

Physiological and Molecular Studies of Deep-Sea Fungi

Thesis submitted for the degree of

Doctor of Philosophy

in

Marine Sciences

to the

Goa University

by

Purnima Singh

National Institute of Oceanography,

Dona Paula, Goa-403004, India

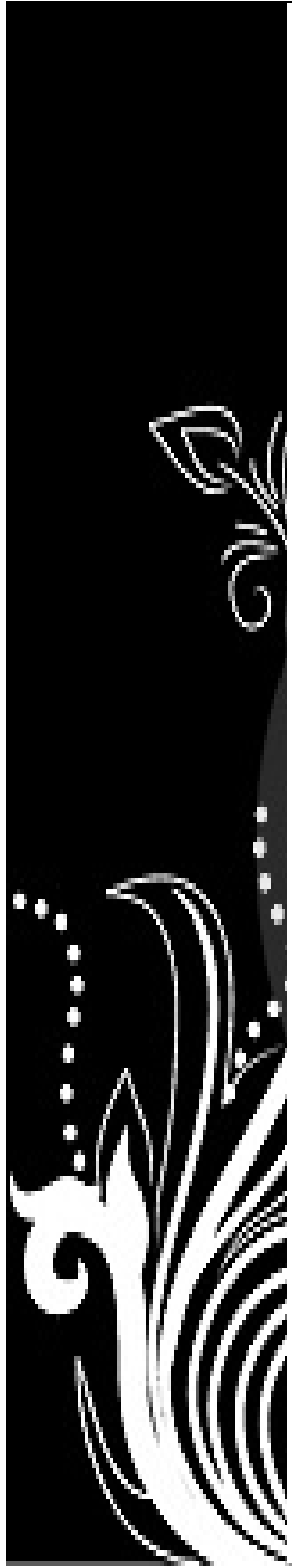
February 2011

STATEMENT

As per requirement, under the University Ordinance 0.19.8 (vi), I state that the present thesis titled “*Physiological and Molecular Studies of Deep-Sea Fungi*” is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities or suggestions have been availed of.

Purnima Singh



Dedicated
To
Lord Shiva

CERTIFICATE

This is to certify that the thesis titled “**Physiological and Molecular Studies of Deep-Sea Fungi**” submitted for the award of the degree of Doctor of Philosophy in the Department of Marine Sciences, Goa University, is the bona fide work of **Ms Purnima Singh**. The work has been carried out under my supervision and the thesis or any part thereof has not been previously submitted for any degree or diploma in any university or institution.

Place:

Date:

Dr. Chandralata Raghukumar

Ph.D. Supervisor,

Marine Biotechnology Laboratory,

National Institute of Oceanography,

Dona Paula, Goa, India.

Acknowledgments

I express my sincere thanks to Dr. Chandralata Raghukumar, my research supervisor. She encouraged me to choose this topic for my thesis and I am very grateful to her for valuable guidance and constant encouragement throughout this study. She always supported patiently and taught me experimental and writing skills. She as a person is always ready to hear and solve the problems of her students in whatever way possible. Working with her was the most memorable achievement for me. I also thank Dr. S. Raghukumar, for his valuable and thoughtful suggestions regarding my work. Both of them have influenced me a lot with their enthusiastic and positive approach.

I wish to thank Dr. S. R. Shetye, Director, NIO for giving me opportunity to work and providing the infrastructure facilities in the institute.

I am thankful to my Co-guide Dr. S. Ghadi, Department of Biotechnology, Goa University for all his support throughout my study.

I would also like to extend my appreciation to all my FRC members, Dr. G. N. Nayak, Dean, Department of Marine Science, Goa University, Dr. H. Menon, Head, Department of Marine Sciences, Goa University and VC's nominee Dr. N. Ramaiah, Scientist, NIO for all their critical comments and for their periodic assessment of my work. I would also thank Dr. D. J. Bhat, Head, Department of Botany, Goa University for helping me in the identification of fungi.

I am thankful to the University Grants Commission, India for the award of Research Fellowship.

I am thankful to Dr. M.P. Tapaswi, Head Librarian, NIO, for providing some important references whenever required. I am also grateful to Mr. Arun Mahale, Mr. Uchil and staff of DTP section to help me in modification of some of the figures in this thesis.

I specially thank Department of Oceanography (DOD) funded PMN-EIA project for providing me opportunity to go for sampling cruises to the Central Indian Basin without which this work would not have been possible. I thank Dr. Rahul Sharma, Project Leader, PMN-EIA, for allowing me to participate in these cruises and all the support for my work. I am also thankful to all the crew members of the Russian ship Akademik Boris Petrov for their co-operation and all the sampling and lab facilities extended on board.

I am thankful to Dr. Yogesh Shouche, Scientist, NCCS, Pune for extending the sequencing facility in his lab. I am also very much grateful to Pankaj Verma, NCCS, Pune for his great help and useful suggestions throughout my thesis.

I am thankful to all my friends in the Marine Biotechnology and Microbial Ecology Lab, NIO for all their timely help. I specially thank Dasu, who was always there to help me in all the possible ways. I thank all my friends from BOD, COD, GOD and POD for the help provided during the study.

I am deeply indebted to all my family members for their support and encouragement.

I am thankful to my loving husband Gaurav for his help, thoughtful suggestions and encouragement throughout my study.

And last but not the least; I am thankful to God for giving me all the strength to achieve this goal.

Purnima Singh

Table of Contents

	Page no.
Chapter 1	Fungi in deep-sea ecosystem: an introduction... .. 1-22
Chapter 2	Isolation of fungi from deep-sea sediments of the Central Indian Basin (CIB) and their identification using molecular approaches..... 23-59
Chapter 3	Fungal Community analysis from deep-sea sediments of CIB using culture-independent approach..... 60-101
Chapter 4	Effect of simulated deep-sea conditions on growth and protein patterns of a few select cultures..... 102-132
Chapter 5	Summary..... 133-142
Appendix 143-147
Bibliography 148-189
Publications 190

Chapter 1

Introduction

1.1 Fungi in deep-sea ecosystem: an introduction

1.1.1 Oceanic environments

The ocean is the entire body of salt water, comprising ~ 70% of the total earth surface to an average depth of 3800 m. The water in the oceans is estimated to have a volume of $1.368 \times 10^9 \text{ km}^3$, and to be equivalent to about 0.24% of the Earth's total mass. The shelf seas 0-200 m deep, that fringe the continental landmasses, extend over about 5% of the Earth's area. At the outer edge of most shelf seas is the shelf-break where the seabed falls quite steeply to depths of around 3000 m, forming the continental slope and rise (Fig 1.1); these continental margins account for a further 13% of the Earth's area. Major oceans are Atlantic, Pacific, Indian, Arctic and Antarctic oceans. There are various types of habitats existing in the oceanic environment differing in their characteristics. Major environments include pelagic that is entire water column and other is benthic, the near bottom water and bottom sediment surface area of the ocean. The open ocean can be divided vertically on the basis of penetration of sunlight into the following zones (Fig. 1.1):

- **Epipelagic zone (ocean surface up to 200 meters deep)**

This is the euphotic zone of the ocean, where sunlight is easily available for photosynthetic processes. This zone is characterized by maximum primary production by the existing organisms which are the main fuel source for all other pelagic and benthic life forms. This zone is inhabited by most of the phytoplankton communities which produce considerable oxygen through photosynthesis. Other organisms which feed on these phytoplanktons include various kinds of zooplanktons (pteropods, salps, foraminifers, copepods, protozoa, larval stages of many crustaceans, worms and fishes), fishes (Sardines, Tuna, sea turtles, dolphins and whales) and swimming mammals. These organisms function as consumers at higher trophic levels.

- **Mesopelagic zone (from 200-1,000 m depth)**

This is the zone where faint sun light reaches and is known as twilight zone. This light is not sufficient for photosynthesis. Therefore the main option for feeding in this zone becomes restricted to detritory or carnivory. Some of the animals inhabiting this zone include octopus, squid and other fishes. Among mesozooplankton, copepods are numerically dominant forms present, but in the upper part of this zone the larger-sized organisms are predominantly gelatinous, particularly siphonophores. These animals have to withstand elevated hydrostatic pressure and low temperature conditions. Some animals develop the property of bioluminescence i.e. production of their own light to sustain in the dark ambient environment. The characteristic morphology of fishes living at mesopelagic depths is exemplified by the myctophids (Lantern fishes) and argyrolepids (hatchetfishes). They have black backs, flanks that are highly reflective and mirror-like and their undersides are lined with photophores (light organs). The silverying of the flanks is produced by regularly spaced platelets of a white pigment, guanine (Denton and Nicol, 1966).

- **Bathypelagic zone (from 1,000-4,000 m depth)**

This zone is characterized by complete darkness and extreme conditions of temperature and pressure. This zone is also known as midnight zone. In the temperate Atlantic, it also approximates to the deep oxygen minimum and the base of the permanent thermocline (Angel, 1989). This zone is inhabited by greatest variety of pelagic species. Among animals, mollusks, some squids, amphipods, octopods, jellies, crustaceans and angler fish are known to be present. Occasionally sperm whales dive in search of food to this zone.

- **Abyssopelagic zone (from 4,000-6,000 m depth)**

This zone is also known as abyss i.e. without bottom. It is characterized by complete darkness, very high pressure and freezing temperature of the surrounding water. The organisms surviving there mostly include some of the tiny squids or basket stars without back bone.

- **Hadalpelagic zone (greater than 6,000 m deep)**

This zone is the deepest part of ocean and is also known as trench. The temperature and darkness are same as abyssopelagic zone but hydrostatic pressure is comparatively high. The deepest trench so far known is the mariana trench of coast Japan which is ~ 11,000 m deep. Some of the organisms surviving in this zone include invertebrates such as star fishes and tubeworms.

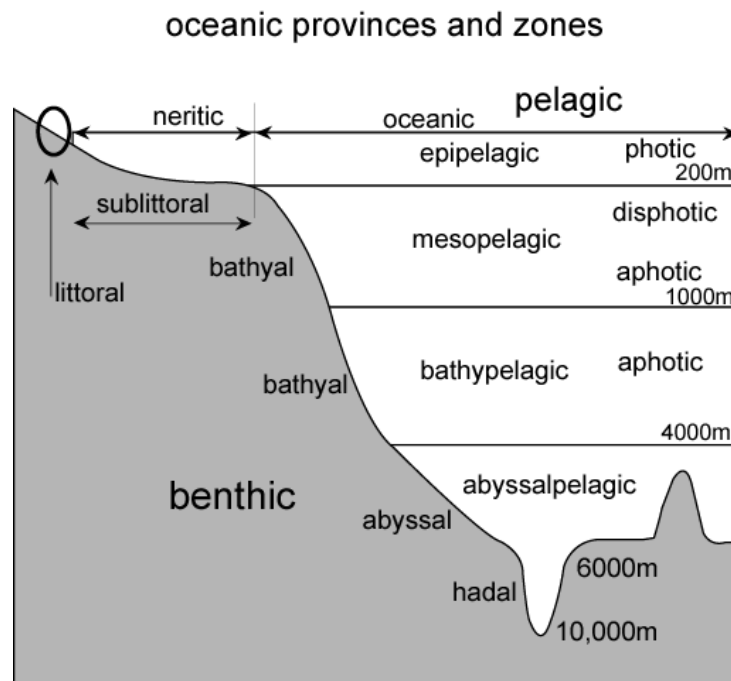


Fig. 1.1 Different zones of the open ocean, Modified from:

(http://www.google.co.in/imgres?imgurl=http://www.coastalwiki.org/w/images/thumb/9/91/Ocean_zones.jpg/300px-Ocean_zones.jpg&imgrefurl=http://www.coastalwiki.org/coastalwiki/Deep_Sea&h=213&w=300&sz=25&tbnid=FAZOW-CfTtv1GM:&tbnh=82&tbnw=116&prev=/images%3Fq%3Docean%2Bzones&zoom=1&q=ocean+zones&hl=en&usg=__ZSxP2OsM9LSPxWiX_8wRwhnBnxM=&sa=X&ei=ZGf3TLuZAsTflgeelLiVAg&ved=0CCgQ9QEwAw)

The open oceanic habitats can be divided horizontally into following two zones:

- **Neritic zone:**

This is the part of the ocean from low tide mark to the edge of continental shelf, extending to a depth of ~ 200 m. The area gets sufficient sunlight and therefore is inhabited by majority of photosynthetic life forms such as phytoplanktons and floating sargassum. Among zooplanktons, the free floating forms e.g., foraminiferans, diatoms, small fishes and shrimps are abundant.

- **Oceanic zone:**

This is the zone beyond the edge of continental shelf and includes ~65% of ocean's completely open water. This zone includes all the oceanic basins and sea mountain areas and varieties of phytoplankton and zooplanktons. Some of the special habitats found in this zone are described as following:

Cold seeps: These are the areas rich in hydrogen sulfide, methane as well as fluid containing other hydrocarbons. Seepage of this fluid in different areas of deep-sea often results in the formation of brine pool. These are depressions formed in the sea floor where salinity is very high in comparison with surrounding water. These habitats are found in the Atlantic, Eastern and Western Pacific Ocean and Mediterranean Sea.

The cold seep habitats are characterized by the occurrence of rocks and reefs composed of carbonates. These rocks are created due to the reactions between methane rich thermogenic or biogenic fluid and surrounding sea water. Apart from this, activities of some bacteria are also responsible for generation of these rocks.

Hydrothermal vents: This environment has similar properties as cold seep areas except for the high temperature of surrounding sea water. In some areas of the sea floor, spreading of tectonic plates occurs due to which cracks and fissures are generated. The surrounding water then seeps down into these cracks, occasionally to an extent of higher depth also. As

the water goes down, it comes into contact with the superheated molten magma present below the earth crust and gets superheated. This hot sea water rises to the surface back through the fissures, carrying with it minerals leached from the crustal rock below.

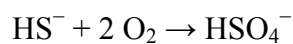
The superheated seawater then ejects out of the holes in the crust, rising quickly above the colder, denser waters of the deep ocean. As the hot seawater and the cold seawater meet, the minerals suspended in the hot water precipitate out right at the vent opening. The temperature of this hot water coming out has been found in the range of 60 to 600°C. In spite of such a high temperature the surrounding water is prevented from boiling due to extreme hydrostatic pressure of the sea water present above. The minerals carried with seawater get deposited and form geologically unique structures called chimneys. The atmosphere and hydrosphere are relatively oxidizing with an abundance of potential electron acceptors (O_2 , SO_4^{2-} , and NO_3^-). In contrast, the basaltic rocks that form the oceanic crust are relatively reduced because of the abundance of ferrous iron. High-temperature fluid/rock interaction form reduced gases (H_2S , H_2 , and CH_4) that dissolve in hydrothermal fluid (Zierenberg et al, 2000).

Anoxic sites: These are the areas of sea or fresh water that is depleted of dissolved oxygen. The lack of dissolved oxygen generally results due to restriction of water exchange in these areas. The other causes for prevention of oxygen from reaching to deeper layers may be some physical barriers also e.g., silt and sometimes the presence of heavier hypersaline waters at lower depths resulting in stratification of densities. Also, the anoxic conditions may arise if the organic matter oxidized by the bacterial communities is at a higher rate than the supply of dissolved oxygen. Some examples of anoxic zones are eastern Pacific Ocean and Arabian Sea. In these areas, being rich in upwelling, surface productivity is exceptionally high, so that an unusually high supply of organic carbon sediments down to thermocline depths.

The oxygen minimum zones (OMZ) present in these areas are resulted due to combination of high productivity described above and

limited mixing or circulation with poorly oxygenated waters (Naqvi et al, 1998). The oxygen minimum zone of the Arabian Sea is largely influenced by the South-west and north-east monsoons resulting in bottom water upwelling and therefore high productivity (Warren, 1994; Naqvi, 1994). This has resulted in the formation of one of the world's largest perennially oxygen-depleted environment in the open ocean and a seasonal hypoxic zone along the western coast of India. The coastal hypoxic condition develops along the continental shelf down to 200 m depth, leading to intense sedimentary denitrification activity (Naqvi et al, 2000). Dissolved oxygen (DO) levels during anoxic conditions drop to a concentration of -20 mM which is around 180 mM during normal conditions (Naqvi et al, 2006).

The bacterial communities present in these areas find suitable substrates and start producing sulfide in the sediments followed by expansion into the water column. When oxygen is depleted in a basin, bacteria first turn to the second-best electron acceptor, which in sea water is nitrate. Denitrification occurs, and the nitrate gets consumed rather rapidly. After reducing some other minor elements, the bacteria turn to reducing sulfate. If anoxic sea water becomes oxygenated, sulfides get oxidized to sulfate according to the chemical equation as following:



1.1.2 Organisms present in oceanic habitats

The organisms present in oceanic habitats can be divided into following groups depending on their site of distribution:

a) **Plankton:** These are drifting forms of organisms such as bacteria, archaea, plants or animals. They inhabit mostly the pelagic zone of Open Ocean and play important role in the food chain as well as in

biogeochemical cycles. Further, these planktonic forms can be divided in following subgroups depending on their trophic levels:

Phytoplankton: These are autotrophic planktons which are found near the water surface. Therefore they get sufficient light for the photosynthetic processes. They function as primary producers in the food chain. Example: diatoms, cyanobacteria, dinoflagellates and coccolithophores.

Zooplankton: Small protozoans or metazoans (e.g. crustaceans and other animals) that feed on other plankton. Some of the eggs and larvae of larger animals, such as fish, crustaceans, and annelids, are included in this subgroup.

Bacterioplankton: Bacteria and Archaea, living in the open ocean, play an important role in remineralising organic material and obtain energy through this process. The diverse forms of bacterioplankton have been reported from marine environments (Pinhassi et al, 1997). They produce organic carbon through oxidation of inorganic compounds like nitrite, ammonia, methane, sulphur. This mechanism is called as chemosynthesis, carried out by a group of bacteria, known as chemoautotrophs. These bacteria can be eaten up directly by other organisms like protozoans and microflagellates. They may also grow in association with particulate materials i.e. marine snow. These particulate materials are mostly organic matter, including dead or dying animals and plants, fecal matter, sand and other inorganic dust. This marine snow is an important source of nutriment for larger zooplankton species. This is also an important vehicle whereby particulate organic matter is exported by sedimentation to the deep ocean (Silver et al, 1978; Lampitt et al, 1993). Sometimes they may be dependent on external supply of organic materials for obtaining the nutrition. Such kind of bacterioplanktons is known as chemoheterotrophs. They absorb organic materials from outside both for body building and liberation of energy.

b) Benthos: The lowest layer of the ocean i.e. sediment surface and subsurface layers are called benthic environment. Organisms inhabiting

this environment are called benthos. They generally live in close relationship with the substrate bottom. Some of them are permanently attached to the bottom. As light does not penetrate deep ocean-water, the energy source for deep benthic ecosystems is often organic matter from higher up in the water column which drifts down to the depths. This dead and decaying matter sustains the benthic food chain. Most organisms in the benthic zone are scavengers or detritivores. They can be divided into following types based on their size:

Microbenthos: Microscopic benthos that are less than 32 μm in size. Some examples are bacteria, diatoms, ciliates, amoeba and flagellates.

Meiobenthos: These are tiny benthos, less than 0.5 mm but greater than 32 μm in size. Some examples are nematodes, gastrotriches and smaller crustaceans such as copepods and ostracodes.

Macrobenthos: These are the larger benthos, greater than 0.5 mm in size. Some examples are polychaete worms, bivalves, echinoderms, sea anemones, corals, sponges, sea squirts, turbellarians and larger crustaceans such as crabs, lobsters and cumaceans.

1.1.3 Deep-sea environment

Deep-sea covers $\sim 65\%$ area of the total earth surface ([Svendrup et al, 1942](#)). Deep-sea environment is characterized by the extreme conditions of temperature, hydrostatic pressure and nutrient conditions. The hydrostatic pressure increases with depth at a rate of 0.1 MPa/10 m (0.1 MPa is equivalent to 1 bar hydrostatic pressure). Therefore, at a 5000 m of depth the hydrostatic pressure is ~ 50 MPa. In contrast, temperature decreases with increasing depth and is around $\sim 2\text{-}3^\circ\text{C}$ at 5000 m depth. This environment is also characterized by low nutrient conditions and is supposed to be physically stable ([Sanders, 1968](#)).

Among other parameters, salinity plays important role which is generally 35 ppt (completely marine) in most of the deep-sea habitats. However, in some hypersaline habitats the salinity value reaches more

than 39 ppt (Mediterranean and Red Sea) as well as 300 ppt (Orca Basin in the Gulf of Mexico) (Shokes et al, 1976). Light intensity is very low at these depths as the penetration of light does not occur below 250 m depth. Thus deep-sea habitats are supposed to be dark and devoid of any photosynthetic activities by the organisms thriving there. The main source of oxygen in these habitats is the surface water which carries dissolved oxygen generated by phytoplanktons in the euphotic zone as well as exchange with the surrounding waters. Deep-sea currents generated due to descending movement of surface water are mainly responsible for bringing this oxygenated surface water to the lower depths. Usually the dissolved oxygen concentration in the bottom water is $\sim 5\text{-}6 \text{ ml L}^{-1}$ whereas in surface waters it is $\sim 5\text{-}8 \text{ ml L}^{-1}$ (Gewin, 2010). Also, it has been found to vary with depth in different deep-sea sediments (Reimers, 1987).

Seafloor sediment is characterized on the basis of the nature of source which causes its origin. The source may be biotic, present in overlying ocean water, eroded material from land transported to the ocean by rivers or wind, ash from volcanoes, and chemical precipitates derived directly from sea water. In short, the particles found in sediment on the seafloor vary considerably in composition depending on the processes that have acted to form, transport, and preserve them. Major proportion of sediments near sea shore is composed of terrigenous sediments. Terrigenous sediments are those which transported to the ocean as particles of gravel, sand or mud by erosion from continents. Some sediments, called as volcanogenic, are formed from material flung into the sea by volcanoes, both those on land and those under water.

The rocky base of the deep-sea floor, distantly located from shore is composed of three major types of sediments i.e., red clays and two biogenic sediments, calcareous and siliceous. The inorganic components of the sediments, which constitute the red clays, consist of finely divided fragments of eroded and transformed rock. They are small enough to drift far from land before they sink to bottom. Drifting in suspension, they may be borne by the currents for years or decades before they are finally

dropped onto the sea floor. The red clay also includes material from outer space. Most of it enters the earth's atmosphere in the form of cosmic dust.

Among biogenic sediments, calcareous sediments are composed of calcium carbonates and are present mostly in sea-mount areas. The sea-mount areas are present at comparatively lower depth and thus are supposed to be favorable for deposition of carbonates. These sediments are also formed by the precipitation of calcium carbonate by organisms in the upper layer of the sea which gets dissolved in the deep ocean. Some examples of the organisms which contribute towards these sediments are foraminifera, coccoliths and pteropods. **Foraminifera** are the benthic and planktonic forms of protozoans with benthic being the most common ones. They have shells composed of calcite. The calcite dissolution starts at a depth of calcite compensation from planktonic forms of these foraminiferans (Gooday et al, 2008). The calcite compensation depth is the depth where rate of carbonate supply is equal to rate of carbonate dissolution. The deep water is usually undersaturated with calcite and therefore its dissolution starts with increasing depth. Also the calcite becomes more soluble with elevated pressure and low temperature.

Coccoliths are phytoplanktons which constitutes of single celled algae. Their cells are surrounded by heavy armour of calcite plates and contribute towards the largest proportion of calcareous sediments in the ocean floor. **Pteropods** are small gastropod mollusks, with their shells composed of aragonite. Their ooze commonly known as pteropod ooze/carbonate ooze contributes towards calcareous sediment of Deep Ocean.

Other type of sediment is the siliceous sediments which are composed of siliceous shells, a form of hydrated silicon dioxide. The distribution pattern of siliceous sediments in the ocean is generally inversely related to calcareous sediments. Their solubility usually decreases with increase of pressure and decrease of temperature. Therefore their concentration is higher in deep-sea environment where calcareous sediments are absent due to Calcite Compensation Depth (CCD).

Radiolarians and diatoms are the major silica producers. Radiolarians are protozoans having silica exoskeleton with many spines extending outwards. Their skeleton remains form radiolarian ooze on deep-sea floor, which sometimes get converted to hard sedimentary rock, called as radiolarian cherts. Diatoms are single celled algae that form frustules by incorporation of silica in their cell walls. Both of them are useful for dating of rocks because their skeleton remains preserved in the sediments for long period of time.

1.1.4 Microbes inhabiting deep-sea environment

There are several reports about the existence of diverse types of microbes from the deep-sea habitats e.g., bacteria (Li et al, 1999a; Kato and Qureshi, 1999; Schauer et al, 2010), archaea (DeLong, 1992; Massana et al, 2000; DeLong, 2004) and protists (Edgcomb et al, 2002; Li et al, 2010). Hongxiang et al. (2008) reported bacterial diversity from deep-sea sediments of northeastern Pacific Ocean by culture-independent approach. Among Archaea, Euryarchaeota (methanogens/extreme halophiles) were found to be abundant in surface waters (Massana et al, 1997; Vetriani et al, 1999), whereas Crenarchaeota (themophilic group) prevalent in the deep (Massana et al, 1997; Vetriani et al, 1999; Massana et al, 2000; Takai et al, 2004). Wang et al. (2005) reported archaeal diversity in the deep-sea sediments of west pacific warm pool by DGGE (Denaturing Gradient Gel Electrophoresis) of the amplified 16S rDNA fragments. Highly diverse forms of these archaea were reported from hydrothermal vent sites by PCR (Polymerase Chain Reaction) mediated SSU (Small Sub Unit) rDNA sequencing analysis using archaeal specific primers (Takai and Horikoshi, 1999).

In general, cold seeps and hydrothermal vent environments are inhabited by chemosynthetic communities. They are unique in that they utilize carbon sources not produced as a result of usual photosynthesis that supports all other life forms on Earth. Through the process of

chemosynthesis these microbial communities can actively support other higher living organisms through symbiotic relationships. Since the surrounding environment has been characterized by extreme conditions of temperature and pressure these organisms are also known as extremophiles.

Archaea and Bacteria present in this environment process sulfides and methane through chemosynthesis into chemical energy. More complex organisms, such as vesicomid clams and tube worms use this energy to sustain their own life processes. In exchange, these complex organisms help the microbes by providing them shelter. Sometimes other microbial communities cover large areas by forming huge mycelial mats. Due to comparatively cooler and stable atmosphere the organisms found in cold seeps have a greater life span than those found in hydrothermal vent habitats. [Li et al. \(1999b\)](#) reported diversity of sulfate reducing bacteria as well as Archaea from sediments of the deepest cold seep area of Japan Trench.

Bacteria are most abundant microbes found in a variety of habitats. These are single-celled organisms without cell nuclei. They are found in all portions of the water column, the sediment surface, and the sediments themselves ([Hartgers et al, 1994](#); [Kuwaie and Hosokawa, 1999](#)). Some are aerobic (requiring oxygen), whereas others are anaerobic (not requiring oxygen). Most bacteria are free-living, but some live as partners (symbionts) within other organisms. For instance, many deep-sea fish harbor symbiotic bacteria that emit light, which the fish use to signal other members of their species. The bacteria's ability to emit light is called bioluminescence. Bioluminescence causes water to glow, a phenomenon which can be noticed most easily at the surface but is also present in all the depths.

Among bacterial communities, cyanobacteria are the unicellular form of bacteria, capable of obtaining energy through the process of photosynthesis. Although all cyanobacteria are photoautotrophic, many can utilize simple dissolved organic carbon (DOC) for heterotrophic

growth or for mixotrophic growth in the light. (Khoja and Whitton, 1971; Fogg et al, 1973; Sahu and Adhikary, 1982; Radwan and Al-Hasan, 2000). They are found to be present in planktonic, (Wille, 1904; Gallon et al, 1996), benthic (Humm and Wicks, 1980) and symbiotic forms in association with lichens, protists and sponges (Carpenter and Janson, 2000, 2001; Carpenter and Foster, 2002). They played an important role in the history of Earth and in ocean processes (Brock 1973; Schopf, 1996). They also helped in the development of stromatolites. Stromatolites are large structures present in shallow water areas which are formed due trapping of sedimentary grains by large biofilms of the cyanobacteria. These structures have important ecological role encoding biological activity present in a particular habitat. Also the cyanobacteria helped in generating the oxygen in earth's atmosphere by the process of photosynthesis (Holland, 1997).

Bacteria also play an important role in the major biogeochemical cycling processes (Pfannkuche, 1992). The other microbial communities similar to bacteria, known as archaea are one of the major domains of life on Earth. Since their discovery in 1970, these microorganisms have been found in many extreme environments on Earth. Apart from bacteria and archaea, viruses are also found to be abundant forms in the benthic environments (Danovaro et al, 2001; Hewson et al, 2001; Breitbart et al, 2004). Ortmann and Suttle. (2005) reported higher abundance of viruses at hydrothermal as well as non-hydrothermal vent sites, suggesting a significant role for viruses in ecological and geochemical processes in the deep-sea.

1.1.5 Fungi in marine environment

Fungi are ubiquitous in the terrestrial and aquatic environments. They occur in the form of unicellular yeasts, polymorphic and filamentous fungi either free living or as symbiotic forms. They are also found to occur as potential pathogenic forms of plants and animals. Various workers have attempted to define a marine fungus (Kohlmeyer and Kohlmeyer, 1979; Jones, 1993).

Early workers determined the physiological requirements for the growth of marine fungi in sea water, or in particular concentrations of sodium chloride (Jones and Jennings, 1964; Meyers, 1968). According to Kohlmeyer and Kohlmeyer. (1979), which is supposed to be the best working definition, marine fungi can be divided into two forms: 1) Obligate species, which exclusively grow and sporulate in a marine habitat and 2) Facultative species, which are able to grow and possibly also sporulate in the marine environment but are also found in terrestrial and freshwater milieus. Another criterion that was accepted is the ability to germinate and to form mycelium under natural marine conditions (Hyde et al, 2000).

Marine fungi are distinct in their physiology, morphology and adaptation to an aquatic habitat (Meyers, 1996). According to the literature, higher marine fungi may be divided into a majority of Ascomycota (97%), a few Basidiomycota (~2%) and anamorphic fungi (< 1%). Several papers have addressed the issue of the diversity and numbers of marine fungi (Jones et al, 1995; Jones and Mitchell, 1996; Jones and Alias, 1997). There are approximately 80,000 species of described fungi (Kirk et al, 2001), representing only about 5% of the estimated 1.5 million species worldwide (Hawksworth, 1991). Jones and Mitchell. (1996) estimated that there are some 1500 species of marine fungi compared with 200,000 marine animals and 20,000 marine plants. The dramatic rise in the numbers of new marine fungi, which has occurred since the statement by Kohlmeyer and Kohlmeyer. (1979), is due to the nature of the substrata examined for the occurrence of fungi. Kohlmeyer and Kohlmeyer. (1979) listed 42 species of mangrove fungi, while the current number approaches 200 species (Jones and Alias, 1997). Similarly the number of fungi recorded by Kohlmeyer and co-workers has also increased dramatically (Kohlmeyer et al, 1996a). It is believed that the documentation of marine fungi is still at the inventory stage and many new taxa await discovery.

Most marine fungi show one of four patterns of distribution: arctic, temperate, tropical, or cosmopolitan. While the majority of fungi may be grouped in to pantemperate and pantropical, there is little evidence of

species being restricted to countries or continents (Jones, 1993). Of the approximately 500 species of marine fungi that have been described, at least 135 are found in the tropics. Water temperature is the most important factor in controlling the geographical distribution of marine fungi (Jones, 1993). Tropical or colder currents often cross boundaries and influence the mycobiota therein. A mixture of temperate and tropical fungi is found at intermediate sea temperature habitat. The probable reason for this may be that these fungi are dispersed by separate propagules or on wood growing in the sea, and therefore sea masses present no barrier to their dispersal.

There exist species that are typically tropical (e.g., *Antennospora quadricornuta* and *Halosarpheia ratnagiriensis*), temperate (e.g., *Ceriosporopsis trullifera* and *Ondiniella torquata*) and arctic (e.g., *Spathulospora antartica* and *Thraustochytrium antarticum*). The intertidal mangrove species *Halosarpheia fibrosa* and *Halosarpheia marina* may have a subtropical distribution (Hyde and Lee, 1995). Some marine fungi are cosmopolitan (Jones, 1993), like *Ceriosporopsis halima*, *Lignicola laevis*, *Corollospora maritime* and *Halosarpheia appendiculata*, which are more or less common in temperate and tropical seas (Jones, 2000). Sometimes the occurrence of fungi in a particular habitat depends on the type of substratum also. For example, fungi occurring on submerged wood in the open sea usually differ from those on intertidal mangrove wood (Hyde, 1989a). Wood in the open ocean tends to favour the growth of members of the *Halosphaeriales* whose species have deliquescent asci, passive ascospores release and variously appendaged ascospores which are involved in flotation and attachment (Jones et al, 1994). In mangrove habitats, loculoascomycetes are especially common on decaying wood in the intertidal zone where they can eject their spores during low tides (Hyde, 1989b).

Over 90% of the higher marine fungi use woody and herbaceous substrata. They are major decomposers of these substrata existing in marine ecosystems. Thus, they exert their influence in areas of large input of litter of vascular plants, especially at some types of terrestrial/marine

ecosystemic interfaces (ecotones) (Hyde, 1989a). The nature of a particular substratum can have a major effect on the fungi colonizing it (Hyde, 1990). Lignocellulosic materials yield the greatest diversity, in contrast to a few species colonizing calcareous materials or sand grains. Competition between fungi can markedly affect fungal diversity and species composition (Tan et al, 1995). Salinity is also important in affecting species composition. Many fungi occur primarily in fully saline waters (e.g., *Lindra inflata* and *Ondiniella torquata*); others are more frequent in brackish water (e.g., *Amylocarpus encephaloides* and *Aniptodera chesapeakenis*), while terrestrial and fresh water species may be able to grow at lower salinities (e.g., *Chytridium citriforme*, *Saprolegnia ferax* and *Stachybotrys atra*) (Jones, 2000). In mangroves many species (e.g., *Halophytophthora* sp.) can tolerate great variation in salinity of the water (Leano et al, 2000).

A range of factors may govern the occurrence of marine fungi in a particular habitat or on a substratum. Some marine fungi are common in occurrence (e.g., *Ceriosporosis halima*, *Lulworthia* spp., and *Zalerion maritimum* in temperate waters and *Antennospora salina*, *Antennospora quadricornuata* and *Lulworthia grandispora* in the tropics), while others are rarely collected (e.g., *Orbimyces spectabilis* and *Torpedospora ambispinosa*). For the latter group, this may be due to seasonality (e.g., *Mycaureola dilsea* on *Dilsea edulis*) or temperature requirements (e.g., *Digitatispora marina* occurring during the winter months when water temperatures are below 10°C). Other species are subjected to a consortium of factors operating together in controlling the biodiversity of fungi in the sea (Dighton, 2007).

1.1.6 Different habitats of marine fungi

Different marine habitats like salt marshes, sea grass beds and mangrove forests produce large amount of dead plant material (litter), much of which enters the system as relatively large detrital particles. In all marine ecotones, the organic matter is rapidly incorporated into a complex

decomposer food web in which fungi are secondary producers. *Antennospora quadricornuata*, *Arenariomyces* species, *Corollospora* species and *Torpedospora radiadata* are typical fungi of coastal waters (Jones, 2000). Following are the habitats in marine ecosystem generally inhabited by fungi:

Saltmarsh habitats: Bays, estuaries and their surrounding saltmarshes and mudflats, are among the most productive systems of the biosphere. Saltmarshes constitute dynamic environments that present characteristics of both marine and terrestrial systems. Due to the limitation of only a few animal species to feed directly on the living plant material in these habitats, fungi and bacteria seem to be the principal competitors for the organic substrates. Microbial production in standing-grass litter is dominated by fungi, mainly by ascomycetes. Some of the examples are *Phaeosphaeria spartinicola* and *Buergenerula spartinae* (Newell, 1996). The importance of fungi in the biogeochemical cycling of carbon and nutrients in coastal saltmarshes has been studied well (Newell, 2001). A principal role of aerobic filamentous fungi is the degradation of lignocelluloses in vascular plants. Ascomycetous fungi have been shown to be capable of degrading both the lignin and polysaccharide moieties of lignocelluloses (Bergbauer and Newell, 1992; Newell, 1996).

Mangrove habitats: Mangrove forests are considered open interface ecosystems connecting upland terrestrial and coastal estuarine ecosystems (Lugo and Snedaker, 1974). Contributors to the geo-aquatic food chain, mangrove forests are important for biomass production and coastline protection. Mangrove fungi may be found in the subtropics and warm temperate regions, but its species diversity is higher in the tropics (Hyde and Lee, 1995). Fungi inhabiting mangrove habitats differ in their distribution. Mangrove fungal diversity is dependent on the diversity, age and abiotic factors, such as salinity and tidal range of the mangrove stand (Hyde and Lee, 1995). A higher percentage of fungi occurring on wood in mangrove ecosystems, indicates their important ecological role as they are able to aggressively degrade lignocelluloses (Hyde et al, 1998). Wood is a

heterogeneous polymer made of polysaccharides, cellulose and hemicelluloses and lignin (Fengel and Wegener, 1989). Some of the commonly found fungal species in these habitats include *Halosarpheia marina*, *Lignincola laevis*, *L. longirostris*, *Lulworthia grandispora*, *Periconia prolifica*, *Savoryella lignicola*, *Trichocladium achrasporum*, *Dactylospora haliotrepha* and *Verruculina enalia*. (Sarma and Vittal, 2000; Alias and Jones, 2000).

Fungal enzymes involved in wood degradation (cellulases and redox enzymes) have been studied at large scale in wood-inhabiting marine fungi. Beta-glucosidase and endoglucanase are the most frequent enzymes of cellulose metabolism. Many of the marine fungi show high cellulase activity. The presence of laccase enzyme has been found to be different among the various fungal systematic groups, with higher percentages in the basidiomycetes and ascomycetes, which mostly belong to the group of white-rot and soft-rot fungi, respectively (Rohrmann and Molitoris, 1992). Laccase belongs to a family of multicopper oxidases that are widespread in fungi. Lignin peroxidase and manganese-dependent peroxidase appear to be relatively less common in the marine fungi (Raghukumar et al, 1994).

Calcareous shells: One of the major habitats for colonization of marine fungi is calcareous shells (Kohlmeyer and Kohlmeyer, 1979; Raghukumar et al, 1992). Endolithic fungi growing within such calcareous shells have been reported from intertidal beaches (Porter and Zebrowski, 1937; Kohlmeyer, 1969; Cavaliere & Alberte, 1970). Coral rocks (Kohlmeyer and Volkmann-Kohlmeyer, 1987, 1989) and also live corals (Kendrick et al, 1981; Ravindran et al, 2001). These fungi are considered to be important as organic eroders of calcium carbonate skeletons and as producers of fine carbonate detritus (Perkins and Halsey, 1971; Tudhope and Risk, 1985). Apart from these habitats presence of fungi has been reported from calcareous sediments and rocks in marine ecosystems.

1.1.7 Fungi in deep-sea environment

Fungi in marine ecosystem occupy an important position as they are ubiquitous and involved in the decomposition and mineralization of organic matter (Kohlmeyer and Kohlmeyer, 1979; Hyde, 1989a; Newell 2001). Deep-sea fungi were first reported in shells collected from deep-sea waters of 4610 m depth (Hohnk, 1961 & 1969). Other report on isolation of fungi is from water samples, collected from subtropical Atlantic Ocean, from the surface to a depth of 4,500 m using sterile van Dorn bags or Niskin samplers (Roth et al, 1964). Fungi from deep-sea were obtained only as preserved specimens by directly submerging wooden panels at 1615-5315 m depth (Kohlmeyer, 1977). Poulicek et al. (1986 & 1988) reported presence of fungi inside molluskan shells. Several marine yeasts such as *Debaryomyces* sp., *Rhodotorula* sp. And *Rhodospiridium* sp. were isolated in culture form over a range of temperature and hydrostatic pressure conditions (Lorenz & Molitoris, 1997). Presence of fungi has also been reported from Mariana trench at a depth of ~ 11,000 m in the Pacific Ocean (Takami et al, 1997).

Various filamentous fungi were isolated from calcareous sediments and were also observed to show germination of spores at elevated hydrostatic pressure (Raghukumar and Raghukumar, 1998). Fungal filaments were directly detected in calcareous fragments using the fluorescent brightner calcofluor under epifluorescence microscope (Raghukumar and Raghukumar, 1998). Isolation and direct detection of fungal filament was reported from the Chagos trench in the Indian Ocean from the deep-sea sediment which is supposed to be oldest in age (Raghukumar et al, 2004). Over 200 fungal isolates were obtained in culture form from deep-sea sediments of the Central Indian Basin using different techniques like dilution plating, particle plating and pressure enrichment techniques (Damare et al, 2006). One of these isolates was confirmed to be native of deep-sea sediment using immunofluorescence technique as well as growth at simulated deep-sea conditions.

1.1.8 Role of fungi in deep-sea ecosystem

Fungi play parasitic, symbiotic or pathogenic role in various associations reported from marine environments (Hyde et al, 1998; Herndl and Weinbauer, 2003). *Mycaureola dilseae*, a marine basidiomycete fungal species has been reported to be the parasitic form of subtidal red algae, *Dilsea carnosa*. *Eurychasma dicksonii* and *Chytridium polysiphoniae* are two widespread, zoosporic fungal pathogens of marine macroalgae (Sparrow, 1960). The symbiotic association of some fungal species with marine sponges has also been reported (Taylor et al, 2007). These fungi secrete useful antimicrobial compounds (Bugni and Ireland, 2004; Bhadury et al, 2006). *Phoma* sp., often isolated from marine and estuarine environment is found as parasites of seaweeds, sea-grasses, mollusks and sponges (Kohlmeyer and Volkmann-Kohlmeyer, 1991). Some marine yeast species such as *Rhodotorula*, *Rhodospiridium* are known to be as potential mycoparasites and phytopathogens. Some cellular structures known as colacosomes are found in these parasitic species which enable them for host-parasite interactions (Bauer and Oberwinkler, 1991). A pathogenic black yeast in mussel and other animals have been reported from hydrothermal vent sites (Moreira and Lopez-Garcia, 2003; Van Dover et al, 2007). High number of *Aspergillus* sp. was isolated from deep-sea sediments of the Central Indian Basin (Damare et al, 2006), which have been reported earlier to be the potential pathogenic forms (Behnsen et al, 2008).

Various studies have reported diverse fungal forms from the Indian Ocean (Raghukumar and Raghukumar, 1998; Damare et al, 2006). The cultures were identified by classical taxonomic methods in these studies. The drawback of the above identification method is that the non-sporulating forms can not be identified on the basis of morphological features. Recently few studies have reported identification of fungal cultures by amplification and sequencing of their 18S rDNA and ITS regions (Malosso et al, 2006; Wang et al, 2008; Burgaud et al, 2009; Liu et

al, 2010; Jebaraj et al, 2010). These molecular methods have enabled the identification of both sporulating as well as non sporulating forms of fungal cultures till generic or species level.

Diverse fungal communities have been reported from various regions across world using culture independent approach also. Bass et al, (2007) reported yeast to be the dominant fungal forms in the deep ocean by using fungal specific primers for SSU rDNA region. Presence of fungal signatures in hydrothermal sediments of the mid-Atlantic ridge was reported by analysis of 18S rDNA sequences amplified with eukaryote-specific primers (Lopez-Garcia et al, 2003). Several other reports have recovered fungal sequences from different deep-sea habitats such as methane hydrate bearing deep-sea sediments (Lai et al, 2007), hydrothermal vents (Calvez et al, 2009), anoxic sites (Jebaraj et al, 2010) etc.

Till date there is no report on identification of fungi using molecular methods from the Central Indian Basin which is the sampling site in the present study. In the earlier reports fungi have been isolated and identified only by classical morphological methods (Damare et al, 2006). These studies were found to give biased results for the diversity estimation because of the presence of non-sporulating forms which could not be identified with classical methods. Therefore, in order to examine the actual diversity of fungi in the deep-sea sediments of the Central Indian Basin, both culture-dependent as well as culture-independent approaches were applied in the present study in addition to the classical methods. The 18S and ITS regions of SSU rDNA were targeted for the culture identification and environmental gene libraries construction. Also, the results were compared with earlier reports on fungal diversity from the same sampling area of the Indian Ocean.

Therefore I propose to study the diversity and growth patterns of deep-sea fungi isolated from Central Indian Basin with the following objectives:

- Isolation of fungi from deep-sea sediments from the Central Indian Basin.
- Analysis of the diversity of fungi from deep-sea sediments through amplification and sequencing of 18S and ITS region of ribosomal DNA.
- To study the effect of different hydrostatic pressure and temperature conditions on growth of a few select cultures.
- Isolation and analysis of stress proteins from a few select cultures, subjected to temperature and pressure shock.