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Study of the Halophilic and Halotolerant Microbial Diversity in Marine Sponge *Callyspongia fallax* collected from Ratnagiri Coast, Maharashtra, India

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Abstract: This research investigates the culturable halotolerant bacterial and haloarchaeal communities associated with the marine sponge *Callyspongia fallax*, collected from the coastal regions of Ratnagiri district, India. Sponge samples (SP2 and SP4) were identified via COI-based DNA barcoding, and microbial isolates were cultured on SG medium with 20% NaCl at 37°C, yielding 63 colony morphotypes: 50 bacterial (white, mucoid) and 13 haloarchaeal (pink-pigmented). Identification employed MALDI-TOF MS and 16S rRNA gene sequencing; database limitations left many unidentified, but confirmed bacteria included *Halomonas elongata* (most abundant at 47.7%) and *Chromohalobacter salexigens*, alongside seven genera total, with haloarchaea like *Haloferax alexandrinus*, *H. lucentensis*, and *H. volcanii*. Results reveal sponge-selective enrichment of seawater microbes, highlighting *H. elongata*'s potential for high exopolysaccharide (EPS) production and novel sponge-archaea interactions, warranting further industrial and ecological studies

Index Terms: Sponge microbial diversity, Symbiont, Halotolerant

INTRODUCTION

Sponges, recognized as the oldest metazoans, form a crucial part of benthic communities across the globe and significantly influence both benthic and pelagic ecological processes [1]**.** Despite their simple body structure, lacking complex tissues and coordination systems, sponges are highly efficient filter feeders with a sessile lifestyle. Tropical sponges are especially notable for their vibrant colors, morphological diversity, and varying shapes and sizes, ranging from a few millimeters to about one meter in length [2]**.** Their adaptability to different ecosystems is largely due to their ability to utilize a wide range of food sources, including dissolved organic matter [3]**,** small crustaceans [4], and plankton sized between 2 and 10 micrometers. [3, 5] These remarkable filter feeders are found throughout tropical, temperate, and polar marine regions, with over 15,000 species reported in both marine and freshwater environments. Sponges are ecologically important not only for their own biological functions but also because of the diverse and complex microbial communities they harbor. Of the 15,000 known species, only about 1% inhabits freshwater environments, highlighting their predominance in marine ecosystems. [6] Marine ecosystems are highly dynamic, and marine sponges exhibit a wide range and diversity compared to their freshwater counterparts. Sponges thrive in nearly every aquatic habitat imaginable, ranging from freshwater lakes and rivers to hypersaline environments like the Dead Sea and saltern ponds, where salt concentrations may reach saturation. The microbial communities associated with sponges in these varying habitats have adapted to local environmental conditions, allowing sponges to inhabit environments such as mangrove ecosystems, tropical seas, polar waters, and deep-sea habitats. Ecologically, sponges fulfill vital functional roles, including bio-erosion—breaking down substrates—participating in nutrient cycles, and interacting with other organisms, all of which contribute to ecosystem health and stability.

The relationship between sponges and microorganisms is an ancient one, dating back approximately 500 million years to Precambrian times. Marine microorganisms exist both freely floating in the plankton and in symbiotic relationships with other microbes, animals, plants, algae, and sponges. [7, 8] This widespread symbiosis suggests that multicellular organisms should not be viewed as independent entities but rather as halobionts—complex meta-organisms composed of the host and its associated microbial communities. These microbes provide essential functions for the host, including nutrition, defense, immunity, and developmental support [9,10]. Within sponges, microorganisms live symbiotically in a gelatinous matrix called the mesohyl, located between the inner choanoderm layer and outer pinacoderm layer of the sponge body. This internal body cavity contains a rich and diverse microflora that can reach densities of 10^8 to 10^9 bacteria per gram of sponge tissue [11]. This unique association is largely due to the structural design of the sponge's body and its aquiferous system, which facilitates constant water flow and nutrient exchange [12]. Among the microorganisms found in and around sponges are halophiles, microbes that require high salt concentrations for growth. Typically, halophiles need at least 1.5 M sodium chloride (about 9% weight/volume) to survive and may grow optimally at concentrations as high as 3 M sodium chloride (18% weight/volume) or more. Microbes capable of surviving and growing across a wide range of salt concentrations, including zero salt conditions, are called halotolerant microbes [13]**.** Moderate halophiles grow best at salt concentrations between 0.5 and 2.5 M (3-15%)**.** Gilmour classified bacteria growing in a range from 0.1 to 4.5 M salt as extremely halotolerant. Furthermore, many bacterial strains that can flourish in salt concentrations from 0% to 17% are termed haloversatile, while microorganisms able to grow in salt ranges from 12% to 32% are mostly archaea. Halophiles exhibit a variety of metabolic adaptations that enable their survival in extreme environments characterized by nutrient scarcity, intense solar radiation, low water availability, and high ionic strength. These adaptations include the accumulation of osmolytes like potassium chloride (KCl) or sulfotrehalose intracellularly to balance osmotic pressure. Haloarchaea, a group of salt-loving archaea, often display these mechanisms, adapting their cellular enzymes to function efficiently in saline conditions**.**

Additionally, halophilic organisms exhibit unique cellular membrane compositions and possess genomes enriched with genes conferring salt resistance. The metabolic activities of halophiles are of significant economic interest due to their production of valuable biomolecules such as carotenoids, enzymes with industrial applications, biopolymers like polyhydroxyalkanoates (PHAs) and polyhydroxybutyrates (PHBs), and halocins—antibacterial proteins produced by haloarchaea. These biomolecules have potential applications in biotechnology, pharmaceuticals, and environmental management. In conclusion, sponges are essential components of aquatic ecosystems due to their ancient lineage, efficient filter-feeding capabilities, and their role as hosts to diverse and functionally important microbial communities. These sponge-microbe associations contribute not only to ecosystem nutrient cycling and bio-erosion but also to broader ecological and biogeochemical processes. The halophilic and halotolerant microbes associated with sponges exemplify the incredible adaptability of life forms in extreme environments and offer promising avenues for biotechnological innovation. This understanding highlights the integral role of sponge holobionts in maintaining marine ecosystem health and their potential utility for human industry.

I. RESEARCH METHODOLOGY

1. Sampling:

The sampling for marine sponge *Callyspongia fallax* was carried out in May 2022 from two different coastal regions of Ratnagiri district, Maharashtra (Fig.1 Anjarle and Harne). In details, fresh sponge samples were collected in 50 ml falcon tubes containing sterile Sehgal and Gibbons medium (SG) having 20% NaCl concentration. All the samples were stored at 4°C until further processing.

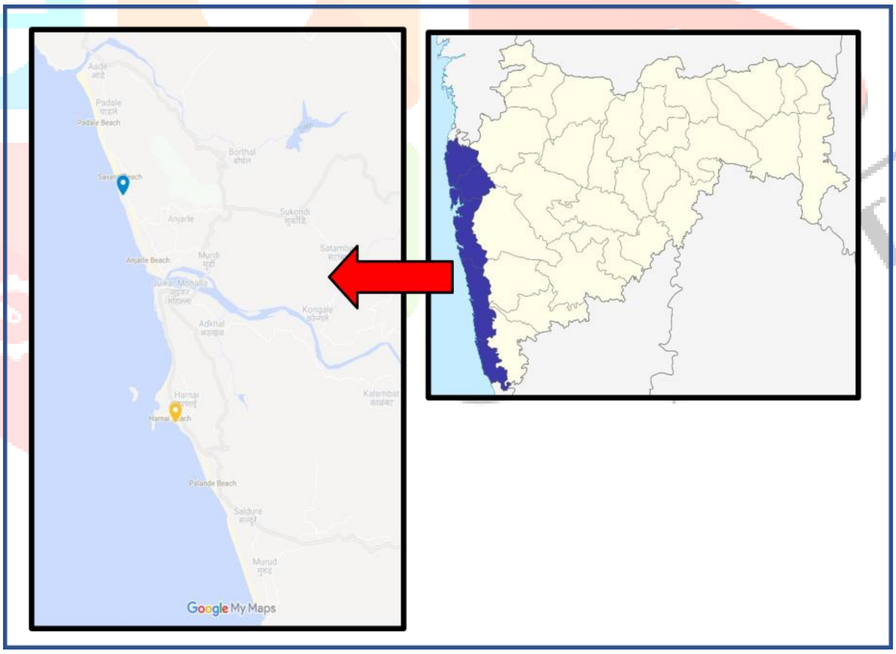

Fig.1: Map showing sampling sites (source: google map)

2. Isolation and purification of halotolerant cultures:

For the isolation of halophilic/halotolerant bacterial and archaeal community associated with the sponges, a direct inoculation method was used. In brief, the individual sponge samples were washed three times with double distilled water to removes un-associated microbial flora from the sponge body. Further, these samples were directly inoculated on SG medium plates containing 20% of NaCl concentration. The plates were incubated for one week 32 at 37˚C, and the microbial growth surrounding the sponge was noted. The representative of each colony morphotype was streaked by sterile toothpick on SG medium with 20% NaCl agar plates until pure cultures were obtained.

3. Identification of isolated cultures by MALDI-TOF MS:

The sample for analysis by MALDI MS is prepared by mixing or coating with a solution of an energy-absorbent, organic compound called the matrix. When the matrix crystallizes on drying, the sample entrapped within the matrix also co-crystallizes. The sample within the matrix is ionized in an automated mode with a laser beam. Desorption and ionization with the laser beam generate singly protonated ions from analytes in the sample. The protonated ions are then accelerated at a fixed potential, where these separate from each other based on their mass-tocharge ratio (m/z). The charged analytes are then detected and measured using different types of mass analyzers like quadrupole mass analyzers, ion trap analyzers, time of flight (TOF) analyzers, etc. For microbiological applications mainly TOF mass analyzers are used. During MALDI-TOF analysis, the m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube. Based on the TOF information, a characteristic spectrum called peptide mass fingerprint (PMF) is generated for analytes in the sample. Identification of microbes by MALDI-TOF MS is done by either comparing the PMF of an unknown organism with the PMFs contained in the database or by matching the masses of biomarkers of an unknown organism with the proteome database. In PMF matching, the MS spectrum of unknown microbial isolates is compared with the MS spectra of known microbial isolates contained in the database.

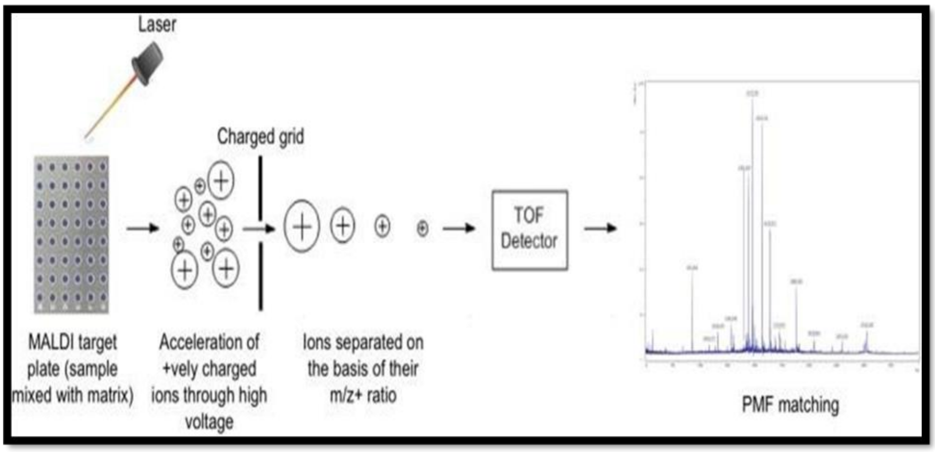

Fig. 2: Schematic representation of MALDI-TOF MS

II. RESULTS AND DISCUSSION

2.1 Identity of collected sponge samples:

In the present study two sponge samples were collected from coastal regions of Ratnagiri district. The identification of these sponge samples was already done by COI-based DNA barcoding in the previous research work of the laboratory. This identification found that all collected sponge samples were identified as marine sponge *Callyspongia fallax*. (Fig.3)

Fig. 3 sponge species *Callyspongia fallax* from sampling site

Table 1: Sponge samples identification

Sample ID	Sponge species identified by DNA barcoding
SP2	*Callyspongia fallax*
SP4	*Callyspongia fallax*

2.2 Isolation and purification of halotolerant bacteria and archaea

After the incubation of one week at 37˚C, the microbial growth was started to appear on SG medium (20% NaCl). Total of 63 colony morphotypes were recovered and further picked for obtaining pure cultures. Out of 63 colonies, 13 were appeared to be forming pink pigmentation, indicating their haloarchaeal identity. While other colonies (50) are white, glistening and mucoid in nature and appeared to be bacterial isolates. (Fig.4 and 5)

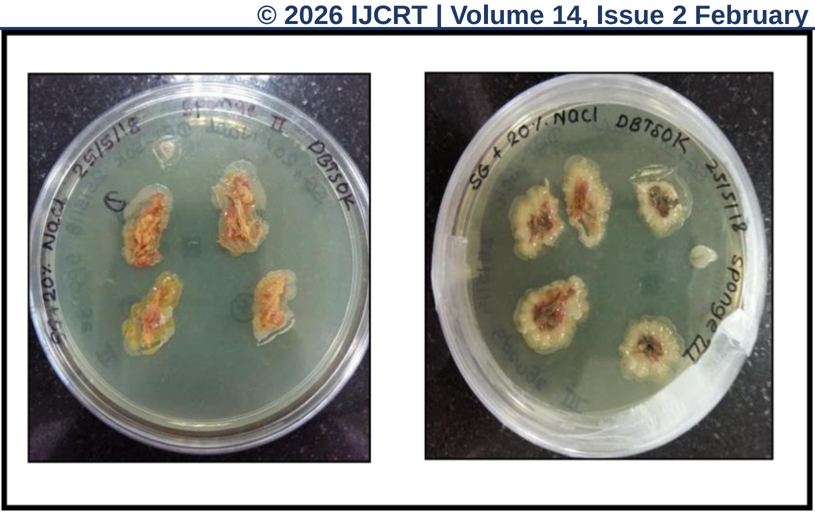

Fig.4: direct inoculation of sponge’s tissue (callyspongia fallax) on sg + 20% nacl medium

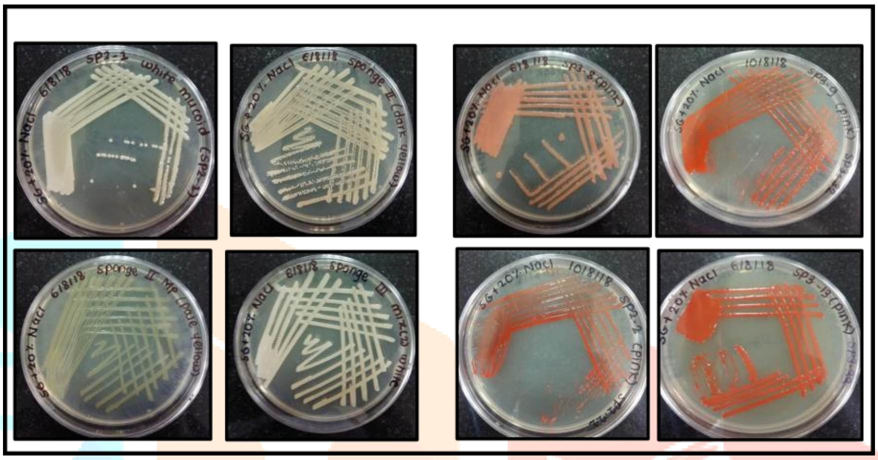

Fig.5: pure cultures of bacterial isolates and pure cultures of archaeal isolates
(On SG+20% NaCl medium)

2.3 Results for MALDI-TOF MS-based identification of isolates

The total protein was extracted from all 63 bacterial isolates for MALDI based identification. However, due to the limitation of unavailability of halophilic/halotolerant bacterial protein mass spectra in Bruker database; large numbers of our isolates remained unidentified in the results. Only five bacterial isolates were identified as tabled follow. In this, *Halomonas elongata* and *Chromohalobacter salexigens* are the most frequent bacterial isolates identified in MALDI-TOF MS. (Table 2) None of the haloarchaeal isolates were identified by MALDI-TOF MS due to the unexpanded archaeal protein mass spectra in Bruker database.

Table 2: MALDI-TOF MS based identification of isolates

Sr.No.	**CultureID**	**MALDI-Score**	**Identified microorganism**
1	SP2-2	1.733	*Chromohalobacter salexigens*
2	SP2-8	2.113	*Chromohalobacter salexigens*
3	SP2-9	2.029	*Chromohalobacte rsalexigens*
4	SP3-15	1.892	*Halomonas elongata*
5	SP3-16	1.725	*Halomonas elongata*

In the present study, the culturable halotolerant bacterial and haloarchaea community associated with the sponge C. fallax was explored by using molecular techniques like 16S rRNA gene sequencing and MALDI-TOF MS. These results show that the sponge *C. fallax* associated with seven different bacterial species; belonged from 7 genera. *Halomonas elongata* (47.7%) is the most abundant bacterial species associated with the sponge *C. fallax*, followed by *Chromohalobacter israelensis* (11.4%) and *Salibacterium halochares*. (11.4%). Although all the species mentioned above were reported to be present in seawater at low abundance; their enrichments in sponge *C. fallax* indicates that sponge is selecting bacterial population from its surroundings in a selective way. The abundance of *H. elongata* species in sponge has special industrial significance as it is reported to be one of the highest exopolysaccharides (EPS) producers. Here we would like to hypothesize that the strain of *H. elongata* originated from our sponge *C. fallax* may have significantly higher EPS-producing ability compared with other stains of *H. elongata*. In the present study, one of the most important findings was the isolation of haloarchaea form sponge *C. fallax*. In this culture dependant study three species of haloarchaea (*Haloferax alexandrines, Haloferax lucetence, and Haloferax volcani*) from genus *Haloferax* were reported from sponge species. This kind of finding suggests the further need for studies on haloarchaea for better understanding of sponge-archaea interactions

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