSAES values of 1.56 and 1.5% were shown by bionts GUVFAM-3 and GUVFAM-1 against *A. aerogenes* and *S. citreus* for GUVFAM-3 and against *S. typhi* and *A. aerogenes* for GUVFAM-1. They were identified as *Corynebacterium* sp. and *Bacillus* sp., respectively. Biont GUVFECM-5 identified as *Chromohalobacter* sp. showed a value of 1.15% against *S. marcesans*, *E. coli* and S. *citreus*.

Bionts with PMSAES values in the range 0.2-1% were GUVFERM-7 at 0.91%, it was identified as *Chromohalobacter* sp. and inhibited *A. aerogenes* only. This was followed by biont GUVFTM-4 at 0.53%, identified as *Bacillus* sp. and active against *A. aerogenes* and *S. marcesans*. Bionts with equal values of 0.45% were GUVFEIM-1, GUVFECM-6 and GUVFECM-9. They were all identified as *Chromohalobacter* sp. GUVFEIM-1 inhibited *S. typhi*, GUVFECM-6 was active against *S. marcesans* whereas GUVFECM-9 inhibited *A. aerogenes* alone. GUVFAM-4 identified as *Bacillus* sp. had a value of 0.37% and was active against *A. aerogenes* and *E. coli*. GUVFTM-5, GUVFTM-1 and GUVFECM-2 had similar PMSAES values of 0.23% and were identified as, *Chromohalobacter* sp., *Bacillus* sp. and *Chromohalobacter* sp. and were all active against *S. typhi* and *A. aerogenes*. Bionts GUVFTM-3, GUVFTM-6, GUVFERM-1, GUVFERM-2, GUVFEIM-7, GUVFEIM-10, GUVFEIM-12, GUVFECM-8, GUVFECM-7 and GUVFECM-8 all showed similar PMSAES value of 0.11%. GUVFTM-3 inhibited *S. typhi* and *A. aerogenes* whereas the rest were active solely against *A. aerogenes*.

Amongst the bivalve bionts GUVFPM-2 identified as *Bacillus* sp. showed the highest PMSAES value at 7.8% and was active against *S. typhi*, *A. aerogenes*, *E. coli* and *S. citreus*. This was followed by biont GUVFPM-1 with a relatively lower value of 2.85%. It was identified as *Bacillus* sp. and was inhibitory against *S. typhi*, *A. aerogenes*, *E. coli* and *P. vulgaris*. Bionts GUVFPM-4 and GUVFPM-5 identified as *Bacillus* sp. and *Psychrobacter* sp., respectively, showed near equal PMSAES values of 1.45 and 1.5%, respectively. GUVFPM-4 inhibited *S. typhi*, *E. coli*, *S. citreus* and *P. vulgaris* whereas GUVFPM-5 was active against *S. typhi* and *A. aerogenes*. Lowest

PMSAES was shown by biont GUVFPM-3 identified as *Bacillus* sp. with a value of 0.73% against *S. typhi*.

The calculated percent overall inhibition efficiency score of bionts (POIES) indicated that 33% of the bionts were active against *A. aerogenes*, 21% inhibited *S. typhi*, 14% were active against *E. coli*, 13% against *S. citreus* and 12% against *P. vulgaris*. The least inhibited was *S. marcesans* at 6%.

Further, the percent overall screening efficiency score of antibacterial activity (POSES) exhibited by bionts was computed by scoring presence or absence of zones by bionts against indicator cultures and depicted in fig. 3. Highest score of 83.33% was given by sponge bionts GUVFCCM-4 and GUVFSM-1 and coral biont GUVFTM-2.

DISCUSSION

The study was an attempt to investigate the antibacterial activity of bacterial bionts from sponges, corals and bivalves thought to be involved in the epibacterial chemical defence of the host[17], in this regard, it was a noteworthy observation that 46 isolates out of the 100 screened, showed promising antibacterial activity. These bionts having activity against multidrug-resistant clinical pathogens, isolated from hospital patients, have potential to serve as drug candidates. The inhibition of the pathogens by the extracts obtained from the invertebrate-associated bacteria strongly supports the hypothesis of the microbial origin of the compounds formerly ascribed to these macro invertebrates as there are several reports on the antibacterial potential of the marine invertebrates used in this study[12,26,27].

Halophilic bacterial strains exhibited a higher antimicrobial activity against the Gram-negative bacteria than against the Gram-positive bacteria. These results are not consistent with previous studies wherein Gram-positive bacteria were more susceptible to antibiotics than Gram-negative bacteria[28]. The probable reason for this finding is that only a single Gram-positive indicator test was included in the test panel.

Our study corroborates with the findings of Chen *et al.*[29] in that approximately 50% of the culturable bionts exhibited antibacterial activity. The results thus confirm that invertebrate-associated microorganisms are highly potential resources of bioactive natural products[30].

The absence of antimicrobial activity in the remaining 50% bionts in the bioassays conducted does not necessarily indicate a lack of antimicrobial chemical defence, as the diffusion assay only measures cell death, however, there are reports of inhibition of other phenotypic characteristics such as chemotaxis, swarming attachment and swimming, which is also a means of counteracting bacterial invasion. Another proposed hypothesis by Geffen and Rosenberg[31] for no bioactivity of some of the isolates could be that the release of the bioactive factor is only seen following induction by deleterious microorganisms and mechanical stress which was not done in our present study. It could also be possible that the bioactive component was not extractable in the EA solvent or that it diffuses poorly in the agar medium employed. The demonstration of poor bioactivity by some bacterial isolates associated with the invertebrates is suggestive that the invertebrates resort to some other means of defence rather then production of chemical compounds as reported by Rublee *et al*[32].

The isolates having the greatest antimicrobial activity belonged to the genus *Chromohalobacter*, followed by *Bacillus* and *Corynebacterium*. The genus *Bacillus* have been well-known to produce lipoproteins, phenolic derivatives, aromatic acids, acetylamino acids (amino acid analogues), peptides[33], isocoumarin antibiotics and[34] bacteriocin like substances[35] having a broad antibiotic spectrum, the genus *Corynebacterium* is also increasingly reported as a source of bioactive agents capable of displaying competitive biosynthetic capabilities[36], however, the potential of the genus *Chromohalobacter* as a promising resource for antimicrobial compounds is fairly recent with no reports on the structure elucidation of the antimicrobial compound and scarce

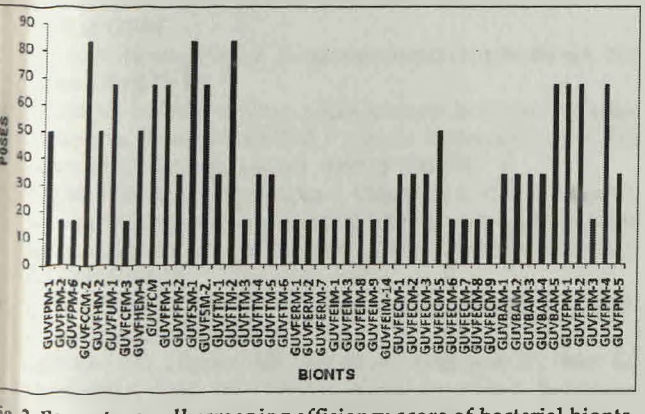


Fig. 3: Percent overall screening efficiency score of bacterial bionts

reports on its antimicrobial activity[29]. Thus, our first report on the isolation of halophilic bacterial strains from marine invertebrates as promising sources for the discovery of novel bioactive compounds is of immense importance.

The high proportion of strains producing antimicrobial compounds may be associated with an ecological role, i.e., a defensive action to maintain their niche, preventing the invasion of microbial competitors into an established microbial community. Thus, marine invertebrates represent an ecological niche harbouring a largely uncharacterised microbial community with unexploited potential sources of new secondary metabolites. Further chemical isolation and characterisation of active compounds from these bacterial extracts is under investigation, and findings will be reported in due course. As yet, there have been no published reports on the antibacterial activity of all the marine organisms discussed so far from the GoM. Thus, the present study is the first report and it proves that the EA extracts of marine bacteria associated with sponges, bivalves and corals are a promising resource having profound antibacterial activity, and thus may have potential use in medicine.

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Retrieval of Euryhaline Eubacterial and Haloarchaeal bionts from nine different benthic Sponges: Reflection of the bacteriological health of waters of Mandapam in India

Velho-Pereira S. & Irene Furtado*

Department of Microbiology, Goa University, Taleigao Plateau, Goa 403 206, India.

***[E-mail: ijfurtado@unigoa.ac.in]**

Abstract:

Bacterial bionts from nine sponges from intertidal reaches of Mandapam (9°17'0" N and 7°7'0"E), namely: were enumerated by the acridine orange and viable count in Tryptone Yeast Extract medium (TYE) with 3-25% NaCl. Forty two pure bionts were characterized to generic level according to Bergey's Systematic Bacteriology and SYSTAT-v.12.01. Diversity, richness and evenness of genera were determined using PRIMER v.5. Direct bacterial counts for sponges, averaged at $5.2\pm2.20\times10^9$ cells/g. Viable counts averaged at $3.31\pm2.60\times10^8$ cfu/g, $3.83\pm2.86\times10^8$ cfu/g and $2.89\pm2.3\times10^8$ cfu/g on TYE, 3% TYE and NTYE, respectively. The Genus *Bacillus*, followed by *Corynebacterium* and *Chromohalobacter* predominated the sponges. Retrieval of bacterial bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* known for pathogenicity to humans, reflects pollution of Mandapam waters by sewage and heralds *Caution* for safeguarding the waters and ensuring sustainability of biota.

[**Keywords**: Extremophilic-Eubacteria, Euryhaline, Haloarchaea, Bionts, Sponges, Mandapam-waters]

Introduction

Marine environments are complex and dynamic largely due to hydrography, changing physico-chemical conditions and biota. Globally marine sponges are reported to be widely distributed in the intertidal regions of estuarine reaches. These regions undergo fluctuations in salinity, temperature and light that are reported to adversely affect the bacterial flora. Among marine invertebrates, sponges of phylum Porifera, during their benthic lifestyle and filter feeding habits encounter micro-organisms inherent or transient in the waters they live in. Microorganisms, that get access into the interior of the sponge during the efflux and influx of water establish therein as endobionts or exobionts[1]. Bacteria are reported to contribute up to 40% of the sponge biomass (equal to about 10^8 to 10^9 bacteria/g of sponge tissue).

Mandapam in India with shallow waters is intertidal and is rich in marine resource[2]. In recent times, the region is reported as a water body for disposal of sewage, industrial effluents and terrestrial runoff [3,4]. Sponges inhabiting such waters due to their feeding habits are expected to sieve in bacteria singly or along with dissolved, particulate organic and inorganic matter, through their ostia into its system of internal channels. The process thus offers an opportunity for the bacteria to establish as bionts of the host tissue. Bacteriological studies related to unveiling the possible role of retrieved bionts, state that they are expected to exhibit activities that are antagonistic/ protogonistic/ remediative. Studies on retrieval of bacteria from sponges inhabiting the GoM-India have been carried out using culture media having 3.5% NaCl incapable of supporting growth of halophiles requiring higher concentrations of NaCl [5,6,7], although moderately halophilic/halotolerant heterotrophic bacteria, requiring 15-20% NaCl, have been reported from various non hypersaline marine habitats [8,9].

In the light of the above, in this study, we selected nine different sponges, collected from Mandapam in India, namely *Petrosia testudinaria, Cinachyra cavernosa, Haliclona sp., Callyspongia fibrosa, Heteronema erecta, Fasciospongia cavernosa, Callyspongia reticutis var solomonensis* and two unidentified sponges to retrieve bacterial bionts, using strategies of manipulation of concentration of NaCl in the nutrient rich growth media and their characterization to generic level using keys of Bergey's Determinative Biology and SYSTAT v.12.01, and evaluation of their predominance and commonality with respect to the hosts through PRIMER v.5.

Material & Methods

Sponge samples

Sponges were collected from Mandapam in Tamil Nadu on the Eastern coast of the Indian peninsula situated at 9°17'0" North and 7°7'0" on East Coast in the Gulf of Mannar (GoM), and deposited under the accession code MAM 1, MAM 2, MAM 4, MAM 5, MAM 6, MAM 10, NIO 1, NIO 2 and NIO 3 in the Biological Chemistry laboratory at the National Institute of Oceanography, Dona Paula, Goa-India.

Enumeration and retrievable of bacteria from sponge tissue

Preparation of sponge macerate

One gram of sponge tissue was excised from the middle of the whole sponge using a sterile scissor, weighed and washed several times in 3% (w/v) NaCl. Following discard of these washings, the sponge samples were homogenized in the same medium using a mortar and pestle in aseptic conditions. The macerate was filtered to remove any solid debris and used for:

i) Enumeration by Acridine Orange Direct Count (AODC) using epifluorescent microscopy

To determine the total bacterial counts, a defined volume of the tissue homogenate was fixed with 2.5% (w/v) SEM grade glutaraldehyde to a final concentration of 4% (w/v) and stored at 4°C until use. It was filtered through a 25 mm diameter polycarbonate filter (GE Osmonics,

Minneapolis, MN) and stained with 200 µl of Acridine Orange (10 mg/ml) solution for 5 mins as described previously[10]. The filters were air dried and mounted with immersion oil onto a microscopic slide. Bacterial numbers were determined using an epifluorescence microscope (Axioplan microscope; Zeiss, Germany). 20-40 quadrats with a minimum of 600 cells were counted, which gives a precision of ±10%. The bacterial cell numbers were calculated using the Generalization: cells/ml = (SC-BC) x CF x F/V wherein, SC is the mean of sample counts/quadrat; BC is the mean of background counts/quadrat; CF is the effective filter area/quadrat area; F is the volume of preservative/volume sample preserved +1; V is the volume of sample filtered.

ii) Retrieval and isolation of pure bacterial bionts

The macerate was serially diluted, and 10 µl of the filterate was plated separately onto i) Tryptone Yeast Extract medium (TYE)[11] consisting of (g/l) $MgS0_4$-20; $CaCl_2$-0.2; Tryptone-5; Yeast Extract-3, pH was adjusted to 7, using 1N NaOH, ii) TYE supplemented with 3% NaCl (3% TYE) and iii) TYE with 25% of NaCl (NTYE)[12]. All the chemicals used were from Himedia-India. Plates were incubated at R.T. until visible colonies were seen on the plates. The bacterial colonies were enumerated to obtain cfu/g of sponge tissue. Morphology of the colonies growing on TYE medium, 3% TYE, and NTYE were recorded. Distinct colonies were purified by repeated streaking on corresponding agar medium, designated and maintained as axenic cultures on agar slopes of the different isolation media at 4°C for a month. Isolates were designated as GUVFPM; GUVFCCM; GUVFHM; GUVFUM; GUVFCFM; GUVFHEM; GUVFCM; GUVFFM; GUVFSM denoting GU (Goa University),V (Velho), F (Furtado), P (*Petrosia*),C (*Callyspongia*), F (*Fasciospongia*),S (Unidentified Sponge), CC (*Cinachyra cavernosa*),H(*Haliclona*),U(Unidentified Sponge),CF(*Callyspongia fibrosa*),HE (*Heteronema erecta*) and M(Mandapam).

Morphological and Biochemical Characterisation of bacterial bionts

Morphological, and biochemical potentials of the isolates were examined using standard bacteriological procedures and individual isolates were identified upto generic level following schemes given by Smibert and Krieg[13].

Statistical Analysis

For every cultured bacterial isolate, the absence and presence of specific phenotypic character, was assessed and rated as 0 (for negative) or 1 (for positive) respectively. Bacterial cultures were sorted out by measuring the similarity and diversity between two bacteria namely A and B, by dividing the size of the intersection by the size of their union as in Jaccard coefficient. Hierarchical rearrangement of clusters attained through group-average linking method based on Jaccard distance, which measures dissimilarity between sample sets and is obtained by subtracting the Jaccard coefficient from 1, by dividing the difference of the sizes of the union and the intersection of two sets by the size of the union. Computations were carried out using the software SYSTAT v.12.01[14] to Generate dendograms which reflect the phenotypic relationships between members and expressed as a *phenogram*.

Statistical measures of diversity

Margalef's species richness (*d*), Pielou's eveness (*J'*) and Shannon-Wiener's diversity index (*H'*) were analyzed using PRIMER v.5, inorder to arrive at univariate measures of bacterial community in sponges.

Results

Enumeration of bacterial bionts by Acridine Orange Direct Count

The Acridine orange direct counts for each of the nine sponges macerates, ranged from 2.3 - 8.7 x 10^9 cells/g. The total bacterial counts averaged at 5.2 ± 2.2 x 10^9 cells/g. The highest count of 8.7 x 10^9 cells/g was observed in unidentified sponge MAM5 and the lowest count of

2.30 x 10^9 cells/g in *C.cavernosa* and *H.erecta*. Bacterial morphotypes resolved were cocci, coccobacilli, curved rods, long rods, short rods (Figure 1).

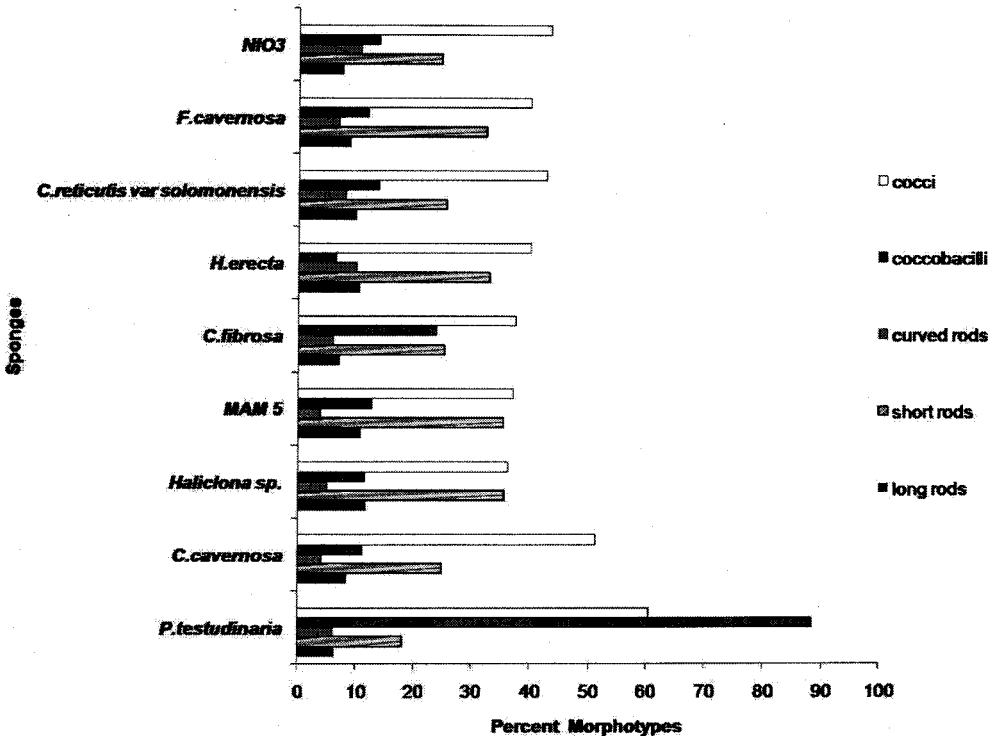

Figure 1- Percent Morphotypes of bacterial bionts revealed by Acridine Orange Direct Count in sponge tissue of Mandapam-India

Retrieval and isolation of pure bacterial bionts

The coccal forms were a maximum of 61% in *P. testudinaria* followed by 51% in *C. cavernosa,* 44% in Unidentified sponge NIO 3, 43% in *C. reticutis var solomonensis,* 40% in *H. erecta* and *F.cavernosa* and a near equal of 38% in *C. fibrosa, Haliclona sp.* and MAM 5. Coccobacillary forms predominated in the sponge *P. testudinaria* at 88% followed by 24% in *C. fibrosa*, 14% in *C. reticutis var solomonensis,* NIO 3 and *F.cavernosa* and a near equal of 12% in sponges *C. cavernosa, Haliclona sp.* and MAM 5. Among the rod morphotypes the proportion of

the long and curved rods ranged from 6-12% and 3-10% respectively. The short rods on the other hand were the highest with an almost equal in sponges MAM 5 and *Haliclona sp.* at 36% followed by 33% in *H. erecta* and *F. cavernosa,* 25% in sponges *C. fibrosa, C.reticutis var solomonensis,* NIO 3 and *C. cavernosa.*

As seen in Figure 2, total viable counts on TYE ranged from $2x10^5$ - 8.32 x 10^8 cfu/g in all the sponges. The counts on TYE averaged at 3.31 ± 2.60 x 10^8 at cfu/g. The total viable counts on 3% TYE on the other hand ranged from $2x10^5$ - 7.99 x 10^8 cfu/g with average counts of 3.83 ± 2.86 x 10^8 cfu/g in all the sponges. Individual sponges gave a total viable count ranging from 1.2 x 10^5 - 6.48 x 10^8 cfu/g on NTYE. The total viable counts on NTYE averaged at 2.89 ± 2.3 x 10^8cfu/g. The highest average counts were obtained on 3% TYE.

Greatest diversity of aerobic flora in terms of morphotypes, was observed in TYE media and gradually decreased with the increasing concentration of NaCl. The cream coloured colonies that dominated in NTYE, showed a reddish orange pigment on prolonged incubation. Long incubation periods maximized the viable counts and diversity of the halophiles. Interestingly despite of high colony count, the bacterial growth from individual sponges largely comprised of restricted colonial morphotypes, which resulted in isolation and purification of only a maximum of 42 different colonies. Of the total isolates 61.9% were Gram positive, and the remaining 38.09% of the isolates were Gram negative.

Individual bacterial bionts, although initially retrieved from growth on TYE/3%TYE/NTYE, were also able to grow at other concentrations of NaCl (Table 1). and hence categorized as: *marine halophiles* growing at 3% NaCl and *halotolerant or euryhaline* growing at 0-25% NaCl. 78.57% of the bionts associated with all the sponges under study were euryhaline while the remaining 21.42% were marine halophiles.

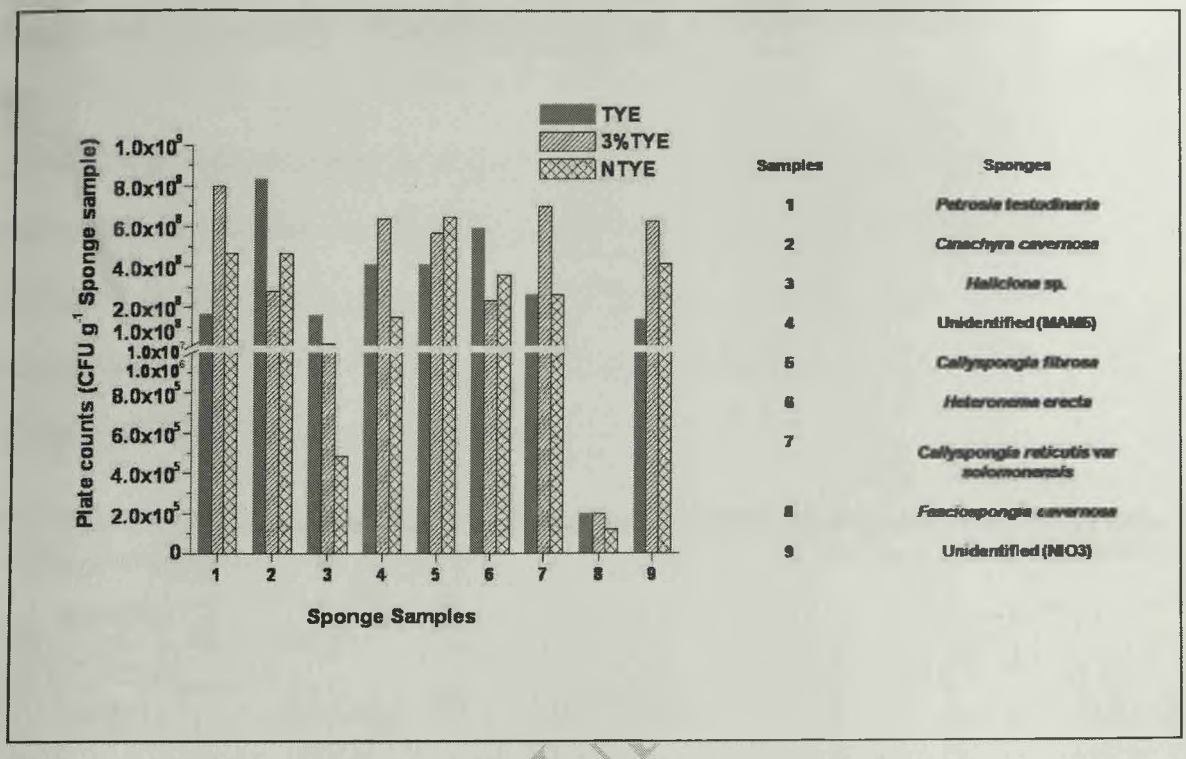

Figure 2 - Total bacterial counts in cfu/g on TYE, 3%TYE and NTYE.

Morphological and Biochemical Characterization of bacterial bionts and Statistical Analysis

Systat analysis further sorted the Gram positive and Gram negative bionts based on their biochemical characteristics (Table 1) using distance matrix and UPGMA clustering into 4 and 1 **Phena** and 1 and 3 **Phena,** respectively as represented in the phenogram (Figure. 3a & b). Thus confirming the identification resolved to generic level, for each retrieved bacterial biont (Table 1) according to keys of Bergey's Systematic Bacteriology [13, 15].

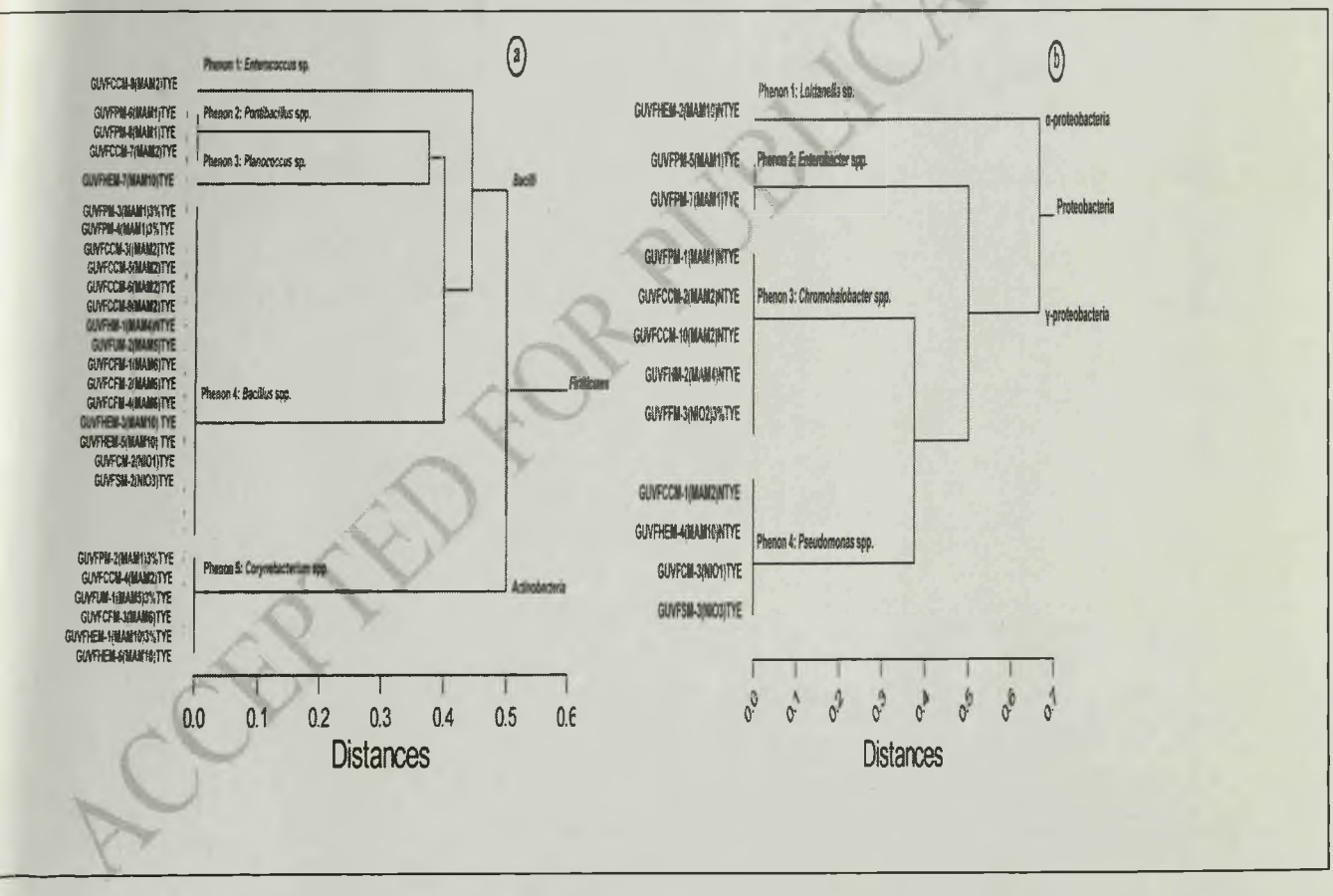

Figure 3 - Phenogram depicting sorting of morphological and biochemical characteristics of eubacterial bionts retrieved from sponges of Mandapam-India using keys of Bergey's Systematic Bacteriology and **SYSTAT v.12.01.**

(a) Gram positive bacteria ≈ **Cluster A** (Phylum: Firmicutes) with **Subcluster IA** (Class: Bacilli) with 4A **Phena** (Genera: *Enterococcus; Pontibacillus; Planococcus* and *Bacillus*) and **Subcluster IIA** (Class: Actinobacteria) with 1A **Phenon** (Genera: *Corynebacterium*). (b) Gram negative bacteria ≈ **Cluster B** (Phylum: Proteobacteria) with **Subcluster IB** (Class: Alphaproteobacteria) with 1B **Phenon** (Genera: *Loktanella*) and **Subcluster IIB**: (Class: Gammaproteobacteria) with 3B **Phena** (Genera: *Enterobacter; Chromohalobacter* and *Pseudomonas*).

Table 1. Biochemical potentials and tentative identification of dominant bacterial bionts retrieved and purified from sponges of Mandapam- India

Sponges	Isolates	Retrieval on TYE with NaCl (%)	Pigmentation	Morphotypes/ spores	Gram Character	Motility	Oxidase	Catalase	Glucose	Sucrose	Lactose	Indole	Methyl Red	VP	Citrate	NR	Growth in NaCl (%)			Genera
																	0	3	25	
MAM 1 *Petrosia testidunaria*	GUVFPM-1	25	cream	R	Nil	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	+	***Chromohalobacter* sp. 1**
	GUVFPM-2	3	cream	SR	+	Nil	+	+	+	Nil	Nil	+	+	Nil	Nil	+	+	+	Nil	***Corynebacterium* sp. 1**
	GUVFPM-3	3	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	***Bacillus* sp. 1**
	GUVFPM-4	3	yellow	RS	+	+	+	+	Nil	Nil	Nil	Nil	Nil	+	Nil	Nil	+	+	+	***Bacillus* sp. 2**
	GUVFPM-5	0	white	SR	Nil	+	Nil	+	+	Nil	Nil	Nil	+	+	Nil	Nil	+	+	Nil	***Enterobacter* sp. 1**
	GUVFPM-6	0	beige	SRS	+	+	Nil	+	+	Nil	Nil	+	+	Nil	Nil	Nil	+	+	+	***Pontibacillus* sp. 1**
	GUVFPM-7	0	yellow	SR	Nil	+	Nil	+	+	Nil	Nil	+	+	Nil	Nil	+	+	+	Nil	***Enterobacter* sp. 2**
	GUVFPM-8	0	beige	RS	+	+	Nil	+	+	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	+	***Pontibacillus* sp. 2**
MAM 2	GUVFCCM-1	25	CL	SLR	Nil	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	***Pseudomonas sp.*1**
	GUVFCCM-2	25	cream	R	Nil	+	+	+	Nil	+	+	+	+	Nil	Nil	+	+	+	+	***Chromohalobacter sp.* 2**

Cinachyra cavernosa	GUVFCCM-3	0	yellow	RS	+	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	+	+	+	+	***Bacillus* sp. 3**
	GUVFCCM-4	0	cream	R	+	Nil	+	+	+	Nil	Nil	+	+	Nil	Nil	Nil	+	+	+	***Corynebacterium* sp. 2**
	GUVFCCM-5	0	orange	RS	+	+	+	+	+	Nil	Nil	+	+	+	Nil	Nil	+	+	Nil	***Bacillus* sp. 4**
	GUVFCCM-6	0	yellow	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	Nil	***Bacillus* sp. 5**
	GUVFCCM-7	0	white	RS	+	+	Nil	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	***Pontibacillus* sp. 3**
	GUVFCCM-8	0	cream	C	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	***Enterococcus* sp. 1**
	GUVFCCM-9	0	white	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	Nil	***Bacillus* sp. 6**
	GUVFCCM-10	25	cream	R	Nil	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	+	+	***Chromohalobacter* sp. 3**
MAM4 ***Haliclona sp***	GUVFHM-1	25	cream	RS	+	+	+	+	Nil	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	***Bacillus* sp. 7**
	GUVFHM-2	25	cream	R	Nil	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	+	+	***Chromohalobacter* sp. 4**
MAM5 Unidentified sponge	GUVFUM-1	3	Pale orange	R	+	Nil	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	Nil	***Corynebacterium* sp. 3**
	GUVFUM-2	0	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	***Bacillus* sp. 8**
MAM6 ***Callyspongia fibrosa***	GUVFCFM-1	0	cream	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	+	Nil	+	+	+	+	***Bacillus* sp. 9**
	GUVFCFM-2	0	cream	RS	+	+	+	Nil	Nil	Nil	Nil	+	+	Nil	Nil	Nil	+	+	+	***Bacillius* sp. 10**
	GUVFCFM-3	0	cream	SR	+	Nil	+	+	+	Nil	Nil	Nil	+	Nil	Nil	+	+	+	+	***Corynebacterium* sp. 4**
	GUVFCFM-4	0	cream	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	+	Nil	+	+	+	+	***Bacillus* sp. 11**
MAM10 ***Heteronema erecta***	GUVFHEM-1	3	cream	R	+	Nil	+	+	Nil	Nil	Nil	+	Nil	+	Nil	+	+	+	+	***Corynebacterium* sp. 5**
	GUVFHEM-2	3	cream	SLR	Nil	Nil	+	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	***Loktanella* sp. 1**
	GUVFHEM-3	0	cream	SLRS	+	+	+	+	Nil	+	+	Nil	Nil	Nil	Nil	Nil	+	+	Nil	***Bacillus* sp. 12**

	GUVFHEM-4	25	CL	SLR	Nil	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	***Pseudomonas* sp. 2**
	GUVFHEM-5	0	cream	RS	+	+	+	+	+	Nil	Nil	+	+	Nil	Nil	+	+	+	+	***Bacillus* sp. 13**
	GUVFHEM-6	0	cream	SR	+	Nil	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	+	***Corynebacterium* sp. 6**
	GUVFHEM-7	0	cream	C	+	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	***Planococcus* sp. 1**
NIO1	GUVFCM-1	25	orange	R	Nil	+	+	+	+	+	+	Nil	Nil	Nil	Nil	+	+	+	+	***Haloarchaea* sp. 1**
Callyspongia	GUVFCM-2	0	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	***Bacillus* sp. 14**
reticutis var																				
solomonensis	GUVFCM-3	0	CL	SLR	Nil	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	***Pseudomonas* sp. 3**
NIO2	GUVFFM-1	25	orange	R	Nil	+	+	+	+	+	+	Nil	Nil	Nil	Nil	+	+	+	+	***Haloarchaea* sp. 2**
Fasciospongi	GUVFFM-2	25	orange	R	Nil	+	+	+	+	+	+	Nil	Nil	Nil	Nil	Nil	+	+	+	***Haloarchaea* sp. 3**
a cavernosa	GUVFFM-3	3	cream	R	Nil	+	+	+	Nil	Nil	Nil	+	Nil	Nil	+	+	+	+	+	***Chromohalobacter* sp. 5**
NIO3	GUVFSM-1	25	orange	R	Nil	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	Nil	***Haloarchaea* sp. 4**
Unidentified	GUVFSM-2	0	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	***Bacillus* sp. 15**
sponge	GUVFSM-3	0	CL	SLR	Nil	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	***Pseudomonas* sp. 4**

CL, colourless; **R**, Rods; **RS**, Rods with spores; **SR**, Short rods; **SLR**, Slender rods; **SRS**, Short rods with spores; **SLRS**, Rods fine slender with spores; **C**, Cocci; **VP**, Voges Proskauer; **NR**, Nitrate reduction; **(+)**, present; **(Nil)**,absent

Accordingly the 4 and 1 **Phena** under Gram positive cluster corresponds to 4 Genera under Class Bacilli and 1 Genus under Class Actinobacteria belonging to Phylum Firmicutis (Figure 3a). The retrieved bionts were distributed as in:

Phenon 1 ≈ Genus *Enterococcus* - GUVFCCM-8 a motile cocci and catalase negative

Phenon 2 ≈ Genera *Pontibacillus* - GUVFPM-6, GUVFPM-8 and GUVFCCM-7 are sporulating motile rods, catalase positive and oxidase negative

Phenon 3 ≈ Genus *Planococcus* - GUVFHEM-7 a motile cocci, catalase and oxidase positive

Phenon 4 ≈ Genera *Bacillus* - GUVFPM-3, GUVFPM-4, GUVFCCM-3, GUVFCCM-5, GUVFCCM-6, GUVFCCM-9, GUVFHM-1, GUVFUM-2, GUVFCFM-1, GUVFCFM-2, GUVFCFM-4, GUVFHEM-3, GUVFHEM-5, GUVFCM-2 and GUVFSM-2 are sporing rods, oxidase and catalase positive

Phenon 5 ≈ Genera *Corynebacterium* - GUVFPM-2, GUVFCCM-4, GUVFUM-1, GUVFCFM-3, GUVFHEM-1 and GUVFHEM-6, are non motile rods, catalase and oxidase positive.

Similarly the 1 and 3 **Phena** obtained under Gram negative cluster corresponds to the 3 Genera under Class Alphaproteobacteria and 1 Genus under Class Gammaproteobacteria belonging to Phylum Proteobacteria (Figure 3b). The retrieved bionts distributed as in:

Phenon 1 ≈ Genus *Loktanella* - GUVFHEM-2 a non motile rod, oxidase and catalase positive, methyl red negative and did not reduce nitrate to nitrite

Phenon 2 ≈ Genera *Enterobacter* - GUVFPM-5 and GUVFPM-7 are motile rods, lactose non fermentors, oxidase negative, catalase positive

Phenon 3 ≈ Genera *Chromohalobacter* - GUVFPM-1, GUVFCCM-2, GUVFCCM-10, GUVFHM-2 and GUVFFM-3 are motile rods, oxidase and catalase positive, indole positive

Phenon 4 ≈ Genera *Pseudomonas* - GUVFCCM-1, GUVFHEM-4, GUVFCM-3 and GUVFSM-3 are non motile rods, oxidase and catalase positive, did not produce acid from glucose

These bionts of the nine Genera showed varied distribution in sponges. The sponge *P. testudinaria,* showed the presence of 62.5% of euryhaline and 37.5% of marine halophilic bionts. As detailed in Table 1, the euryhaline bacteria retrieved from *P.testudinaria* were identified as *Chromohalobacter* spp., *Bacillus* spp. and *Pontibacillus* spp. while the marine halophiles were identified as *Corynebacterium* spp. and *Enterobacter* spp. 70% of isolates associated with sponge *C.cavernosa* were euryhaline bacteria, identified as *Pseudomonas* spp., *Chromohalobacter* spp., *Bacillus* spp., *Corynebacterium* spp., *Pontibacillus* spp. and *Enterococcus* sp. 1, while the remaining 30% were marine halophiles belonging to the Genus *Bacillus* spp. All the isolates associated with sponge *Haliclona* sp. were euryhaline bacteria identified as *Bacillus* spp. and *Chromohalobacter* spp. One of the isolates retrieved from the unidentified sponge MAM 5 was an euryhaline bacterium identified as *Bacillus* spp. while the other, was a marine halophile identified as *Corynebacterium* spp.

All the isolates retrieved from sponge *C. fibrosa* were euryhaline bacteria belonging to the Genus *Bacillus* spp. and *Corynebacterium* spp. 85.7% of the isolates associated with sponge *H. erecta* were euryhaline bacteria identified as *Corynebacterium* spp., *Loktanella* sp. 1, *Pseudomonas* spp., *Bacillus* spp. and *Planococcus* sp. 1, while the only marine halophile belonged to the Genus *Bacillus*.

Isolates GUVFCM-2 and GUVFCM-3 from *C. reticutis var solomonensis* and isolates GUVFSM-2 and GUVFSM-3 from Unidentified sponge NIO 3 were euryhaline bacteria belonging to the Genus *Bacillus* and *Pseudomonas*. Isolate GUVFFM-3 from sponge *F.cavernosa* belonged to the Genus *Chromohalobacter* and was an euryhaline bacterium.

Isolate GUVFCM from the sponge *C. reticutis var solomonensis,* GUVFFM-1 and GUVFFM-2 from the sponge *F.cavernosa* and the single isolate GUVFSM from the unidentified sponge NIO 3, were tentatively identified as Haloarchaea as they were tolerant to salinities higher than 25%,

produced the orange pigments and showed presence of Glycerol diether moieties, which are chemotaxonomic markers for ascribing the isolates as members of the third domain.

As recorded in Figure 4, bionts of genera *Bacillus,* followed by those of genera *Corynebacterium* predominated 88.9% and 55.6% of sponge samples respectively. *F.cavernosa* is the only sponge having *Chromohalobacter* as its lone Genera.

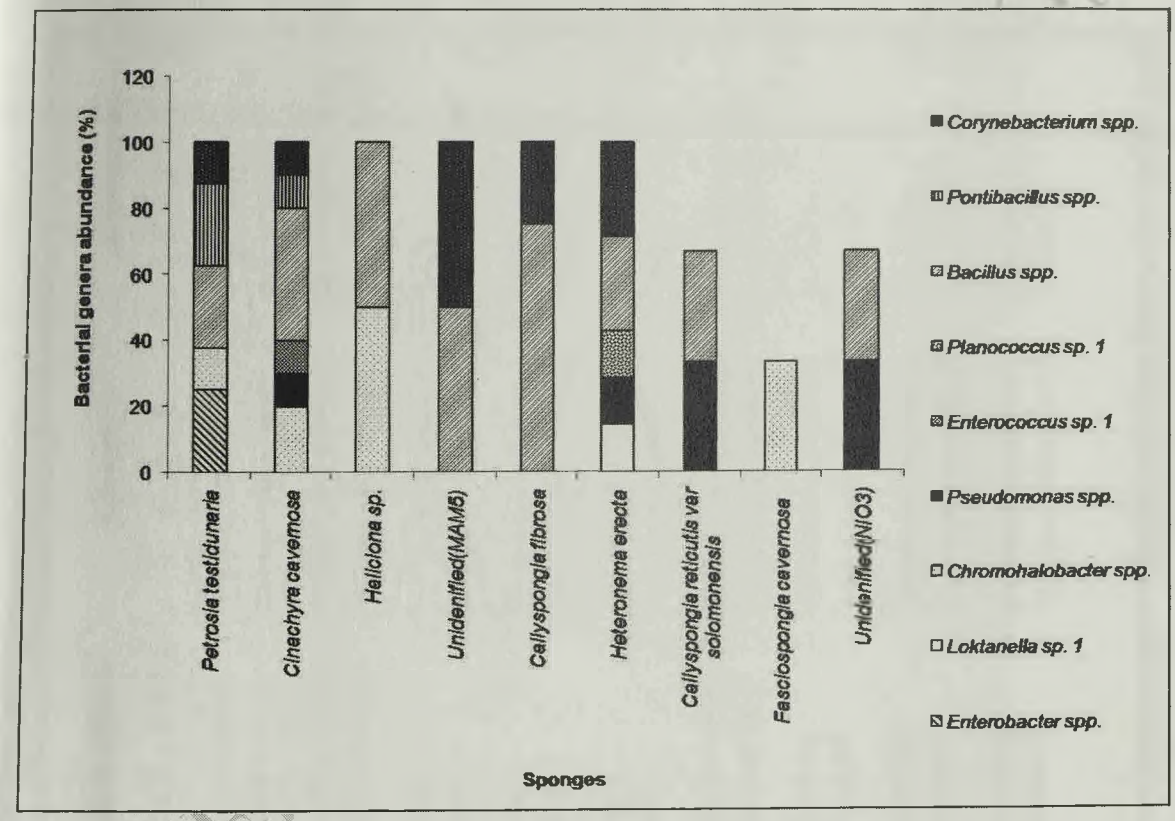

Figure 4 - Dominant Genera of culturable bacterial bionts from different sponges.

Statistical measures of diversity

PRIMER v.5 showed highest Genera richness (*d*) and diversity (*H'*) in sponge *C.cavernosa* followed by *Heteronema erecta* and *Petrosia testudinaria* (Figure 5a & c).

Interestingly the bacterial bionts were evenly distributed in sponges namely *Haliclona* sp., Unidentified sponge (MAM 5), *Callyspongia reticutis var solomonensis, Fasciospongia cavernosa* and Unidentified sponge (NIO 3) as seen in Figure 5b.

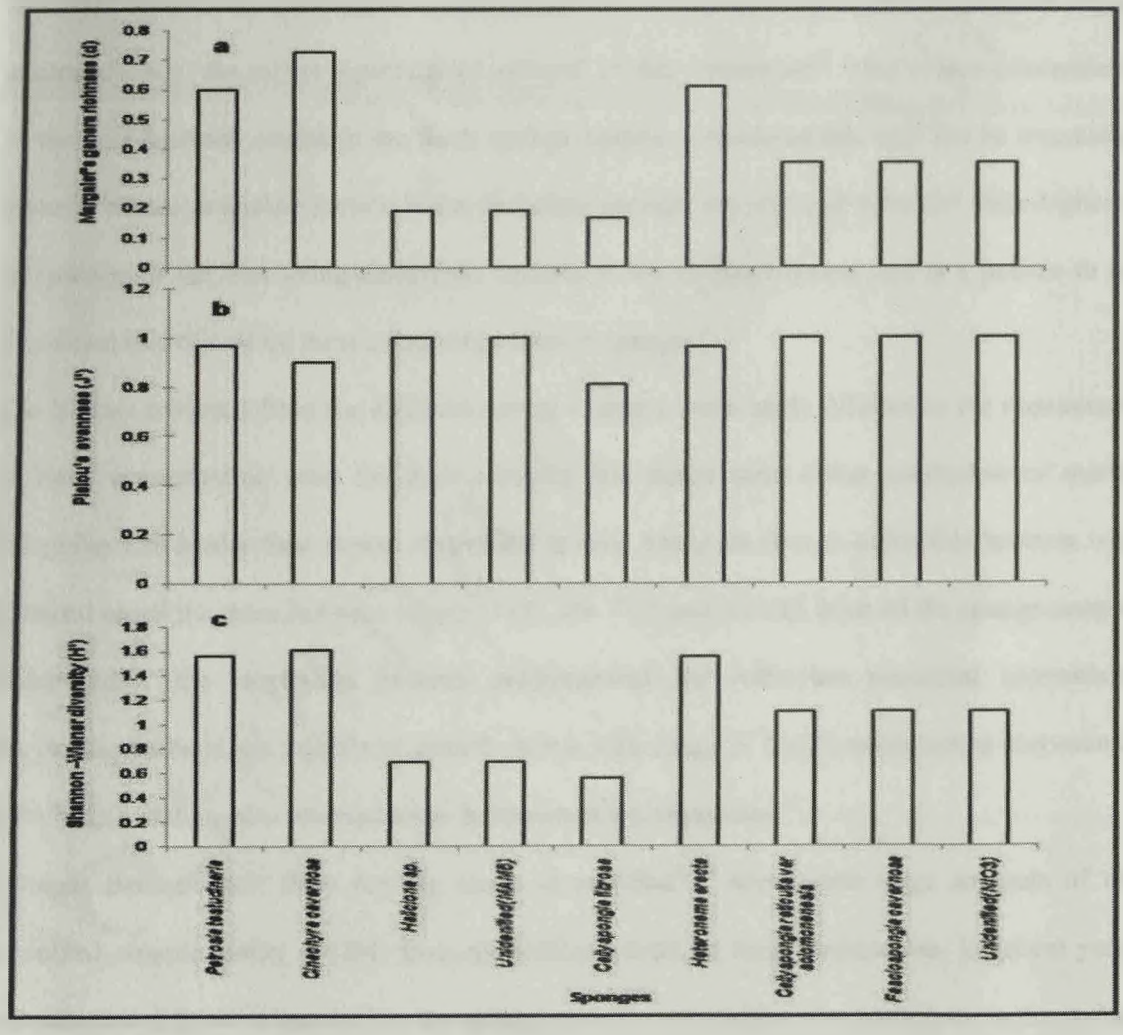

Figure 5- (a) Genera richness, (b) Pielou's evenness and (c) Shannon-Wiener diversity of the bacterial community in the different sponges.

Discussion

The total bacterial counts determined using AODC method, averaged at, $5.20 \pm 2.2 \times 10^9$ cells/g and are comparable with counts $2.8 \pm 0.4 \times 10^9$ cells/ml reported from other marine sponges[16]. This result clearly supports the finding that sponges provide an econiche wherein several bacterial types dock for safety against predators or harsh environmental conditions and for nutrition [17-20].

Even though our sponges were washed repeatedly with 3% NaCl to get rid of the surface associated bacteria the total viable counts of intimate associates of sponges are 10^3 times higher in comparison to the earlier reports from sponges, of this embayment[7]. This is also substantiated by the total bacterial counts in the same sponge samples. However this may not be unusual as bacterial counts in marine inverterbrates including sponges are reported to be 2-3 folds higher in comparison to the free living/planktonic bacteria in the ambient waters and is a pointer to the significant role played by these micro-organisms in sponges[21].

The isolates retrieved from the different sponge samples under study differed in the requirement of NaCl concentration used for their recovery and hence were either euryhaline or marine halophiles and haloarchea. It was interesting to note that even though culturable bacteria were obtained on all the three isolation media (TYE, 3% TYE and NTYE) from all the sponge samples under study, the euryhaline bacteria predominated the culturable microbial assemblage. Euryhaline bacteria are capable of growth over a wide range of NaCl concentration (between 0-25% NaCl), and are also referred to as 'halotolerant extremophiles'[22].

Sponges through their filter feeding mode of nutrition[23,24] accumulate large amounts of this dissolved organic matter (DOM) from the habitat waters, in their choanocytes. In recent years, assimilation of DOM is ascribed to the sponge–microbe association who participate in the sponge nutrition by secreting extracellular enzymes that act on the particulate organic matter accumulated within the sponge body, and thereby provide assimilable carbon to the sponge host[25-27]. The relenting osmolal stress owing to the increased concentration of organic compounds in the sponge interiors due to the sequestering of DOM creates a selection pressure on euryhaline bacteria.

Given to understand, the tolerance of euryhaline halophiles to high or fluctuating salinities[28], it is reasonable to consider that we found euryhaline bacteria to predominate the cultural assemblage in intertidal sponge species which face abrupt and short term salinity fluctuations.

The physiological flexibility of these isolates[22] and their ability to resist the changing osmolar conditions also adds to the selection pressure. The retrieval of euryhaline archaea from these sponges in our study corroborates with the findings of Martins *et al.*[29] who demonstrated the production of compatible solutes to cope osmotic stress which is possibly responsible for establishment of bionts of genera not known to produce osmolytes.

In the present study, we demonstrated *Bacillus* spp. and *Corynebacterium* spp., as the major culturable bacterial community in all the sponges under study. The greater predominance of Gram-positive bacteria is probably attributable to their ability to produce spores under adverse conditions; a physiological adaptation enabling eubacterial bionts to survive within the sponge interiors.

Studies by Devi *et al.*[30] ; Feby and Nair[27]; Vasanthbharathi and Jayalakshmi[6] and other workers have demonstrated the presence of bacteria in Indian sponges. The single report on bacterial isolation from sponges of Mandapam is by Saravanakumar *et al.*[7]. This is the first record of retrieval of euryhaline, halotolerant extremophilic eubacteria (0-20%) and haloarchaeal bionts from nine different benthic sponges of Mandapam in India. Earlier studies on Indian sponges have reported bacteria growing in 0.5-3% NaCl [5-7] whereas in the present study bacterial bionts grew at 0-25% NaCl.

To our knowledge eubacteria from Genera *Loktanella* sp. 1, *Pontibacillus* sp., *Planococcus* sp. 1, *Enterococcus* sp.1 retrieved by us have not been reported from Mandapam waters possibly, as they could not withstand tidal fluctuations. Retrieval of sponge bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* known for their pathogenicity to humans and others, reflects their occurrence in the habitat waters, possibly accounting to reports describing Mandapam in the GoM, as a sink, receiving high load of organic matter through the

indiscriminate discharge of industrial effluents, household wastes, leacheate of solid waste dumps and garbage, animal and human excreta etc. [3,4,31]

The retrieval of bacteria from nine Genera indicates that the sponge tissue provides a suitable substratum for settlement of bacteria reflecting their occurrence in habitat waters.

Conclusion

The study clearly reveals:

(1) The presence of cohabitating marine halophiles (3% NaCl), halotolerant or euryhaline halophiles (0-25% NaCl) and haloarchaea.

(2) The predominance of euryhaline bacteria, capable of coping with osmolal stress in the sponge interiors, possibly due to the sequestration of DOM from the ambient waters of the sponge habitat, which inturn supports co-existence of bionts of Genera unknown to produce osmolytes.

(3) That retrieval of sponge bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* reflects the entry of bacteria, known for pathogenicity to humans, into waters of Mandapam, through sewage and other pollutants.

(4) Microbial pollution of Mandapam waters and *Heralds Caution* for taking measures for safeguarding the waters and ensuring sustainability of biota.

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Figures

Figure 1 - Percent Morphotypes of bacterial bionts revealed by Acridine Orange Direct Count in sponge tissue of Mandapam-India.

Figure 2 - Total bacterial counts in cfu/g on TYE, 3%TYE and NTYE.

Figure 3 - Phenogram depicting sorting of morphological and biochemical characteristics of eubacterial bionts retrieved from sponges of Mandapam-India using keys of Bergey's Systematic Bacteriology and **SYSTAT v.12.01.**

(a) Gram positive bacteria ≈ **Cluster A** (Phylum: Firmicutes) with **Subcluster IA** (Class: Bacilli) with 4A **Phena** (Genera: *Enterococcus; Pontibacillus; Planococcus* and *Bacillus*) and **Subcluster IIA** (Class: Actinobacteria) with 1A **Phenon** (Genera: *Corynebacterium*). (b) Gram negative bacteria ≈ **Cluster B** (Phylum: Proteobacteria) with **Subcluster IB** (Class: Alphaproteobacteria) with 1B **Phenon** (Genera: *Loktanella*) and **Subcluster IIB**: (Class: Gammaproteobacteria) with 3B **Phena** (Genera: *Enterobacter; Chromohalobacter* and *Pseudomonas*).

Figure 4 - Dominant Genera of culturable bacterial bionts from different sponges.

Figure 5- (a) Genera richness, (b) Pielou's evenness and (c) Shannon-Wiener diversity of the bacterial community in the different sponges.