Physiological and Molecular Studies of Deep-Sea Fungi

Thesis submitted for the degree of

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in

Marine Sciences

to the

Goa University

by

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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

Purnima Singh

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 **Mrs 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.

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Date: Nov 29, '11

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All the corrections suggested by referees have been incorporated.

22.12.2011

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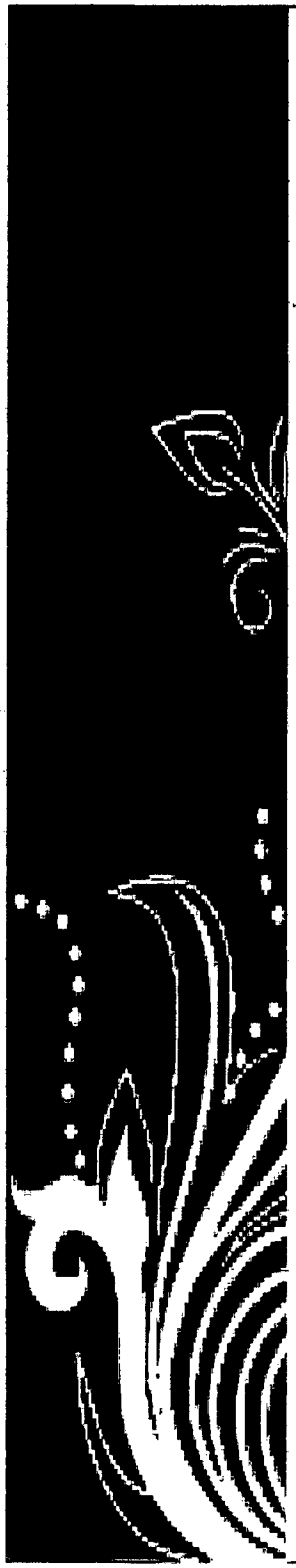

Dedicated

To

Lord Shiva

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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 $1.368*10^9$ 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 detrivory 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 argyropelecids (hatchetfishes). They have black backs, flanks that are highly reflective and mirror-like and their undersides are lined with photophores (light organs). The silvering 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.

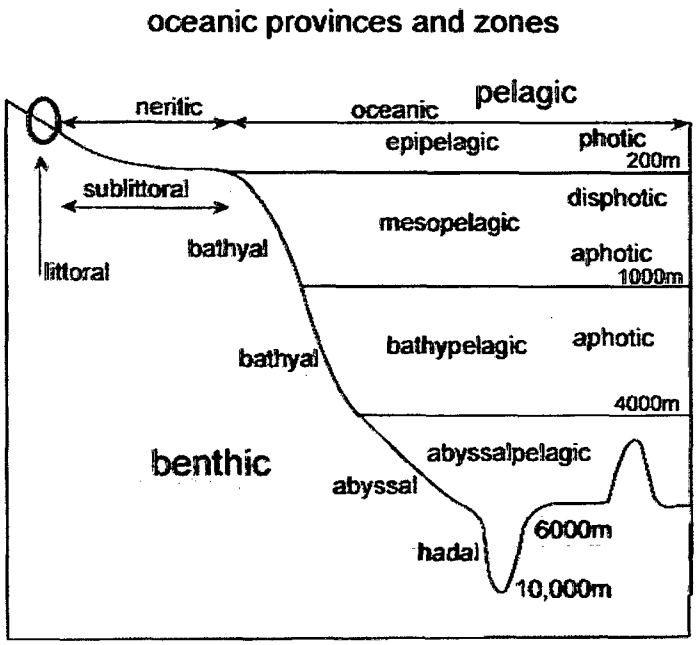


Fig. 1.1 Different zones of the open ocean, Modified from:

(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^-, 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:

$HS^- + 2\ O_2 \rightarrow HSO_4^-$

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 ~ 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 ~ 2-3°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 ~ 5-6 ml L^{-1} whereas in surface waters it is ~ 5-8 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 vesicomyid 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; Kuwae 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 Basidiomycoa (~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, Lignincola 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 deliquescing 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 geoaquatic 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 *Rhodosporidium* 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, Rhodosporidium* 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.

Chapter 2

Isolation of fungi from deep-sea sediments of the Central Indian Basin (CIB) and their identification using molecular approaches.

2.1 Introduction

Deep-sea harbors various kinds of microorganisms comprising bacteria, archaea and fungi, known to play an important role in recycling of nutrients (Snelgrove et al, 1997). Among these, bacteria and archaea have been studied in detail (Stackebrandt et al, 1993; Urakawa et al, 1999; Li et al, 1999a; Takai and Horikoshi, 1999; Delong and Pace, 2001; Sogin et al, 2006; Hongxiang et al, 2008; Luna et al, 2009). Fungi have also been reported in several deep-sea environments (Nagahama et al, 2006), including hydrocasts near hydrothermal plumes from the Mid-Atlantic Ridge near the Azores (Gadanho and Sampaio, 2005) and in Pacific sea-floor sediments (Nagahama et al, 2001b). Fungi from both of these habitats were dominated by unicellular forms, commonly designated as yeasts. Many of the fungi isolated from the deep ocean have been previously undescribed species. For example, Nagahama and coworkers have described a number of novel species of yeasts from deep-sea sediments (Nagahama et al. 2001a, 2003, 2006). Undescribed species of yeasts were also identified from Atlantic hydrothermal plume waters (Gadanho and Sampaio, 2005).

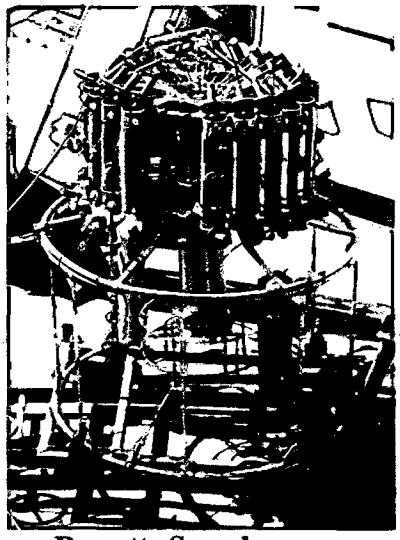
Presence of fungi in deep-sea sediments of the Central Indian Basin was reported by direct detection and immuno-fluorescence techniques (Damare et al, 2006). Fungi were also isolated from these deep-sea sediments using various isolation techniques. These cultures showed increase in biomass and germination of their spores under simulated deep-sea conditions of elevated hydrostatic pressure and low temperature (Damare, 2006). Numerous new compounds and antibiotics have also been discovered from fungi isolated from marine organisms (see Raghukumar, 2008). Also, some plant associated fungi have been reported to produce esters, alcohols, and small molecular weight acids (Mitchell et al, 2008). Considering the role of fungi as a source of useful bioactive compounds (Bhadury et al, 2006; Paramaporn et al, 2010; Konishi et al, 2010) and their involvements in various biogeochemical cycles, the diversity of fungi from deep-sea sediments needs to be examined.

2.1.1 Methods for Collection of water and sediment samples

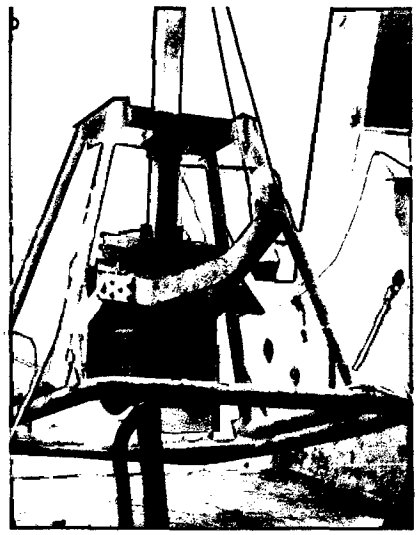
Fungi in deep-sea waters may be collected using routine oceanographic samplers such as Niskin or Nansen bottles or a Rossette or ZoBell sampler (Fig. 2.1a). These samplers do not maintain *in-situ* hydrostatic pressure. First water sampler, used to collect water sample for isolation of piezophilic bacteria which was able to retain deep-sea pressure was reported by Jannasch et al. (1973). In general, mycelial fungi draw nutrients from organic matter in the sediment and produce hyphae, which spread out and grow in between the sediment particles (Raghukumar, 1990). Therefore it is more appropriate to look for fungi in the sediments as they are not well adapted as free living forms. Several types of sampling devices for collection of sediment samples have been used successfully by mycologists.

A deep-sea workstation was developed under the Deep-Star program at JAMSTEC, Japan, for retrieving and culturing deep-sea organisms under simulated deep-sea conditions. This system also contains a pressure-retaining sediment sampler, which is able to obtain sediment sample while maintaining ambient pressure and low temperature after sampling at the deep-sea bottom (Yanagibayashi et al, 1999). For routine microbiological sampling of deep-sea sediments, multiple corers, long gravity corers or box corers may be used (Fig 2.1b). Box corers may be used efficiently for more or less flat oceanic floors for sampling. The box corer is lowered on a ship's trawl wire till it penetrates the bottom. As the corer is pulled out of the seabed, the top and bottom of the sample box are closed. The advantage of a box corer is that it collects a sample which is generally $\geq$20 cm in length which encompasses the bulk of the vertical distribution of deep-sea organisms.

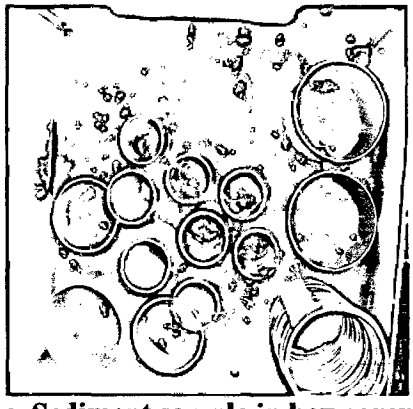
Deep-sea sediments can also be collected using research submarines. A research submarine is comparatively bigger in size and can carry a pilot and one or two scientists in a pressure sphere about 2 m in diameter.



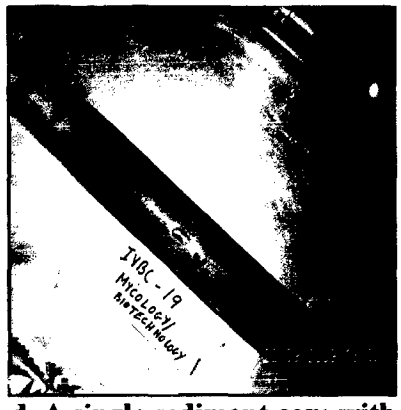
a. Rossette Sampler



b. USNEL Box Corer



c. Sediment sample in box corer



d. A single sediment core with sample

e. Collecting sediment sub-cores in the lab

Fig. 2.1 Water and sediment sampling methods during cruises #ABP26 and #ABP38 from the Central Indian Basin.

Surrounding the sphere is equipment for life support, propulsion, ascent and descent, and equipments for scientific purposes such as manipulator arms, cameras and specialized payload in a carrying basket (Heirtzler and Grassle, 1976).

Another method for sampling is remotely operated vehicles (ROVs) which are self propelled instrument packages. Some operate at the end of a cable that provides power and hosts a two-way communications link; others are untethered, carrying their own power and recording images and data. The instrument package consists of a propulsion unit, sensors (particularly television), and, in some cases, manipulator arms. These are designed either to fly or crawl, e.g. the Remote Underwater Manipulator (Thiel and Hessler, 1974) over sea bottom surface and are more suitable for seabed sampling and experimentation.

2.1.2 Methods for studying microbial communities

Microbial communities play pivotal role in various biogeochemical cycles in different habitats (Molin and Molin, 1997; Trevors, 1998; Wall and Virginia, 1999). The major problem in correct estimation of microbes at a particular habitat is sampling and analyzing comparatively very small proportion of total area. In addition, due to spatial heterogeneity of the species or the habitat, diversity analysis of the microbes may be underestimated. Other factor for inability of correct estimation of microbial communities at a particular habitat is their low culturability. It has been suggested that at least 99% of bacteria observed under a microscope are not cultured by common laboratory techniques (Borneman et al, 1996; Giller et al, 1997; Pace, 1997; Torsvik et al, 1998; Trevors, 1998). Most of the fungal species also elude culturing in the laboratory (van Elsas et al, 2000).

Species diversity consists of species richness, the total number of species present, species evenness, and the distribution of species (Trevors, 1998; Ovreas, 2000). Some of the methods used for the measurement of cultured microbial diversity are as following:

a) **Direct plate count method:** In this method the samples containing microbes are spread plated on selective media plates, followed by the counting of the colonies appearing after an incubation period. This method has limitations in terms of selection of appropriate media, pH and incubation temperature, colony-colony inhibition (Trevors, 1998) etc. Sometimes the fast growing forms can overtake slow growing ones, often resulting in biased results (Dix and Webster, 1995). In addition, conidiation may also give false over-estimate of some of the sporulating fungi.

b) **Community level physiological profiling:** In this method the culturable microorganisms can be identified on the basis of different carbon source utilization patterns. Choi and Dobbs (1999) introduced Biolog plates containing all the known carbon sources for this purpose. For analysis, samples containing microbes are inoculated and monitored over time for their ability to utilize substrates and the speed at which these substrates are utilized. Multivariate analysis is applied to the data and relative differences between soil functional diversity can be assessed.

c) **Fatty acid methyl ester (FAME) analysis:** Fatty acids make up a relatively constant proportion of the cell biomass and signature fatty acids exist that can differentiate major taxonomic groups within a community. Therefore, a change in the fatty acid profile would represent a change in the microbial population. The fatty acid profiles can be used to study microbial community composition and population changes (Siciliano and Germida, 1998; Kelly et al., 1999). Studies of bacteria have, in general, shown that phenotypic relationships among organisms as indicated by fatty acid composition are in agreement with phylogenetic associations based on DNA and rRNA homology (Nichols et al, 1986; Sasser and Smith, 1987; Guckert et al, 1991).

Individual species and strains of bacteria have been shown to have characteristic fatty acid profiles and are now being identified on this basis (Mayberry et al, 1982; Sasser and Fieldhouse, 1984; Veys et al, 1989). This method can also be used for the identification of yeasts (Gunasekaran and Hugh, 1980; Tredoux et al, 1987; Marumo and Aoki, 1990). Stahl and Klug. (1996) reported different biomarkers for different phyla of fungi which can be used for their identification. For example, fungi belonging to Oomycota were found to differentially express 20:5 fatty acids which were absent in fungi from other phyla. Zygomycota were characterized by production of higher number of fatty acids than Dikaryotic forms i.e. Ascomycota and Basidiomycota. Dikaryotic forms never produce 18:3 fatty acids which are major fatty acids for Zygomycetes. There was no difference in fatty acid profiles between Ascomycota and Basidiomycota. Sterile forms of fungi were found to produce 22:0 fatty acids, which was not found in any other taxon, and relatively high amounts of 18:0(3OH) fatty acid and 20:4 fatty acid, which were produced only in small amounts or not at all by other fungi.

a) **Molecular based techniques:** Polymerase Chain Reaction (PCR) targeting the 16S rDNA has been used extensively to study the identification and diversity of prokaryotes (Pace, 1996, 1997, 1999). Among eukaryotes, 18S and internal transcribed spacer (ITS) regions of SSU rDNA are increasingly used to study fungal communities (Fig 2.2). However, the available databases are not as extensive as for prokaryotes (Prosser, 2002). In addition, the D1/D2 domain as well as other regions of Large Sub Unit (LSU) of 28S rDNA of eukaryotes can also be used for identification and diversity analysis (Pang and Mitchell, 2005; Gadanho and Sampaio, 2005; Burgaud et al, 2010). Restriction fragment length polymorphism (RFLP) for the above amplified products can also be applied for the diversity analysis on the basis of DNA polymorphisms. RFLP is the variation in DNA fragment

banding patterns of electrophoresed restriction digests of DNA from different individuals of a species. The reason for the different banding patterns may be the presence of a restriction enzyme cleavage site at one place in the genome in one individual and the absence of that specific site in another individual. Therefore, on the basis of these specific banding patterns after cleavage with a particular restriction enzyme different strains can be identified or compared.

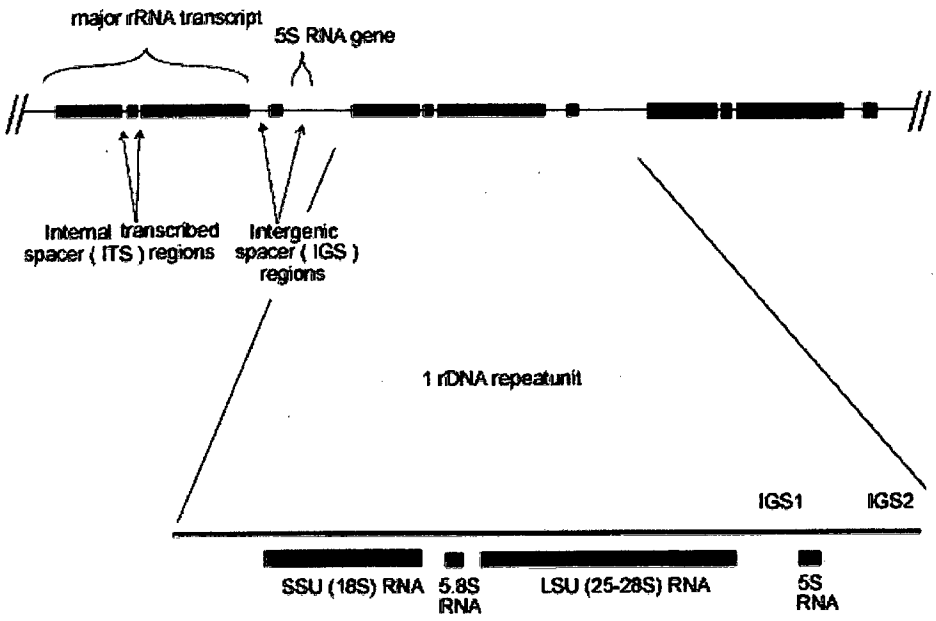


Fig 2.2 SSU rDNA region of eukaryotes displaying locations of 18S and ITS regions

Modified from: (http://www.biology.duke.edu/fungi/mycolab/primers.htm)

2.1.3 Fungal diversity from deep-sea habitats using culture-dependent approach

Recently some reports have described presence of fungi in deep-sea habitats using culture- dependent approaches. Several fungi isolated from various marine habitats using culture based technology, however, have also been found to be taxonomically closely related to species from genera that are well known from terrestrial environments, such as *Acremonium, Aspergillus,*

Penicillium and *Trichoderma* (Raghukumar et al, 1992; Holler et al, 2000; Wang et al, 2008; Jones et al, 2009). Diversity of culturable marine fungi was reported from deep-sea hydrothermal vents (Burgaud et al, 2009). Their physiological characterization revealed them to be more or less adapted to deep-sea conditions. Burgaud et al. (2010), isolated yeast species belonging to genera, *Rhodotorula, Rhodosporidium, Candida, Debaryomyces* and *Cryptococcus* from hydrothermal vent animals and identified them by analysis of 26 rRNA gene sequences. Fungi were isolated in the earlier reports from the deep-sea sediments of the Central Indian Basin from different sites with geographical locations of ~10-16°S and 73-77°E (Raghukumar et al, 2004; Damare et al, 2006). The sampling sites in the present study were also from the Central Indian Basin with almost the same geographic locations reported in previous studies (Table 2.1). Earlier studies have reported the identification of fungi only on the basis of classical taxonomic method, which may give biased results about actual fungal diversity in this region (Raghukumar et al, 2004; Damare et al, 2006). The present study was carried out in the same location of CIB where the culturable fungi were identified by molecular techniques.

Objectives

Isolation of fungi from deep-sea sediment samples of the Central Indian Basin using various techniques and their identification by amplification and sequencing of their 18S and ITS regions of SSU rDNA.

2.2 Materials and Methods

2.2.1 Collection of Sediment samples

Sediment samples were collected from an average depth of ~ 5000 m from different locations of the Central Indian Basin (Fig. 2.3). Twenty box core samples were collected during cruise # ABP26 in December 2006 and 2

during # ABP38 in September 2009 (10-16.5°S and 72-77°E) (Table 2.1 and 2.2) on board the Russian research vessel Akademic Boris Petrov (ABP). These sediment cores were collected with an USNEL-type box corer of 50 cm^3 size (Fig 2.1b). Sediments thus collected were mostly undisturbed and compact. Sub-cores of sediments were collected from a box corer using an alcohol-sterilized PVC cylinder (of 5 cm inner diameter. Subsections of 2 cm down to a depth of 10 cm and thereafter every 5 cm length down to 35-40 cm depth were cut from the above sediment and directly introduced into sterile plastic bags to avoid any aerial contamination (Figs. 2.1 d and e). The bags were closed and carried to the microbiology laboratory on board for further processing.

2.2.2 Isolation of fungi

An aliquot of sediment from the central part of the subsection was removed with an alcohol-sterilized spatula and placed in sterile vials for isolation (Raghukumar et al, 2004). In order to isolate maximum number of fungi, four different media were used during cruise # ABP26 and they were 1) Malt Extract Agar (MEA), 2) Czapek Dox Agar (CDA), 3) Corn Meal Agar (CMA) and 4) Malt Extract Broth (MEB) (Damare et al, 2006). The above media were used at 1/5 strength to simulate low nutrient conditions of deep-sea sediments. During cruise # ABP38, two more media i.e., Sabaraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) (Appendix 6.1a-f) were also used in addition to the above four media. They were prepared in seawater and supplemented with Penicillin (40,000 units in 100 ml medium) and streptomycin (0.1 g in 100 ml medium) to inhibit bacterial growth. Isolation of fungi from sediments was done by four different methods:

1) Particle plating method of Bills and Polishook (1994): Approximately 1 g of sediment was sieved through 2 meshes of 200 and 100 microns using sterile seawater. Particles which passed through 200 microns and remained on 100-micron mesh were spread plated on various media plates (1:5 times diluted).

2) Dilution plating: Approximately 0.1 g of sediment was suspended in 9 ml of sterile seawater and vortexed for 1 min. An aliquot of 100 µl was spread plated on various media plates.

3) Pressure Incubation: Approximately 0.1 g of sediment was suspended in 4 ml of Malt Extract Broth (1:5 times diluted and amended with antibiotics) and sealed in plastic bags. These bags were incubated in pressure vessels at a pressure of 30 MPa and at a temperature of 5°C for 30 days. After incubation bags were opened and 100 µl was spread plated on media plates.

4) Enrichment method. Approximately 0.1 g of sediment was directly suspended in 5 ml of Malt Extract Broth media (1:5 times diluted and amended with antibiotics) and incubated at 5°C for 30 days.

All the media plates were incubated at 5°C for 30 days. Air-borne fungi present in the ship laboratory and deck, contaminating the culture plates were monitored by exposing various media plates for 10 min on the deck of the research vessel where the cores were received, the microbiology laboratory on board the research vessel and the inoculation hood. This was repeated during every sampling station.

After isolation, fungal cultures were maintained on MEA slants at 5°C. The isolates were originally numbered as NIOCC (National Institute of Culture Collection). The sporulating fungi were deposited in a recognized microbial culture repository, Microbial Type Culture Collection (MTCC) at Chandigarh, India, under the accession number MTCC.

2.2.3 Isolation of DNA from the cultures

Fungi isolated by various techniques were identified by amplification and sequencing of their 18S and ITS region of SSU rDNA. DNA was isolated from all the filamentous fungi and yeast for the above purpose. The fungi were grown in MEB for 4-5 days for DNA isolation. Yeasts were grown in yeast extract peptone and dextrose (YPD) (Appendix 6.1g) medium and shaken at 170 rpm for 3-4 days. Mycelia and cells were harvested, lyophilized and crushed in a mortar and pestle to fine powder. Isolation of DNA was carried

out following the modified form of standard procedure (Stoeck and Epstein, 2003).

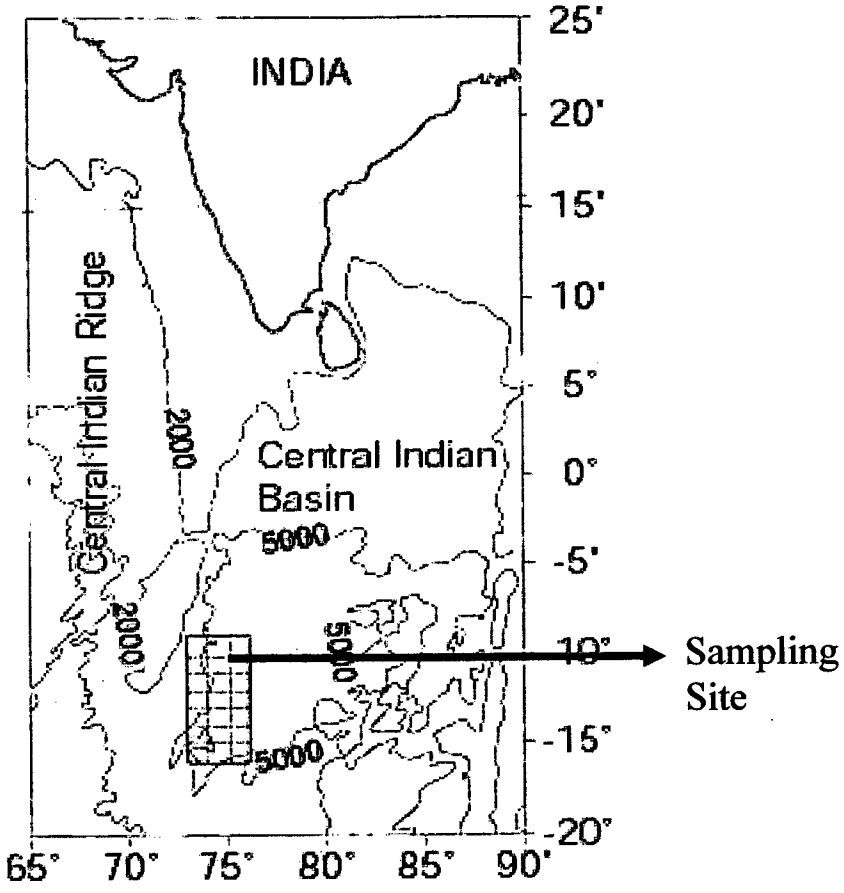


Fig. 2.3 Sampling sites (within inset) in the Central Indian Basin

The above samples were incubated at 65°C for two hours in a high salt extraction buffer containing 100 mM Tris-HCl buffer with 8.0 pH containing 100 mM Na_2EDTA, 100 mM $NaPO_4$, 1.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate, Proteinase K, 100 μg ml^{-1} for obtaining fungal DNA. A low salt extraction buffer containing 10 mM Tris-HCl with pH 8.0, 10 mM Na_2EDTA pH 8.0, 0.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate and Proteinase K, 100 μg ml^{-1} was used for isolating yeast DNA.

Table 2.1 Details of sediment collection sites from CIB during cruise # ABP26.

Sr. No.	Station No.	Date	Touch Positions		Water depth (m)
			Lat (°S)	Long (°E)	
1	SVBC-03	12.12.06	12 00.000	76 29.900	5373
2	SVBC-04	15.12.06	13 00.000	76 29.950	5377
3	SVBC-13	21.12.06	14 59.919	75 29.849	4894
4	SVBC-18	19.12.06	12 59.747	74 30.150	5122
5	SVBC-19	16.12.06	13 00.070	75 29.900	5104
6	SVBC-20	13.12.06	11 59.900	75 29.900	5223
7	SVBC-25	10.12.06	11 00.000	75 29.800	5303
8	SVBC-26	09.12.06	09 59.848	75 30.085	5339
9	SVBC-27	12.12.06	11 30.130	75 59.600	5274
10	SVBC-28	11.12.06	11 30.000	75 30.000	5263
11	SVBC-29	14.12.06	12 29.900	74 59.900	5174
12	SVBC-30	14.12.06	12 30.056	75 29.983	5189
13	SVBC-31	13.12.06	12 00.000	74 59.900	5153
14	SVBC-32	15.12.06	12 29.853	75 59.950	5309
15	SVBC-33	17.12.06	12 59.980	74 59.960	5265
16	SVBC-34	19.12.06	13 30.033	75 29.845	5219
17	SVBC-35	21.12.06	14 30.081	75 29.792	5211
18	SVBC-36	23.12.06	16 01.887	75 28.770	5042
19	SVBC-37	23.12.06	16 06.891	75 24.828	3992
20	SVBC-38	24.12.06	15 57.590	75 31.950	5642

Table 2.2 Details of sediment collection sites from CIB during cruise # ABP38.

Sr. No.	Station No.	Date	Touch Positions		Water depth (m)
			Lat (°S)	Long (°E)	
1	IVBC-18C	23.09.09	12 59.747	74 30.150	5122
2	IVBC-20A	30.09.09	11 59.900	75 29.900	5223

2.2.4 PCR amplification of 18S and ITS regions of rDNA

For amplification of 18S and ITS regions of the fungal SSU rDNA, specific primers were used. Partial region of 18S rDNA was PCR amplified by using primers, NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') of ~1100 bp. Full length of ITS region of ~600 bp was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al, 1990). The conditions for PCR included an initial hot start incubation (5 min at 94°C) followed by 34 cycles (denaturation at 94° C for 30 sec, annealing at 55° C for 30 sec and extension at 72° C for 1 min) and a final extension at 72°C for 5 min.

2.2.5 Sequencing and phylogenetic analyses

Fresh PCR products were purified by using gel extraction kit (Sigma, Genosys, USA) and sequenced at National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3730 DNA Analyzer, Applied Biosystems, USA). Sequence data were edited using Chromas Pro version 1.34. For tentative identification, fungal 18S and ITS rDNA sequences were compared with NCBI (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov)

database. Fungal rDNA -18S and ITS sequences in this study and the matched sequences from GenBank were edited and aligned using CLUSTAL-X, version 1.81 (Thompson et al, 1997). The aligned sequences were imported into DAMBE 4.5.47 (Xia and Xie, 2001). Neighbour-joining (NJ) trees were created using pairwise genetic distances using MEGA 3.1 (kumar et al, 2008). The quality of the branching patterns for NJ was assessed by bootstrap resampling of the data sets with 1,000 replications.

2.2.7 Statistical analyses

The statistical analyses were carried out in Microsoft Excel program and PRIMER 5 software for calculation of diversity indices.

2.3 Results

2.3.1 Physical parameters of the sampling site

The sediment samples collected for the present study were occasionally characterized by the presence of polymetallic nodules. The nodule abundance was found to be ~ 0.4 Kg m^{-2}. Moderate brown sediments dominated the core tops followed by yellow-brown clayish sediments. Intercalations and mottling were seen in most of the sediment cores. Majority of the sediment cores were siliceous in texture. One core (BC-37) was fully calcareous in composition. The approximate salinity, temperature and pH were found to be 35 PSU (practical salinity units), 3°C and 7.0 respectively. Among other parameters, the total proteins, carbohydrates and lipids were approximately 1.5, 1.0 and 1.0 mg g^{-1} sediment respectively. Total bacterial counts in this area ranged from 10^7-10^9 cells g^{-1} dry sediment. Organic carbon ranged from 0.3-0.4 mg g^{-1} dry sediment (Sharma, 2008, 2010).

2.3.2 Isolation of cultures using different techniques

A total of 28 and 19 cultures were isolated during cruises #ABP26 and # ABP38 respectively with majority being the filamentous fungi. Pressure Incubation method for isolation yielded recovery of maximum number of culturable forms (Tables 2.3 and 2.4). The percentage frequency of occurrence of fungi obtained by particle plating and pressure incubation techniques was higher during cruise #ABP38 than the cruise #ABP26. Only these two methods yielded recovery of fungal cultures during cruise #ABP38. In contrast fungi could be obtained by all the four isolation techniques during the cruise #ABP26 (Tables 2.3 and 2.4).

The number of fungal isolates recovered during cruise #ABP26 was maximum with dilution plating technique whereas pressure incubation was found to be the best during #ABP38 (Tables 2.5 and 2.6). Cultures could be isolated from all the depths of the sediment cores including 35-40 cm depth (Table 2.6). Best media for isolation was found to be MEA during cruise #ABP26 whereas both CDA and PDA were found to be good during #ABP38 (Tables 2.5 and 2.6).

Table 2.3 Details of fungi isolated during cruise #ABP26

	Particle plating	Dilution plating	Pressure incubation at 30 MPa/5°C	Direct incubation in MEB at 5°C
Total no. of sediment samples used	116	188	39	153
No. of fungi isolated by using all the four media	7	12	6	3
% frequency of occurrence	6	6	15	2

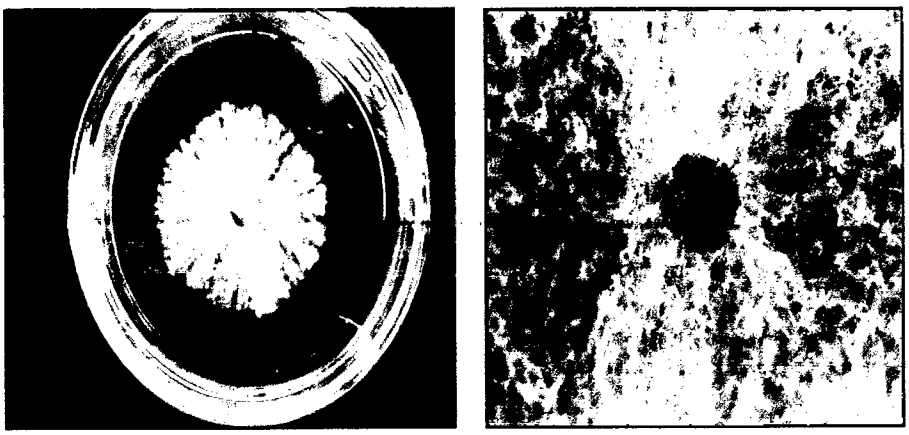
Table 2.4 Details of fungi isolated during cruise #ABP38

	Particle plating	Dilution plating	Pressure incubation at 30 MPa/5°C	Direct incubation in MEB at 5°C
Total no. of sediment samples used	19	19	19	19
No. of fungi isolated by using all the six media	6	0	13	0
% frequency of occurrence	31.6	0	68.4	0

2.3.3 Diversity of fungi during cruise #ABP26

A total of 28 cultures were isolated during cruise #ABP26 including 16 filamentous fungi and 12 yeast isolates. The cultures were identified by classical taxonomic method as well as by amplification and sequencing of their 18S and ITS regions of SSU rDNA (Table 2.7). Only sporulating forms could be identified by classical taxonomic method. The identification of sporulating cultures carried out by classical morphological taxonomy matched with molecular identification based on either 18S or ITS sequences and only seldom with both these sequences (Table 2.7). The 18S and ITS sequences of all the cultures were deposited in GenBank and accession numbers obtained (Table 2.7). Most of the fungi belonged to the phylum Ascomycota and based on 18S sequences clustered into 7 subgroups, namely *Aspergillus* sp., *Sagenomella* sp. (Fig 2.4), *Exophiala* sp. (Fig 2.5), *Capronia* sp., *Cladosporium* sp., *Acremonium* sp. and *Tritirachium* sp. (Fig. 2.6).

Based on ITS sequences they grouped into 6 clusters showing maximum similarity to *Aspergillus* sp., uncultured member of Hypocreaceae family, *Exophiala* sp., an uncultured soil fungus clone DQ682584.1, *Hypocreales* and *Trichothecium* sp. (Fig. 2.7). The two basidiomycetes



A. Culture grown on MEA plate **B. Mycelia observed under Stereo-microscope**

Fig 2.4 *Sagenomella* sp. (NIOCC#F15, #F23, #F65)

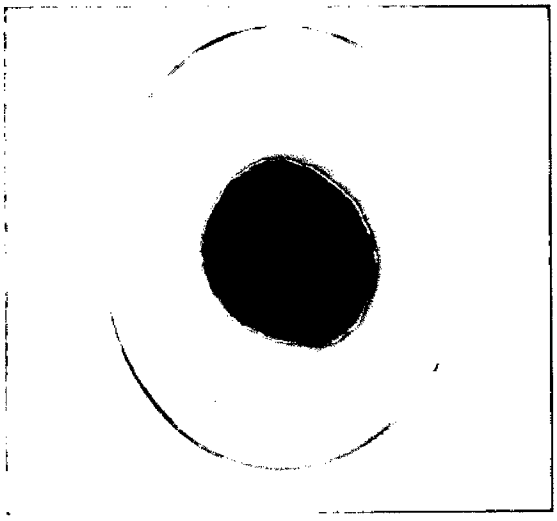


Fig 2.5 *Exophiala* sp. (NIOCC#F16)
(Culture grown on MEA plate)

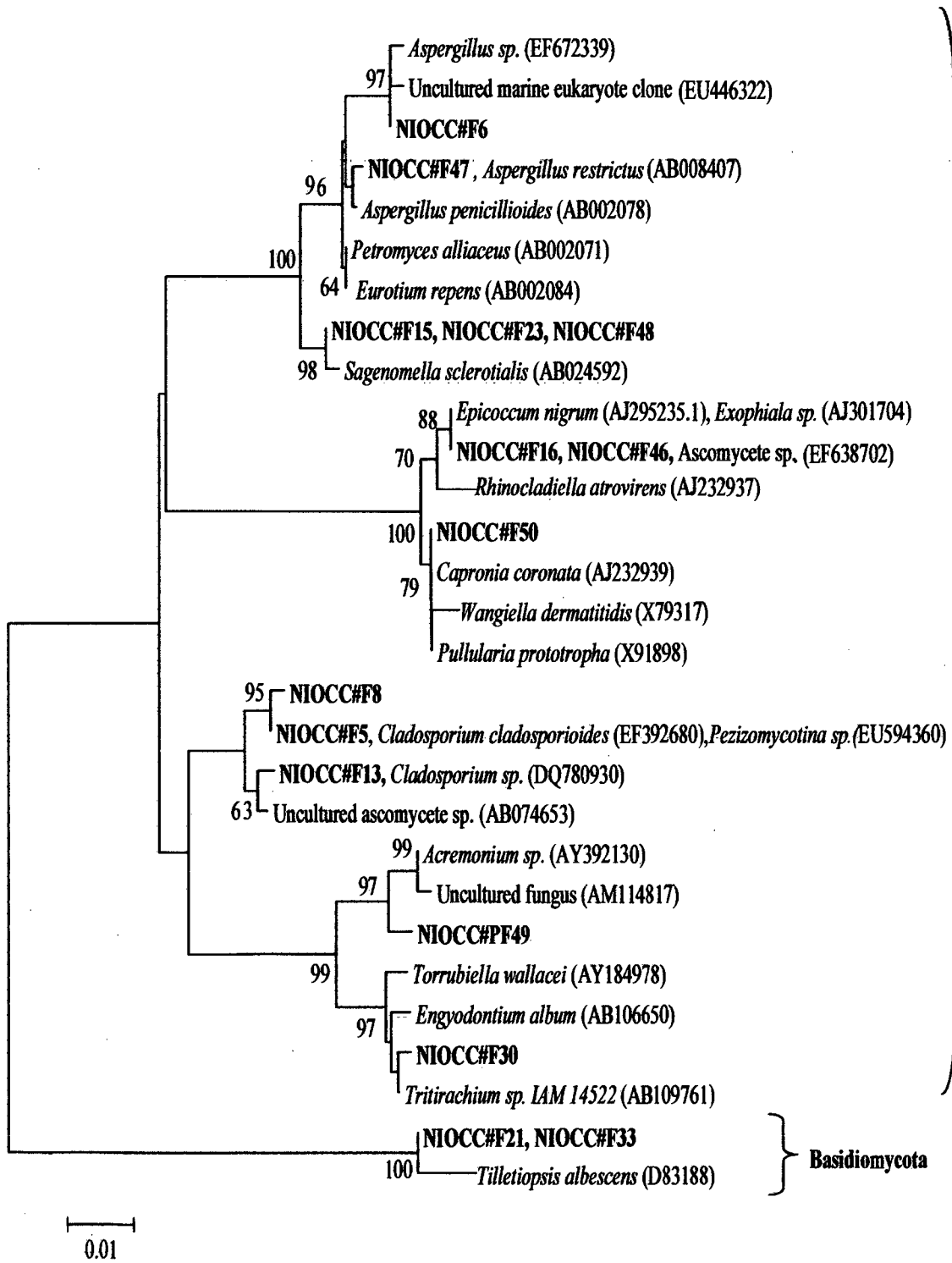


Fig 2.6 Neighbor-joining phylogenetic tree for mycelial fungi isolated during cruise #ABP26 based on rDNA-18S sequences (~1100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

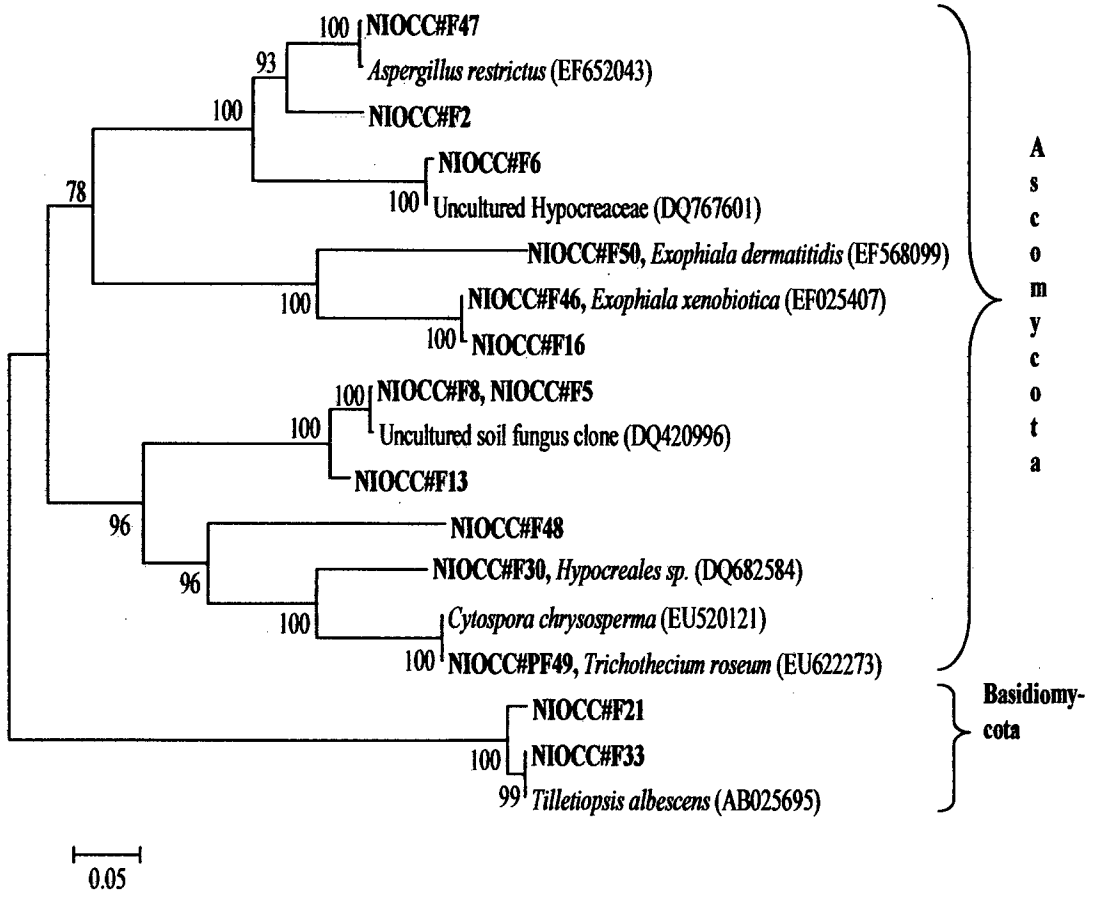


Fig 2.7 Neighbor-joining phylogenetic tree for mycelial fungi isolated during cruise #ABP26 based on rDNA-ITS sequences (~600 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

Table 2.5: Distribution of fungi in various depths and effect of different methods and media on isolation during cruise #ABP26

Isolate	Core	Section of the core (cm)	Method of isolation*	Medium used**
NIOCC#F2	BC-33	8-10	DP	CMA
NIOCC#F5	BC-25	0-2	PI	MEA
NIOCC#F6	BC-25	4-6	PI	CMA
NIOCC#F8	BC-20	15-20	PP	MEA
NIOCC#F13	BC-19	10-15	PI	MEA
NIOCC#F15	BC-26	4-6	PP	MEA
NIOCC#F16	BC-29	6-8	PP	CDA
NIOCC#F21	BC-26	25-30	PP	MEA
NIOCC#F23	BC-26	0-2	DP	MEA
NIOCC#F30	BC-37	0-2	PI	CDA
NIOCC#F33	BC-36	30-35	PI	MEA
NIOCC#F46	BC-29	6-8	DP	CMA
NIOCC#F47	BC-38	20-25	DP	CDA
NIOCC#F48	BC-26	25-30	PP	MEA
NIOCC#F50	BC-25	20-25	DP	MEA
NIOCC#PF49	BC-29	4-6	DI	MEB
NIOCC#Y1	BC-29	2-4	DP	MEA
NIOCC#Y2	BC-18	30-35	DP	CDA
NIOCC#Y3	BC-29	10-15	DP	CDA
NIOCC#Y4	BC-37	4-6	PP	MEA
NIOCC#Y5	BC-04	20-25	DP	MEA
NIOCC#Y6	BC-19	20-25	PP	MEA
NIOCC#Y7	BC-31	2-4	DP	MEA
NIOCC#Y8	BC-29	4-6	DP	CMA
NIOCC#Y9	BC-29	0-2	DP	MEA
NIOCC#Y10	BC-36	8-10	PI	MEA
NIOCC#PY12	BC-29	4-6	DI	MEB
NIOCC#PY13	BC-29	8-10	DI	MEB

*PI: Pressure Incubation, PP: Particle Plating, DP: Dilution Plating, DI: Direct Incubation,

**MEB: Malt Extract Broth, MEA: Malt Extract Agar, CDA: Czapek Dox Agar, CMA: Corn Meal Agar

(NIOCC#F21 and #F33) belonged to *Tilletiopsis* sp. as identified by 18S as well as ITS sequencing (Figs. 2.6, 2.7 and Table 2.7). Species of filamentous fungi *Sagenomella*, *Exophiala*, *Capronia*, and *Tilletiopsis* are being reported for the first time from the deep-sea sediments. The majority of the yeast isolates belonged to the phylum Basidiomycota (Figs. 2.8 and 2.9) and only

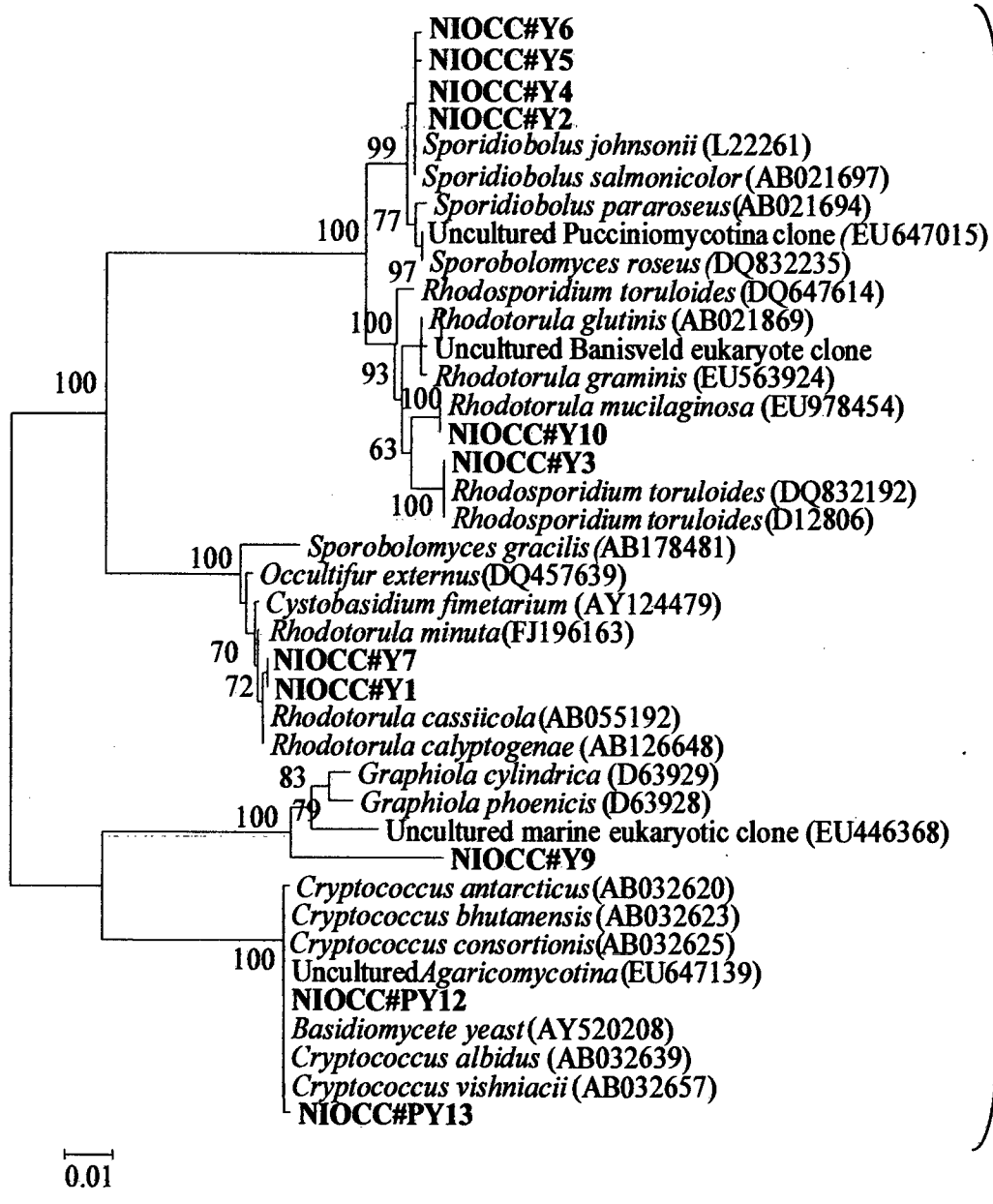


Fig 2.8 Neighbor-joining phylogenetic tree for yeasts isolated during cruise #ABP26 based on rDNA-18S sequences (~1100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

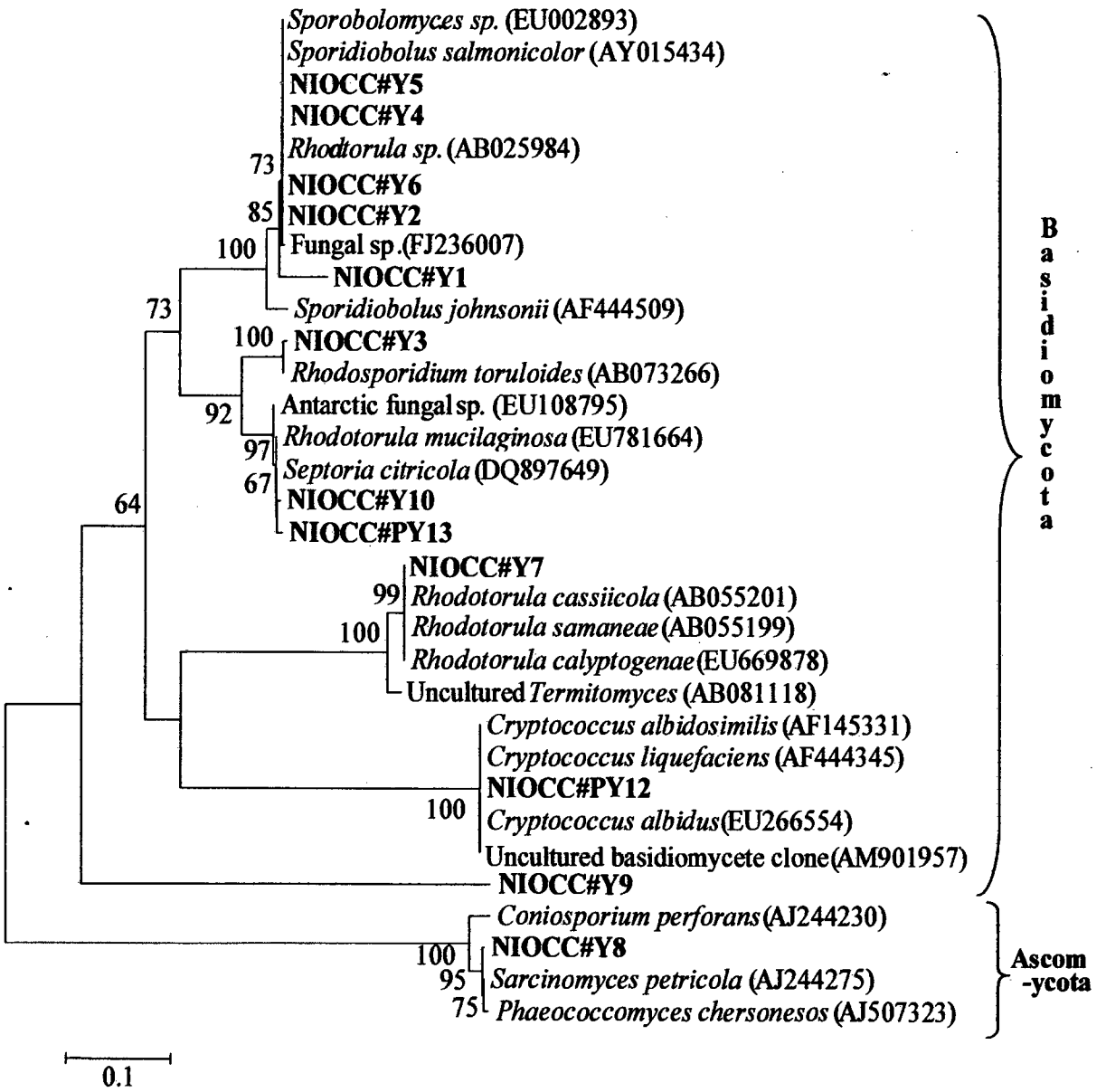


Fig 2.9 Neighbor-joining phylogenetic tree for yeasts isolated during cruise #ABP26 based on rDNA-ITS sequences (~600 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

one isolate, NIOCC # Y8 belonged to Ascomycota identified both by 18S and ITS sequencing (Fig. 2.9 and Table 2.7). Sequences of 18S as well as ITS gave matching identification of yeasts (Table 2.7). The basidiomycetous yeast belonged to the genera *Sporobolomyces* sp., *Sporidiobolus*, *Rhodosporidium*, *Rhodotorula* and *Cryptococcus* sp. A 100% consensus in identification was seen between 18S and ITS sequences of the isolates NIOCC#Y2, Y3, Y6, Y7 and PY12 up to generic level (Table 2.7).

Table 2.6: Distribution of fungi in various depths and effect of different methods and media on isolation during cruise #ABP38

Isolate	Core	Section of the core (cm)	Method of isolation*	Medium used**
NIOCC#F51	BC-18C	8-10	PI	MEA
NIOCC#F52	BC-18C	10-15	PI	CDA
NIOCC#F53	BC-18C	0-2	PI	MEA
NIOCC#F54	BC-18C	8-10	PI	PDA
NIOCC#F55	BC-18C	4-6	PI	CDA
NIOCC#F56	BC-18C	10-15	PI	MEA
NIOCC#F57	BC-18C	20-25	PI	PDA
NIOCC#F58	BC-18C	2-4	PI	PDA
NIOCC#F59	BC-18C	0-2	PI	MEA
NIOCC#F60	BC-18C	30-35	PI	PDA
NIOCC#F61	BC-18C	20-25	PI	SDA
NIOCC#F62	BC-20A	20-25	PP	CMA
NIOCC#F63	BC-18C	35-40	PP	PDA
NIOCC#F64	BC-18C	4-6	PP	CDA
NIOCC#F65	BC-18C	6-8	PP	MEA
NIOCC#F66	BC-20A	4-6	PI	MEA
NIOCC#F67	BC-20A	15-20	PI	SDA
NIOCC#F68	BC-20A	15-20	PP	CMA
NIOCC#Y14	BC-20A	6-8	PP	PDA

*PI: Pressure Incubation, PP: Particle Plating,
**MEA: Malt Extract Agar, CDA: Czapek Dox Agar, SDA: Sabourauds Dextrose Agar, PDA: Potato Dextrose Agar, CMA: Corn Meal Agar

2.3.4 Diversity of fungi during cruise #ABP38

Nineteen fungi were isolated during cruise #ABP38 resulting in a total of 12 distinct fungal genera (Table 2.8).

Table 2.7 Identification of fungi by morphological and molecular approaches and their accession numbers isolated during the cruise #ABP26

Fungal Isolate			Identification		
Designation	Accession number		Microscopic	18S (Closest identified relative)	ITS (Closest identified relative)
	18S	ITS			
NIOCC#F2	FJ357792	EU729705	*Penicillium* sp.	*Penicillium* sp.	*Penicillium citreonigrum*
NIOCC#F5	EU723484	EU729711	*Cladosporium* sp.	Pezizomycotina sp.	*Cladosporium cladosporioides*
NIOCC#F6 (**MTCC9336**)	EU723485	EU729718	*Aspergillus* sp.	Uncultured marine eukaryote clone U112H09	*Aspergillus* sp.
NIOCC#F8	EU723486	EU729712	*Cladosporium* sp.	Pezizomycotina sp.	*Cladosporium cladosporioides*
NIOCC#F13 (**MTCC9333**)	EU723487	EU729719	*Cladosporium* sp.	*Cladosporium* sp.	*Cladosporium* sp.
NIOCC#F15	EU723488	FJ357782	*Sagenomella* sp.	*Sagenomella sclerotialis* sp.	Uncultured fungus clone
NIOCC#F16 (**MTCC9332**)	EU723489	FJ357783	*Rhinocladiella* sp.	Ascomycete MV_26C	*Exophiala spinifera*
NIOCC#F21 (**MTCC9339**)	EU723490	FJ357784	Sporulating, unidentified sp.	*Tilletiopsis albescens*	*Tilletiopsis albescens*
NIOCC#F23	EU723491	FJ357785	*Sagenomella* sp.	*Sagenomella sclerotialis* sp.	Uncultured fungus clone
NIOCC#F30 (**MTCC9337**)	EU723492	EU729707	*Acremonium* sp.	Ascomycete MV_25C	Hypocreales sp.
NIOCC#F33	EU723493	EU729706	Sporulating, unidentified sp.	*Tilletiopsis albescens*	*Tilletiopsis albescens*
NIOCC#F46 (**MTCC9334**)	EU723494	EU729708	*Exophiala* sp.	Ascomycete MV_26C	*Exophiala xenobiotica*
NIOCC#F47	EU723495	EU729709	*Aspergillus* sp.	*Aspergillus restrictus*	*Aspergillus caesiellus*
NIOCC#F48 (**MTCC9341**)	EU723496	FJ357786	*Sagenomella* sp.	*Sagenomella sclerotialis*	*Phialemonium dimorphosporum*
NIOCC#F49 (**MTCC9340**)	EU723497	EU729713	*Acremonium* sp.	*Acremonium* sp.	*Trichothecium roseum*
NIOCC#F50	EU723498	EU729710	Non -sporulating sp.	*Capronia coronata*	*Exophiala dermatitidis*

Table 2.7 contd.

Fungal isolate			Identification		
Designation	Accession number		Microscopic	18S (Closest identified relative)	ITS (Closest identified relative)
	18S	ITS			
NIOCC#Y1	EU723499	FJ357787	Yeast sp.	*Rhodotorula cassiicola*	*Sporobolomyces* sp.
NIOCC#Y2	EU723500	FJ357788	Yeast sp.	*Sporidiobolus johnsonii*	*Sporidiobolus salmonicolor*
NIOCC#Y3	EU723501	EU729715	Yeast sp.	*Rhodosporidium toruloides*	*Rhodosporidium toruloides*
NIOCC#Y4	EU723502	EU729716	Yeast sp.	*Sporidiobolus johnsonii*	*Sporobolomyces* sp.
NIOCC#Y5	EU723503	FJ357793	Yeast sp.	*Sporidiobolus johnsonii*	*Rhodotorula* sp.
NIOCC#Y6	EU723504	EU72972	Yeast sp.	*Sporidiobolus johnsonii*	*Sporidiobolus* sp.
NIOCC#Y7	EU723505	FJ357789	Yeast sp.	*Rhodotorula cassiicola*	*Rhodotorula calyptogenae*
NIOCC#Y8	EU723506	EU729717	Black Yeast sp.	*Coniosporium perforans*	*Sarcinomyces petricola*
NIOCC#Y9	EU723507	FJ357790	Filamentous Yeast sp.	*Graphiola cylindrica*	*Tilletiopsis oryzicola*
NIOCC#Y10	EU723508	EU729721	Yeast sp.	*Rhodotorula mucilaginosa*	*Cryptococcus albidosimilis*
NIOCC#PY12	EU723509	FJ357791	Yeast sp.	*Cryptococcus vishniacii*	*Cryptococcus albidosimilis*
NIOCC#PY13	EU723510	EU729714	Yeast sp.	*Cryptococcus vishniacii*	*Rhodotorula mucilaginosa*

MTCC-Microbial Type Culture Collection, Chandigarh, NIOCC= National Institute of Oceanography Culture Collection

Table 2.8 Phylogenetic affiliations of fungal cultures isolated during cruise #ABP38 on the basis of 18S sequences

Isolate ID	Closest Identified relative (Accession number)	Source of Isolation of the closest relative	% identity
NIOCC#F51	*Nigrospora oryzae*, Ascomycota (AB220233)	Not known	99
NIOCC#F52	*Cladosporium* sp., Ascomycota (GU322367)	Soil	99
NIOCC#F53	*Trametes versicolor*, Basidiomycota (AY336751)	Not known	99
NIOCC#F54	*Chaetomium elatum*, Ascomycota (M83257)	Not known	99
NIOCC#F55	***Aspergillus versicolor*, Ascomycota (AB002064)**	**Not known**	**95**
NIOCC#F56	*Ascotricha lusitanica*, Ascomycota (AB048282)	Leaf	99
NIOCC#F57	*Pleospora herbarum*, Ascomycota (GU238232)	Not known	98
NIOCC#F58	*Cladosporium* sp., Ascomycota (GU322367)	Soil	99
NIOCC#F59	*Eurotium herbariorum*, Ascomycota (AF548072)	Air	99
NIOCC#F60	*Cerrena sp.* NIOCC#2a, Basidiomycota (FJ010210)	Mangrove wood	99
NIOCC#F61	*Cerrena sp.* NIOCC#2a, Basidiomycota (FJ010210)	Mangrove wood	99
NIOCC#F62	*Penicillium griseofulvum*, Ascomycota (FJ717697)	Atmosphere of porto	100
NIOCC#F63	*Penicillium griseofulvum*, Ascomycota (FJ717697)	Atmosphere of porto	99
NIOCC#F64	*Aspergillus versicolor*, Ascomycota (GU227343)	Not known	99
NIOCC#F65	*Sagenomella sp.* , Ascomycota (EU140822)	Not known	99
NIOCC#F66	*Cerrena sp.* NIOCC#2a, Basidiomycota (FJ010210)	Mangrove wood	99
NIOCC#F67	*Cerrena sp.* NIOCC#2a, Basidiomycota (FJ010210)	Mangrove wood	99
NIOCC#F68	*Hortaea werneckii*, Ascomycota (GU296153)	Culture collection	99
NIOCC#Y14	***Hortaea werneckii*, Ascomycota (GU296153)**	**Culture collection**	**97**

NIOCC= National Institute of Oceanography Culture Collection

The identification of these isolates was done on the basis of 18S rDNA region amplification and sequencing. Majority of the forms belonged to Ascomycota. Among these ascomycetous forms, no single species was found to be dominating. It included members from *Aspergillus* sp., *Eurotium* sp., *Cladosporium* sp., *Pleospora* sp., *Chaetomium* sp., *Ascotricha* sp., *Penicillium* sp., *Sagenomella* sp. among filamentous fungi (Fig. 2.10). Two ascomycetous culture sequences were found to cluster with black yeast i.e. *Hortaea* sp. Most of these fungal sequences showed >98% similarity with the sequences of their closest relative species. However two ascomycetous fungal genotypes i.e. NIOCC#F55 and #Y14 showed only 95 and 97 % homology with the existing sequences of the database.

Among basidiomycota, two fungal genera belonging to *Cerrena* and *Trametes* sp. were identified (Table 2.8). Out of 5 basidiomycetous fungi, four showed similarity with *Cerrena* sp. with a percentage similarity of 99. The number of cultures isolated during this cruise was comparatively higher than the previous cruise in spite of less number of samples and also belonged to more diverse fungal species. The isolation of *Cerrena* and *Tremetes* sp. is being reported for the first time from the deep-sea sediments of the Central Indian Basin. Some of the fungi such as *Aspergillus, Cladosporium, Penicillium* and *Sagenomella* sp. were isolated during both the cruises. Whereas other fungi such as *Nigrospora, Trametes, Chaetomium, Ascotricha, Pleospora, Eurotium, Cerrena* and *Hortaea* sp. were isolated exclusively during the cruise #ABP38.

2.3.5 Species distribution and richness at different depths

Fungal species distribution varied among different depths of the sediments cores. The Shannon-Wiener diversity indices values were higher during cruise #ABP26 than the cruise #ABP38 (Tables 2.9 and 2.10). The values were found to be different for different depths, highest being 1.8 for depth 4-6 cm in cruise #ABP26 (Table 2.9). During cruise #ABP38 the highest Shannon-Wiener diversity indices value was 1.0 for the depth 20-25 cm (Table 2.10).

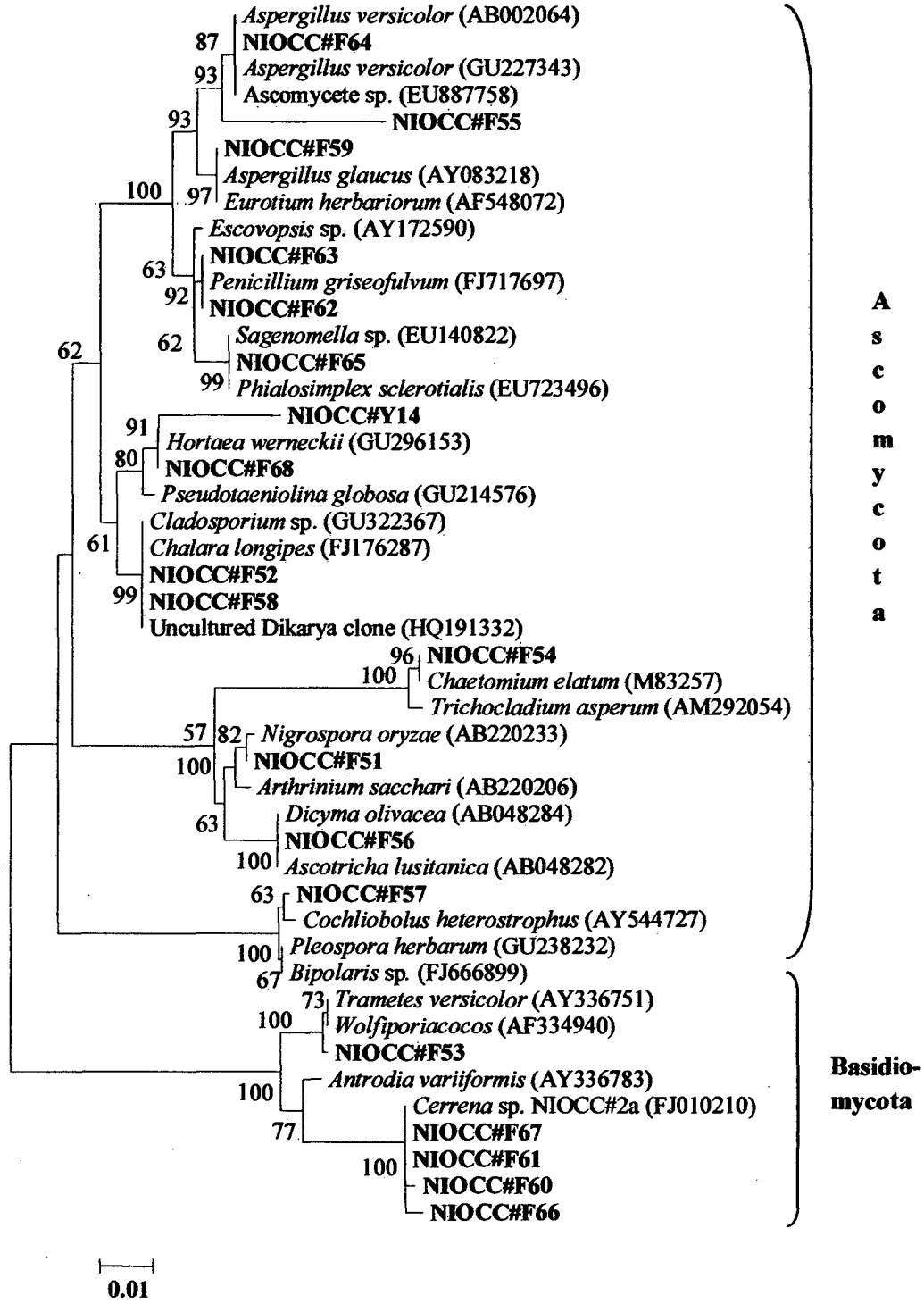


Fig 2.10 Neighbor-joining phylogenetic tree for mycelial fungi isolated during cruise #ABP38 based on rDNA-18S sequences (~1100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates (values below 50% not shown).

Table 2.9 Species distribution at different depths in the sediment core during the cruise #ABP26

Genera (Represented with closest identified relative)	Depths (cm)										
	0-2	2-4	4-6	6-8	8-10	10-15	15-20	20-25	25-30	30-35	35-40
Total species isolate	4	1	6	1	3	2	1	3	2	2	0
Total sediment sections used	20	20	20	20	20	20	20	20	17	8	4
Penicillium sp. (NIOCC#F2)	-	-	-	-	1	-	-	-	-	-	-
Cladosporium sp. (NIOCC#F5, F8, F13)	1	-	-	-	-	1	1	-	-	-	-
Aspergillus sp. (NIOCC#F6, F47)	-	-	1	-	-	-	-	1	-	-	-
Sagenomella sp. (NIOCC#F15, F23, F48)	1	-	1	-	-	-	-	-	1	-	-
Ascomycete sp. (NIOCC#F16, F30, F46)	1	-	-	2	-	-	-	-	-	-	-
Tilletiopsis sp. (NIOCC#F21, F33)	-	-	-	-	-	-	-	-	1	1	-
Capronia coronata (NIOCC#F50)	-	-	-	-	-	-	-	1	-	-	-
Acremonium sp. (NIOCC#PF49)	-	-	1	-	-	-	-	-	-	-	-
Sporidiobolus johnsonii (NIOCC#Y2, Y4, Y5, Y6)	-	-	1	-	-	-	-	2	-	1	-
Rhodosporidium toruloides (NIOCC#Y3)	-	-	-	-	-	1	-	-	-	-	-
Rhodotorula sp.(NIOCC#Y1, Y7, Y10)	-	2	-	-	1	-	-	-	-	-	-
Coniosporium perforans (NIOCC#Y8)	-	-	1	-	-	-	-	-	-	-	-
Graphiola cylindrica (NIOCC#Y9)	1	-	-	-	-	-	-	-	-	-	-
Cryptococcus sp. (NIOCC#PY12, PY13)	-	-	1	-	1	-	-	-	-	-	-
Shannon index (log e)	1.4	0	1.8	0	1.1	0.7	0	1	0.7	0.7	0
Pielou's eveness	1	-	1	-	1	1	-	0.9	1	1	-
Species richness	2.2	0	2.8	0	1.8	1.4	-	1.4	1.4	1.4	-

Table 2.10 Species distribution at different depths in the sediment core during the cruise #ABP38

Genera (Represented with closest identified relative)	Depth (cm)										
	0-2	**2-4**	**4-6**	**6-8**	**8-10**	**10-15**	**15-20**	**20-25**	**25-30**	**30-35**	**35-40**
Total species isolate	2	1	3	2	2	2	2	3	0	1	1
Total sediment sections used	2	2	2	2	2	2	2	2	1	1	1
Nigrospora sp. (NIOCC#F51)	-	-	-	-	1	-	-	-	-	-	-
Cladosporium sp. (NIOCC#F52, F58)	-	1	-	-	-	1	-	-	-	-	-
Trametes sp. (NIOCC#F53)	1	-	-	-	-	-	-	-	-	-	-
Chaetomium sp. (NIOCC#F54)	-	-	-	-	1	-	-	-	-	-	-
Aspergillus sp. (NIOCC#F55, F64)	-	-	2	-	-	-	-	-	-	-	-
Ascotricha sp. (NIOCC#F56)	-	-	-	-	-	1	-	-	-	-	-
Pleospora sp. (NIOCC#F57)	-	-	-	-	-	-	-	1	-	-	-
Eurotium sp. (NIOCC#F59)	1	-	-	-	-	-	-	-	-	-	-
Cerrena sp. (NIOCC#F60, F61, F66, F67)	-	-	1	-	-	-	1	1	-	1	-
Penicillium sp. (NIOCC#F62, F63)	-	-	-	-	-	-	-	1	-	-	1
Sagenomella sp. (NIOCC#F65)	-	-	-	1	-	-	-	-	-	-	-
Hortaea sp. (NIOCC#F68, Y14)	-	-	-	1	-	-	1	-	-	-	-
Shannon index (log e)	0.7	0	0.6	0.7	0.7	0.7	0.7	1	0	0	0
Pielou's eveness	1	-	0.9	1	1	1	1	1	-	-	-
Species richness	1.4	-	0.9	1.4	1.4	1.4	1.4	1.8	-	-	-

Species richness was also higher during cruise #ABP26 with a maximum value of 2.8 at 4-6 cm depth than the cruise #ABP38, with a maximum value of 1.8 at 20-25 cm depth (Tables 2.9 and 2.10).

2.4 Discussion

2.4.1 Distribution of fungi in deep-sea sediments

The fungi isolated mostly belonged to Ascomycota and Basidiomycota. Ascomycetes dominated among the filamentous fungi whereas basidiomycetes were dominant among the yeast (unicellular fungi). Bass et al. (2007) observed that yeast sequences dominated in deep-sea sediments of the Pacific Ocean at 1,500–4,000 m depth. On the other hand, in the present study as well as that of Damare et al. (2006) filamentous fungi were the dominant culturable fungi. It is possible that deep-sea yeasts may require more specialized media for culturing. The ITS and 18S sequences gave matching results for identification for only some of the non-sporulating and sporulating mycelia cultures (Table 2.7). On the other hand, there was a 100% consensus between these two techniques while identifying most of the yeast cultures (Table 2.7).

These results indicate that identification up to species level should be based on more than two techniques. Using a few other fungal specific primers as reported by Pang and Mitchell (2005), this problem could be resolved. The presence of fast growing sporulating forms such as *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Curvularia* sp., *Acremonium* sp., and *Fusarium* sp., have been reported as culturable forms (Roth et al, 1964; Raghukumar et al, 1992; Damare et al, 2006) and their molecular sequences have been detected in the oceanic environment (Bass et al, 2007). We are reporting for the first time the presence of *Sagenomella* sp., *Capronia coronata*, *Exophiala* sp., *Tilletiopsis* sp., and *Cerrena* sp. from deep-sea sediments. Several of the cultured fungi in our studies showed phylogenetic similarity to fungi reported in marine environment. A *Capronia*-like fungus (order *Chaetothyriales*), isolated from deep-sea hydrothermal vent elicited a host immune response in mussels and was associated with tissue deterioration

(Burgaud et al, 2009). The 18S sequences of the filamentous fungi NIOCC#F5 and #F8 showed 99-100% homology to Pezizomycotina isolate EU594360, originally reported to have been isolated from a marine sponge *Haliclona simulans* (Table 2.11). The 18S sequences of the isolate NIOCC#F16 and #F30 showed homology to the isolates obtained from deep hydrothermal ecosystem of Mid-Atlantic Ridge and the isolate NIOCC#F13 to *Cladosporium* sp., reported from hypersaline environment (Table 2.11). The ITS sequences of the isolates NIOCC#F5, #F15, #F16, #Y5, and #Y7 showed homology to the cultures either from deep-sea or marine environment (Table 2.12).

2.4.2 Diversity of fungi during cruise #ABP26

The low recovery of culturable fungi from deep-sea sediments was the most striking feature of the sampling carried out during the cruise #ABP26. A total of 28 fungi, inclusive of filamentous and unicellular yeasts were recovered from 496 sediment samples (5.6% frequency) by methods such as particle plating, dilution plating, pressure incubation and low temperature incubation and four different culture media. At the same geographical positions i.e. 10–15°S and 73-76°E of the Indian Ocean Damare et al. (2006) isolated.a total of 181 fungi from 672 sediment samples collected during three cruises from 2001–2003 (9% frequency). Almost similar methods and culture media for isolation of fungi were used by Damare et al. (2006). Such a difference in recovery of fungi may indicate large temporal variation or possibility of extremely patchy distribution of fungi in deep-sea sediments with pockets of high fungal population. To isolate more diverse forms of culturable fungi from deep-sea sediments, optimization of media and nutrient conditions may be applied in future. Turner (1973) reported sinking waterlogged wood originating from offshore during monsoon runoffs creating "islands of wood" in the deep sea. Such persistent but shifting islands of wood might bring in saprophytic species, serve as dispersal centres and contribute to habitat diversity, niche specialization and enrichment. Similar route for several mussels in the deep-sea vents has been hypothesized (Distel et al, 2000).

Patchy distribution of fungi seen in the Central Indian Basin appears to be similar to the scenario of high bacterial population associated with "whale fall" in deep sea (Smith and Baco, 2003). The sinking particulate organic matter from the dynamic surface waters might also be contributing to this vast temporal difference in the almost static deep-sea sediments.

Table 2.11 Phylogenetic affiliations of fungi and yeast isolated during cruise #ABP26 on the basis of 18S sequences

Isolate ID	Closest identified relative, phylum (GenBank Accession no:)	% identity	Source of isolation of Closest relative
(NIOCC#F2)	*Penicillium phialosporum*, Ascomycota (AF245266)	84	Not known
(NIOCC#F5)	Pezizomycotina , Ascomycota (EU594360)	100	*Haliclona simulans* (a marine sponge)
(NIOCC#F6)	Uncultured marine eukaryote clone UI12H09, Ascomycota (EU446322)	99	"Anoxic deep hypersaline L'Atalante Basin, eastern Mediterranean Sea"
(NIOCC#F8)	Pezizomycotina sp., Ascomycota (EU594360)	99	*Haliclona simulans* (a marine sponge)
(NIOCC#F13)	*Cladosporium* sp. EXF228, Ascomycota (DQ780930)	99	Hypersaline environments
(NIOCC#F15)	*Sagenomella sclerotialis*, Ascomycota (AB024592)	99	Not known
(NIOCC#F16)	Ascomycete sp. MV_26C, Ascomycota (EF638702)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
(NIOCC#F21)	*Tilletiopsis albescens*, Basidiomycota (D83188)	99	Not known
(NIOCC#F23)	*Sagenomella sclerotialis*, Ascomycota (AB024592)	99	Not known
(NIOCC#F30)	Ascomycete sp. MV_25C, Ascomycota (EF638701)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
(NIOCC#F33)	*Tilletiopsis albescens*, Basidiomycota (D83188)	100	Not known

Table 2.11 contd.

Isolate ID	Closest identified relative, phylum (GenBank Accession no.)	% identity	Source of isolation of Closest relative
(NIOCC#F46)	Ascomycete sp. MV_26C, Ascomycota (EF638702)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
(NIOCC#F47)	*Aspergillus restrictus*, Ascomycota (AB008407)	100	Culture collection
(NIOCC#F48)	*Sagenomella sclerotialis*, Ascomycota (AB024592)	99	Culture collection
(NIOCC#F50)	*Capronia coronata*, Ascomycota (AJ232939)	99	Not known
(NIOCC#PF49)	*Acremonium* sp., Ascomycota (AY392130)	99	Onychomyces
(NIOCC#Y1)	*Rhodotorula cassiicola*, Basidiomycota (AB055191)	99	Thailand
(NIOCC#Y2)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Dead pustule of *Phragmidium rubi-idaei*, on leaf of Rubus
(NIOCC#Y3)	*Rhodosporidium toruloides* , Basidiomycota (DQ832192)	100	Wood pulp from Coniferae, Sweden
(NIOCC#Y4)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Dead pustule of *Phragmidium rubi-idaei*, on leaf of *Rubus*
(NIOCC#Y5)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Dead pustule of *Phragmidium rubi-idaei*, on leaf of *Rubus*
(NIOCC#Y6)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Dead pustule of *Phragmidium rubi-idaei*, on leaf of *Rubus*
(NIOCC#Y7)	*Rhodotorula cassiicola*, Basidiomycota (AB055191)	99	Thailand
(NIOCC#Y8)	*Coniosporium perforans*, Ascomycota (EF137365)	100	Rocks
(NIOCC#Y9)	*Graphiola cylindrica*, Basidiomycota (D63929)	95	Not known
(NIOCC#Y10)	*Rhodotorula mucilaginosa*, Basidiomycota (EF218987)	100	Not known
(NIOCC#PY12)	*Cryptococcus vishniacii*, Basidiomycota (AB032657)	99	Culture collection
(NIOCC#PY13)	*Cryptococcus vishniacii*, Basidiomycota (AB032657)	99	Culture collection

Table 2.12 Phylogenetic affiliations of fungi and yeast isolated during cruise #ABP26 on the basis of ITS sequences

Isolate ID	Closest identified relative, Phylum (GenBank Accession no.)	% identity	Source of isolation of Closest relative
(NIOCC#F2)	*Penicillium citreonigrum*, Ascomycota (AY373908)	99	Dust
(NIOCC#F5)	*Cladosporium cladosporioides*, Ascomycota (EU497957)	100	Deep-sea
(NIOCC#F6)	*Aspergillus* sp., Ascomycota (EF672304)	99	*Coffea arabica*
(NIOCC#F8)	*Cladosporium cladosporioides*, Ascomycota (EF136373)	99	Rice wine, wheat Qu
(NIOCC#F13)	*Cladosporium* sp., Ascomycota (EF105367)	99	Toxic mold *Stachybotrys chartarum*
(NIOCC#F15)	Uncultured fungus clone, Eukaryota (DQ279836)	91	Deep-sea marine sediments in South China Sea
(NIOCC#F16)	*Exophiala spinifera*, Ascomycota (AM176734)	99	Sediment from deep sea of Pacific Ocean
(NIOCC#F21)	*Tilletiopsis albescens*, Basidiomycota (AB025695)	98	Culture collection, Tokyo
(NIOCC#F23)	Doubtful, Ascomycota	-	-
(NIOCC#F30)	Hypocreales sp., Ascomycota (DQ682584)	100	Coffee plants with the fungal entomopathogen *Beauveria bassiana*
(NIOCC#F33)	*Tilletiopsis albescens*, Basidiomycota (AB025695)	98	Culture collection, Tokyo
(NIOCC#F46)	*Exophiala xenobiotica*, Ascomycota (EF025407)	99	Fungus Testing Laboratory, Texas, US
(NIOCC#F47)	*Aspergillus caesiellus*, Ascomycota (AY373865)	100	Not known
(NIOCC#F48)	*Phialemonium dimorphosporum*, Ascomycota (DQ403199)	99	Lesions excised from *Mugil gyrans*

Table 2.12 contd.

Isolate ID	Closest identified relative, Phylum (GenBank Accession no.)	% identity	Source of isolation of Closest relative
(NIOCC#F50)	*Exophiala dermatitidis*, Ascomycota (EF568099)	100	Clinical sample
(NIOCC#PF49)	*Trichothecium roseum*, Ascomycota (EU622273)	100	Culture collection, china
(NIOCC#Y1)	*Sporobolomyces* sp., Basidiomycota (EU002893)	95	Peduncle endophyte
(NIOCC#Y2)	*Sporidiobolus salmonicolor*, Basidiomycota (AY015434)	100	Not known
(NIOCC#Y3)	*Rhodosporidium toruloides*, Basidiomycota (AB073266)	99	Not known
(NIOCC#Y4)	*Sporobolomyces* sp., Basidiomycota (EU002893)	100	Coffee plants
(NIOCC#Y5)	*Rhodotorula* sp., Basidiomycota (AB025984)	99	Deep-sea environments around the northwest Pacific Ocean
(NIOCC#Y6)	*Sporidiobolus* sp., Basidiomycota (DQ317366)	100	Soils and historic wood from the Ross Sea Region of Antarctica
(NIOCC#Y7)	*Rhodotorula calyptogenae*, Basidiomycota (EU669878)	99	Seawater
(NIOCC#Y8)	*Sarcinomyces petricola*, Ascomycota (AJ244275)	98	Meristematic tissues
(NIOCC#Y9)	*Tilletiopsis oryzicola*, Basidiomycota (AB045708)	95	Leaves
(NIOCC#Y10)	*Rhodotorula mucilaginosa* , Basidiomycota (EU781664)	98	Stem from 5 year old tree growing in a subtropical monsoon climate at an elevation of 600-800m
(NIOCC#PY12)	*Cryptococcus albidosimilis*, Basidiomycota (AF145331)	100	Antarctica
(NIOCC#PY13)	*Rhodotorula mucilaginosa*, Basidiomycota (EU781664)	99	Stem from 5 year old tree growing in a subtropical monsoon climate at an elevation of 600-800m

A total of 12 and 8 distinct species of filamentous and unicellular fungi respectively were identified in the present study. Using classical morphology-based identification system Damare et al. (2006) reported only 8 identified filamentous fungi and several unidentified sporulating and non-sporulating fungi. Therefore, although total number of fungi isolated in the present study was lower than that reported by Damare et al. (2006), the species diversity was comparatively higher.

Both sporulating as well as non-sporulating fungi could be identified by molecular-based identification system in the present study. However, insufficient database for ITS sequences also is one of the reasons for reduced diversity assessment (Anderson et al, 2003a; Zachow et al, 2009). Using 18S and ITS sequences, all the fungi isolated during cruise #ABP26 were identified, sequences and cultures deposited in respective repositories, thus adding to the data base for fungi from extreme environment. Culture-based diversity assessments suffer from biases in isolate recovery, unknown culture conditions, competitive interference and over-growth by other fungi (Panebianco et al, 2002; Pang and Mitchell, 2005). These factors might have played a role in underestimating fungal diversity in deep-sea sediments in the present study.

2.3.4 Diversity of fungi during cruise #ABP38

During this cruise the fungal isolates were classified only based on sequencing a region within the 18S rRNA gene which is generally considered to yield good comparison with the existing database (Guarro et al, 1999). Although the taxonomic resolution of 18S rDNA may not always be sufficient to identify fungal species and strains, this gene is conserved enough to allow comparison across a wide range of fungal taxa. The results obtained for the identification of fungal communities during cruise #ABP26 were found to be more reliable using 18S than ITS primers for SSU rDNA. Therefore, only 18S region was selected for the identification of fungi isolated during cruise #ABP38. During this cruise out of the total 76 sediment samples used for isolation, nineteen fungal cultures were recovered. The percentage frequency of isolation is

~25%, which is comparatively higher than the cruise #ABP26. The probable reason for the above may be the use of two more media i.e. PDA and SDA during cruise #ABP38 for isolation methods. A total of six and two fungal cultures were isolated from the PDA and SDA media plates respectively after incubation of sediment samples using various isolation techniques (Table 2.6). However the species richness was found to be lower than that found in cruise #ABP26 (Tables 2.7 and 2.8). The two stations sampled during this cruise were among the 20 stations of the cruise #ABP26 having same geographical locations (Tables 2.1 and 2.2). During cruise #ABP26 only one culture was isolated from each of these two stations (Table 2.5). However, a total of 19 fungi could be isolated from the same stations during cruise #ABP38 suggesting temporal variation playing a significant role on diversity of fungi in these sediments. The isolation of *Sagenomella* sp., *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. during both the cruises suggests their frequent occurrence, and larger distribution in these deep-sea sediments. These sporulating species except *Sagenomella* have also been reported to be present in deep-sea sediments in previous studies (Damare et al, 2006; Burgaud et al, 2009). The recovery of *Aspergillus, Penicillium and Cladosporium* sp. during both the cruises, suggested them to be natural inhabitant of deep-sea. These species may have a role in deep-sea environments in spite of not being indigenous and without exhibiting optimal growth conditions. Occurrence of fungi in deep-sea sediments from the Chagos Trench in the Indian Ocean at 5000 m depth was reported (Raghukumar et al, 2004). In this study, *Aspergillus* sp., a mitosporic fungus was isolated as a dominant form from a 450 cm long sediment core. Spores of *Aspergillus sydowii* from these core samples germinated and grew at elevated hydrostatic pressures and low temperatures. It has been hypothesized that such spores might eventually sink to the deep-sea surficial sediments, undergo natural selection mechanisms with time and acquire capabilities to grow and multiply in the presence of suitable nutrient sources (Raghukumar et al, 2004). *Sagenomella* species has been reported as a pathogenic fungus in terrestrial environments causing infection

in dogs (Gene et al, 2003) suggesting its possible role as pathogen of deep-sea organisms.

Two of the isolates (NIOCC#F68, #Y14) showing homology to *Hortaea* sp. were isolated. This species has been reported to be potential pathogenic form of mussel from hydrothermal vent site (Van Dover et al, 2007). Also, this black yeast-like fungus has been characterized as halophilic or extremely halotolerant in different studies (Gunde-Cimerman et al, 2000; Kogej et al, 2006). Two interesting fungi isolated during this cruise were *Trametes* (NIOCC#F53) and *Cerrena* sp. (NIOCC#F60, #61, #66 and #67) as these have been reported earlier as potential source of lignin-degrading enzymes (Cullen, 1997; D'Souza-Ticlo et al, 2006; Verma et al, 2010). This is the first report of isolation of these two species from deep-sea sediments. The white-rot fungus *Cerrena unicolor* was found to cause canker rot on two northern hardwood tree species, sugar maple and paper birch (Enebak and Blanchette, 1989). Later this species was found to be an efficient producer of laccase, a well known lignin degrading enzyme (Leonowicz et al, 1997; Rogalski et al, 1999).Theses two species have a powerful extracellular enzymatic complex, able to de-polymerize this aromatic polymer i.e. lignin into lower molecular weight compounds (Rogalski et al, 1999; Kim et al, 2002; Bajpai, 2004). Degradation of lignin and, more specifically, the regulation of the production of individual ligninolytic enzymes, is a complex phenomenon (Liers et al, 2006) that needs to be studied, in order to find out the correct way to obtain efficient biotechnological applications. The isolation of such ligninolytic species from extreme environment of deep-sea suggests their significant role in ecological cycles.

Two fungi i.e., NIOCC#F51 and NIOCC#F57, identified as *Nigrospora* and *Pleospora* sp. respectively have also been isolated from marine sponge (Ding et al, 2010). These species have been reported to be marine in nature, producing useful antimicrobial compounds and thus playing important ecological role in chemical defense in sponges (Bugni and Ireland 2004). Among other cultures, *Chaetomium* sp. has been reported earlier as terrestrial pathogenic or symbiotic forms, colonizing plants roots (Violi et al,

2007). Isolation of other terrestrial species such as *Eurotium* and *Ascotricha* sp. from deep-sea sediments of the Central Indian Basin suggests their transport and gradual adaptation under such extreme conditions. In general, these two species have been known to exhibit pathogenic properties in terrestrial plants and animals (Valldosera and Guarro, 1988; Roussel et al, 2010; Popov et al, 2010). In addition, *Eurotium* species has also been reported to produce secondary metabolites having useful chemical and biological activities (Slack et al, 2009) suggesting the possibility of this species being an efficient source of marine bioactive compounds.

All the fungal genotypes obtained during this cruise showed >98% similarity with the existing fungal sequences in the database except NIOCC#F55 and #Y14. These sequences showed a similarity of <97 % with their closest match suggesting their possibility of being novel forms of *Aspergillus* and *Hortaea* sp. respectively (Table 2.8). Their physiological characteristics such as GC content, fatty acid profiles, RFLP and Randomly amplified polymorphic DNA (RAPD) patterns needs to be analyzed in order to prove them as novel marine forms of the above species. Deep-sea fungi were found to generate different RFLP patterns compared to their terrestrial counterparts after digesting with similar restriction enzymes (Damare et al, 2006) suggesting them to be different from the terrestrial isolates.. Randomly amplified polymorphic DNA (Williams et al, 1990) is also used to construct RFLP maps that are useful in genetic analysis and may be helpful for population studies at the intra-specific level (Goodwin and Annis, 1991; Guthrie et al, 1992; Ouellet and Seifert, 1993).

The fungal diversity obtained using culture-dependent approach during both the cruises was found to be low. Therefore, in order to isolate hidden diverse forms of fungal cultures, new culture media and methodologies need to be used as is done for bacterial diversity studies. This study is the first report of molecular phylogenetics to assess the diversity of culturable fungi in deep-sea sediments. Molecular identification facilitated in identifying non-sporulating species which would not have been possible by classical taxonomic method. Low diversity of culturable fungi might also be due to the

failure to culture the yet-uncultured fungi, a problem similar to the bacterial diversity in deep-sea environment and many other environments. Direct sequencing of fungal genotypes from environmental samples would improve our assessment of fungal diversity in deep seas.

In the subsequent chapter, diversity of fungi is assessed applying a culture-independent approach using different fungal as well as eukaryotic primers.

Chapter 3

Fungal community analysis from deep-sea sediments of CIB using culture-independent approach

3.1 Introduction

Microbial communities existing in deep-sea oceanic environments account for a total cellular carbon content of ~$3*10^{17}$ g (Whitman et al, 1998). Little is known about fungal contribution towards these microbial communities. Being a significant part of the marine ecosystems either as saprobes, pathogens or symbionts, the actual diversity assessment of fungal communities is crucial from ecological point of view. There are several reports on diverse forms of fungi from terrestrial environment (Smit et al, 1999; O'Brien et al, 2005; Gadd et al, 2007). The problems associated with determining fungal diversity accurately have been highlighted in a number of reviews dealing with terrestrial systems (Kowalchuk, 1999; Bridge and Spooner, 2001; Horton and Bruns, 2001; Anderson and Cairney, 2004). Similar limitations also apply to the identification, isolation and quantification of fungi from marine environments, with the additional complication of distinguishing between transitory and native forms.

Diversity assessment of fungi from marine environment using culture-dependent approaches has been applied in previous studies (Damare et al, 2006; Wang et al, 2008). These methods can suffer from spore-related identification problems, as well as exhibiting biases in isolate recovery. Such biases result from unknown culturing conditions, competitive interference and over-growth by other fungi (Hyde et al, 2000; Panebianco et al, 2002). The situation is further aggravated by the presence of sterile mycelial forms that cannot be identified using traditional methods. Many reports have highlighted these and other problems associated with traditional mycological approaches when studying interference competition (Panebianco et al, 2002), the delimitation of geographical distributions (Steinke and Jones, 1993; Volkmann-Kohlmeyer and Kohlmeyer, 1993; Schmit and Shearer, 2004), the definition of host specificity or substratum preference (Steinke and Jones, 1993; Tan et al, 1995; Petersen and Koch, 1997; Schmit and Shearer, 2004), and succession patterns of marine fungi (Booth, 1979; Vrijmoed et al, 1986; Tan et al, 1989; Sarma and Hyde, 2001).

As an alternative, culture-independent techniques (Amann et al, 1995) provide ways for fungal identification and for monitoring communities. These methods are entirely molecular and as such they provide a potential link between ecological processes and the organisms involved. Diversity assessments by using molecular approaches offers the possibility of defining fungal communities accurately, studying fungal-host interactions directly in the environment and identifying ecologically active groups (Edgcomb et al, 2010).

3.1.1 Methods for studying fungal diversity by culture-independent approaches

Various molecular methods can be applied for the assessment of fungal communities directly from environmental samples. In particular, comparison of PCR-amplified ribosomal RNA genes sequences in conjunction with screening strategies such as restriction profile analysis and/or nucleic acid hybridization analysis permits rapid assessment of diversity (Fernandez et al, 1998). A variety of molecular techniques are available to separate PCR-amplified fungal genotypes from environmental sources (Fig 3.1). In order to apply these techniques for diversity assessment, DNA or RNA can be extracted from environmental samples (soils, sediments and water) using a variety of purpose-designed kits (Yeats et al, 1998; Griffiths et al, 2000). These extracted nucleic acids are of mixed origin, complex, comprising DNA or RNA from bacteria, animals, plants, fungi and other microeukaryotes. The efficiency with which the nucleic acids are extracted depends upon the species present, the environmental substrata sampled, as well as the method used.

Obtaining PCR amplifications from environmental sequences often depends upon the purity of the environmental nucleic acids. During extraction PCR-inhibitory components, such as humic acids, polysaccharides and tannins can be co-precipitated with the DNA and RNA. Removal of these impurities may be achieved by dilution or the inclusion of selective detergents, such as cetyltrimethylammonium bromide (CTAB) for the removal of

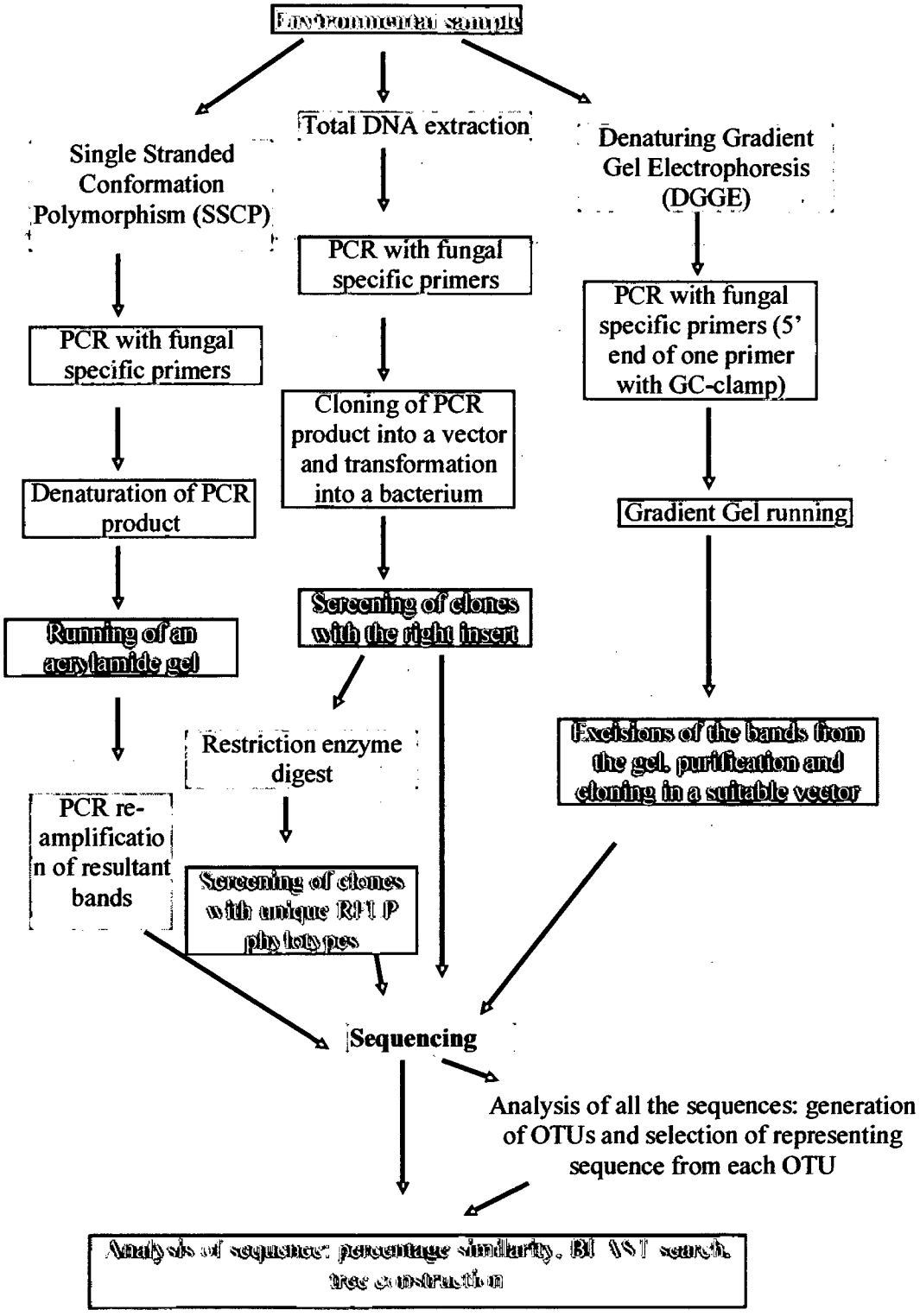


Fig 3.1 Schematic diagram showing different culture-independent approaches used for fungal diversity analysis from environmental samples (modified from Pang and Mitchell, 2005)

polysaccharides, or polyvinylpolypyrolidone (PVPP), which binds polyphenols, in the extraction buffer. Brief description of some of the molecular methods used for fungal diversity assessment from environmental DNA sample is as follows:

a) Amplified ribosomal DNA restriction analysis: In this technique ribosomal genes are PCR amplified using fungal specific primers. These amplicons are digested with restriction enzymes and run on the agarose gel. Community profiling can be deduced using this technique for an environmental sample (Schmidt and Moreth, 2000; Gich et al, 2000).

b) Automated ribosomal intergenic spacer analysis (ARISA): A fluorescent primer is used for PCR amplification which amplifies both the internal transcribed spacer (ITS) regions along with 5.8S rRNA gene. After running in an automated DNA sequencer, fragments of different sizes show different peaks and thus different species can be identified (Ranjard et al, 2001).

c) Denaturing (temperature) gradient gel electrophoresis (DGGE/TGGE): Target gene is amplified by PCR with one primer attached to a GC-clamp. The products (200–650 bp) are run in a polyacrylamine gel with increasing denaturant gradient (DGGE) or temperature gradient (TGGE). The double helix of the PCR products melts in the gel and stops migrating depending on the nucleotide sequence. Therefore, different sequences (species) with identical lengths stop at different positions in the gel. The pattern can be used to reflect community structure. Individual bands can be sequenced and analyzed phylogenetically (Kowalchuk et al, 1997; Daniell et al, 2001; Anderson et al, 2003b; Zuccaro et al, 2003; Green et al, 2004).

d) Gene cloning: Target gene is PCR amplified using specific primers. Amplified products are directly cloned in a suitable vector. The clones are sequenced and analyzed phylogenetically (Birch et al, 1993; Viaud et al, 2000).

e) Single strand conformation polymorphism (SSCP): In this technique the primer used for amplification is phosphorylated at 5' end. Target gene is amplified and the products are digested to destroy one of the strands. Resultant single stranded products are run in a non-denaturing acrylamide gel. Individual bands can be sequenced and analyzed phylogenetically which provides information about the community structure (Simon et al, 1993; Jansa et al, 2003; Nielsen et al, 2004).

f) Terminal restriction fragment length polymorphism (T-RFLP): Target gene is amplified by PCR with one fluorescent primer. The amplicons are then digested with restriction enzymes. After sequencing one peak appears for one organism as only the terminal bands with the fluorescent primer are detected (Nikolcheva et al, 2003; Edwards and Turco, 2005).

3.1.2 Primers used for amplification of fungal genotypes from environmental samples

The amplification or detection of fungal sequences, particularly rare ones, from mixed-origin samples requires that the primers exhibit fungal specificity. Many primer combinations have not been fully validated against other organisms, and some, particularly the nuclear SSU primers, can also amplify sequences from a variety of plants, animals and other microeukaryotes (Lord et al, 2003; Anderson et al, 2003a; Zuccaro et al, 2003). The nuclear LSU and ITS primers appear to be more fungal specific, although, as these have not been fully validated against other organisms, it is possible that they might allow cross-kingdom amplifications. To rectify these problems, a nested or semi-nested PCR approach may be used where the first set of primers exhibits a broad host-template annealing range while the second set is more fungal-specific (Kjøller and Rosendahl, 2000, Anderson and Cairney, 2004).

Some of the primers used commonly for PCR amplification of fungal sequences from environmental samples have been described by Pang and Mitchell. (2005). The primers described by White et al. (1990) were designed

to be generic, whereas other primer combinations were constructed for use in particular environments or with specific molecular techniques, such as denaturing gradient electrophoresis (DGE). Zuccaro and Mitchell. (2005) listed the nested combinations and the molecular conditions used to separate the mixed PCR products. The primer combinations routinely used for amplification of ITS-5.8S rDNA region as described by Gardes and Bruns. (1993) and Larena et al. (1999) were designed to preferentially amplify basidiomycetous and ascomycetous sequences respectively from plant material, particularly roots. The primers (ITS1-F, ITS4-A and ITS4-B) have also been successfully adopted for use with techniques such as ARDRA, DGE and T-RFLP (Klamer et al, 2002; Anderson et al, 2003b). Martin and Rygiewicz. (2005) described novel primer combinations for this region that co-amplified sequences from the Dikaryomycota, thereby allowing a simultaneous assessment of basidiomycetes and ascomycetes. Other primers targeting the ITS-5.8S rDNA regions from the Zygomycota and Oomycota have also been published (Nikolcheva and Barlocher, 2004).

3.1.3 Fungal diversity from deep-sea habitats using culture-independent approaches

Recently, culture-independent molecular analyses have demonstrated the diversity and ecological importance of fungi in various marine environments (Bass et al, 2007; Edgcomb et al, 2010). In particular, there has been increasing interest in the diversity of fungi from extreme marine environments. A few recent studies have reported fungal diversity from extreme environments such as hydrothermal vents (López-García et al, 2003, 2007), anoxic environments (Stoeck and Epstein, 2003, Stoeck et al, 2003, 2006; Jebaraj et al, 2010) and deep-sea sediments (Bass et al, 2007; Edgcomb et al. 2010; Nagano et al, 2010). Sporadic reports of fungi cultured from deep-sea sediments from various locations exist. Species of *Phoma, Lodderomyces, Malassezia, Cryptococcus, Cylindrocarpon, Hortaea, Pichia, Aspergillus,* and *Candida* have been reported from methane hydrate bearing deep-sea

sediments collected from water depths down to ~3,000 m in South China (Lai et al, 2007). Using fungal specific primers for construction of 18S rDNA gene libraries, Bass et al. (2007) showed yeasts to be the dominant forms at several locations in the deep oceans. 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).

Furthermore, the use of different primers has been shown to impact evaluation of fungal diversity from different habitats. Using eukaryotic specific primers, several studies have reported only a small fraction of total rDNA sequences affiliating with fungi in comparison with other eukaryotic lineages (López-García et al, 2001; Stoeck et al, 2003, 2006). However, Edgcomb et al. (2010) found fungal forms in majority from marine deep-sea subsurface by using eukaryotic specific primers. The use of multiple primer approach for studying diversity has revealed recovery of diverse fungal forms from oxygen depleted marine environments (Jebaraj et al, 2010). While studying microeukaryotic diversity in an acidic iron river, Gadanho and Sampaio. (2006) reported new fungal phylotypes with fungal-specific primers that were not detected when universal eukaryotic primers were used. These studies suggested that fungal diversity could have been underestimated in such extreme environments.

In view of the above, environmental gene libraries were constructed with universal and fungal-specific primer sets to assess the fungal diversity in the deep-sea sediments of the Central Indian Basin (CIB) in the present study. Such primers have been known to amplify a variety of fungal sequences from mixed environmental samples (O'Brien et al, 2005). Furthermore, fungal community composition analysis was carried out for distantly located stations as well as two depths at each of these stations. In addition, as fungal recovery from environmental samples have been reported to show primer bias (Jebraj et al, 2010), a multiple-primer approach was used to study fungal diversity for sediment sample of a single location. The efficiency of primers used for this study was evaluated for amplification of fungal as well as other eukaryotic

sequences from mixed environmental samples by targeting ITS and 18S rDNA regions. The primer set used for ITS region was fungal specific, whereas, out of the three 18S rDNA primer sets used, two were eukaryotic. The reason for this was to widely cover the fungal diversity from a single sediment sample. Three different analyses, carried out with different primer sets with samples of two cruises are as following.

1. The sediment samples from three stations i.e. **SVBC-04 (station A), SVBC-31 (station B) and SVBC-37 (station C)** of **Cruise #ABP26** were amplified with three primer sets i.e. a, b and c each. Where primer set **a) ITS1F/ITS4, b) ITS1/ITS4 and c) NS1/NS2.** A total of 18 environmental gene libraries were constructed during this analysis.

2. The sediment sample from one station i.e. **SVBC-33** of **Cruise #ABP26** was amplified with four primer sets. The primer sets were **1. ITS1F/ITS4, 2) NS1/NS2 3) Euk18S-42F/Euk18S-1492RE and 4) Euk18S-555F/Euk18S-1269R.** A total of 4 environmental gene libraries were constructed during this analysis.

3. The sediment samples from two stations i.e. **IVBC- 18C and IVBC-20A** of **Cruise #ABP38** were amplified with two primer sets i.e. **ITS1F/ITS4 and NS1/NS2.** A total of 8 environmental gene libraries were constructed during this analysis (Tables 3.1 and 3.2).

Objectives: The objectives were to evaluate fungal diversity from deep-sea sediment samples of the Central Indian Basin using culture-independent approach. For environmental gene library construction, fungal specific as well as universal primers were used targeting 18S and ITS regions of SSU rDNA

and comparison was made between samples of different locations as well as different depths of the same location.

3.2 Materials and Methods

3.2.1 Sampling methods

Sediment samples were collected during the cruise #ABP-26 on board the Russian research vessel Akademik Boris Petrov in December 2006 from three stations namely, SVBC-04 (station A), SVBC-31 (station B), SVBC-37 (station C), and SVBC-33 of the Central Indian Basin at depths of ~5,000 m (Table 3.1). During the cruise #ABP-38, sediment samples were collected from 2 locations of the CIB in September 2009 (Table 3.2). The sampling procedure described by Raghukumar et al. (2004) and Damare et al. (2006) was followed. The samples were collected with an USNEL type box corer of 50 cm^3 size. Thus, the collected sediments were mostly undisturbed and compact. The average length of sediment cores obtained from these locations was ~30 cm.

Sub-cores of sediments were collected using an alcohol-sterilized PVC cylinder of 5-cm inner diameter. Subsections of 2 cm down to a depth of 10 cm and thereafter every 5 cm length down to 30 cm depth were cut from the sediment cores and directly introduced into sterile plastic bags to avoid any aerial contamination. The sediments were stored at −20°C immediately after sampling. DNA was isolated from each frozen sub-section of the sediment cores under sterile conditions to avoid cross-contamination. The DNA samples from the subsections were pooled into two parts of 0–15 and 15–30 cm for each of the three stations (A, B and C) in order to minimize the number of clone libraries. Thus, finally a total of six DNA samples were processed for these stations. For the Station number SVBC-33, the DNA samples from all the subsections were pooled together as one sample. During cruise #ABP-38 also the DNA samples were pooled into two parts of 0–15 and 15–30 cm for each of the two cores and processed as four samples. In order to monitor the

contamination by air-borne fungi, control media plates were exposed for 10 min on the deck of the research vessel where the cores were received and in the microbiology laboratory on board the research vessel during every sampling.

3.2.2 DNA extraction, environmental PCR and clone library analyses

For amplification of fungal genotypes from environmental sediment samples using specific primers, DNA was isolated from each core as described in materials and methods. DNA was isolated from 0.5 g of the sediment sample from each subsection using the Q-Bio gene Soil DNA extraction kit (MP Biomedicals, OH, US) according to the manufacturer's instructions. DNA samples were pooled for each station described in materials and methods. DNA samples from the three stations (A, B and C) were amplified using fungal-specific ITS1F/ITS4 (Gardes and Bruns, 1993), "primer pair a" as well as universal ITS1/ITS4, "primer pair b", and universal 18S rDNA NS1/NS2 primer set (White et al, 1990), "primer pair c". For the station SVBC-33, DNA sample was amplified using fungal-specific primer pair, ITS1F/ITS4 as well as universal 18S rDNA primers, NS1/NS2, Euk18S-42F/Euk18S-1492RE and Euk18S-555F/Euk18S-1269R (Tables 3.1 and 3.3). DNA samples isolated during the cruise #ABP38 were amplified using

Table 3.1 Details of sediment collection sites from CIB during cruise # ABP26.

Station number	Touch Positions		Water depth (m)	Primer pair used
	Lat (°S)	Long (°E)		
SVBC-04 (Station A)	13 00.000	76 29.950	5377	ITS1F/ITS4, ITS1/ITS4 and NS1/NS2
SVBC-31 (Station B)	12 00.000	74 59.900	5153	
SVBC-37 (Station C)	16 06.891	75 24.828	3992	
SVBC-33	12 59.980	74 59.960	5265	ITS1F/ITS4, NS1/NS2, Euk18S-42F/Euk18S-1492RE and Euk18S-555F/Euk18S-1269R

Table 3.2 Details of sediment collection sites from CIB during cruise # ABP38.

Station Number	Touch Positions		Date	Primer set used
	Lat (°S)	Long (°E)		
IVBC-18C	12 59.747	74 30.150	23.09.09	ITS1F/ITS4 and NS1/NS2
IVBC-20A	11 59.900	75 29.900	30.09.09	

fungal specific ITS1F/ITS4 and universal 18S rDNA NS1/NS2 primer set (Tables 3.2 and 3.3).

The conditions for PCR included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 72°C for 15 min. The PCR reaction mixture (50 µl) consisted of 50 µg bovine serum albumin (New England Biolab), 0.6 U Taq DNA polymerase (Bangalore Genei, India), 1.5 mM $MgCl_2$, dNTPs (0.2 mM each), primers (0.5 µM each), and 1× PCR buffer (Roche, Switzerland). Reaction mixture without template DNA was used as a negative control, and sediments spiked with fungal DNA was used as a positive control. Amplified products were gel purified and ligated with pGEM-T easy vector (Promega, USA) and transformed into *E. coli* cells (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Transformants were grown overnight at 37°C in Luria-Bertani broth containing 100 µg ml^{-1} of ampicillin. The presence of insert was confirmed by PCR with M13 forward (5'-GTTTTCCCAGTCACGAC-3') and M13 reverse (5'-CAGGAAACAGCTATGACC-3') primers. One µl of the broth containing the clone was added to 25 µl of PCR reaction mixture. PCR protocol included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. Clones containing positive insert were further processed for plasmid isolation and purification using Millipore plasmid preparation kit (Millipore, USA).

Sequencing of the plasmids was done at the National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3,730 DNA Analyzer, Applied Biosystems, USA). A total of 18 environmental gene libraries were constructed from the DNA samples of three stations (A, B and C), each with two depths and three primer pairs (3×2×3) during cruise #ABP26. Four environmental gene libraries were constructed from the DNA sample from station SVBC-33 during cruise #ABP26 and eight during the cruise #ABP38. A total of 48 clones were screened from each library.

3.2.3 Phylogenetic analyses

Forward and reverse sequences were assembled using Chromas Pro version 1.34 (Technelysium Pty Ltd, Tewantia, Queensland, Australia). Sequences obtained with ITS and 18S rDNA primers were analyzed separately. All the sequences were checked with Ribosomal Database Project (Cole et al, 2004) for the presence of chimeras. These chimeric sequences were eliminated from subsequent analyses. Pairwise alignment of the sequences was carried out using Clustal W2 software (Thompson et al, 1994). Conserved motifs were identified, and sequences were trimmed manually. Clones were grouped into operational taxonomic units (OTUs) by using sequence similarity cut-off value of 98% (O'Brien et al, 2005) by using MOTHUR software version 1.4.1 (Schloss et al, 2009). For the 3 stations (A, B and C) of the cruise #ABP 26, the results are presented as OTU_01 to OTU_09 (blue fonts) obtained with fungal-specific ITS primer pair, OTU_10 to OTU_22 (green fonts) retrieved with universal ITS primer pair and OTU_23- to OTU_39 (red fonts) with universal 18S rDNA primer pair. Out of the 18 environmental gene libraries, for these stations, two showing biased amplification of a single sequence type due to PCR artifact, were eliminated from the further analyses. For the station SVBC-33, the OTUs are represented as OTU_01 to OTU_08 (blue fonts) obtained with fungal-specific ITS primer pair, OTU_9 and OTU_10 (pink

fonts) retrieved with universal 18S rDNA primer pairs, OTU_11 to OTU_20 (green fonts) with eukaryotic primer sets 42F/Euk18S-1492RE and OTU_21 to OTU_27 (red fonts) with eukaryotic primer sets Euk18S-555F/Euk18S-1269R. For the environmental gene libraries prepared during cruise #ABP38, the OTU details are shown as OTU_01 to OTU_18 (pink fonts) obtained with universal 18S rDNA primer pairs and OTU_19 to OTU_46 (blue fonts) retrieved with fungal-specific ITS primer pair sets. A representative sequence from each OTU was queried against NCBI-GenBank BLASTN search (Altschul et al, 1990).

Table 3.3 Details of the primers used for construction of environmental libraries.

Primer name	Sequences	Reference
ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Lai et al. 2007
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	Lai et al. 2007
NS1	5'-GTAGTCATATGCTTGTCTC-3'	O Brien et al. 2005
NS2	5'-GGCTGCTGGCACCAGACTTGC-3'	O Brien et al. 2005
Euk18S-42F	5'-CTCAARGAYTAAGCCATGCA-3'	Lopez-Garcia et al. 2003
Euk18S-1492R	5'-ACCTTGTTACGRCTT-3'	Stoeck et al. 2006
Euk18S-555F	5'-AGTCTGGTGCCAGCAGCCGC-3'	Lopez-Garcia et al. 2003
Euk18S-1269R	5'-AAGAACGGCCATGCACCAC-3'	Lopez-Garcia et al. 2003

Multiple alignments were done for all the sequences along with their closest match in ClustalW (Thompson et al, 1994). Gaps and ambiguously aligned sequences were removed from further analyses. A phylogenetic analysis was conducted using distance setting (Maximum Parsimony) in MEGA 4.1 (Kumar et al, 2008) with 1,000 bootstrap replicates. Phylogenetic trees were constructed with sequences obtained with ITS and 18S rDNA primer sets individually for each analyses. All diversity analyses were carried out using MOTHUR software. Shannon–Wiener diversity indices were calculated for OTUs obtained from each library constructed with three individual primer sets. Community composition was plotted as relative abundance of taxonomic

subclasses at different stations and depths. Representative sequences of each OTU were deposited in NCBI-GenBank under the accession numbers GU370716 to GU370774 for the 3 stations (A, B and C) of the cruise #ABP 26 (Table 3.4). For station SVBC-33, accession numbers for the OTUs are shown in Table 3.5.

3.3 Results:

3.3.1 Physico-chemical Properties of the Sediments

The area of sampling was characterized by the presence of polymetallic nodules. The nodule abundance ranged 0–10.7 kg m^{-2}. The approximate salinity, temperature, and pH were found to be 35 PSU (practical salinity units), 3°C, and ~7.0 respectively. Among other parameters, the total proteins, carbohydrates, and lipids ranged from 0–1.5, 0–1, and 0–1 mg g^{-1} dry sediment, respectively. Total bacterial counts in this area ranged from 10^7–10^9 cells g^{-1} dry sediment. Organic carbon ranged from 0.3–0.4 mg g^{-1} dry sediment. The seafloor of the CIB is characterized by the presence of homogenous, soft to slightly compact, dark brown sediments with yellowish sediments at deeper depths. The station SVBC-37 was in a seamount area and the sediment texture was calcareous. All the other stations were characterized by the presence of siliceous sediments (Sharma, 2008, 2010).

3.3.2 Environmental Libraries

No growth of aerial mycoflora was observed on media plates that were exposed on the deck and in the microbiology laboratory on board the research vessel. A total of 768 clones were sequenced from the 16 environmental libraries **for the 3 stations (A, B and C) of the cruise #ABP26**. Of the resultant sequences, 257 sequences were found to be fungal, resulting in a total of 39 OTUs, after clustering on a basis of 98% sequence identity criterion.

Table 3.4 Phylogenetic affiliation of the Fungal OTUs obtained with different primer sets for the stations A, B and C of the cruise #ABP26 (Sequences showing <97 % identity are represented in bold)

OTUs with Fungal Specific ITS primers, ITS1F/ITS4 (a)	OTUs with Universal ITS primers, ITS1/ITS4 (b)	OTUs with Universal 18S rDNA primers, NS1/NS2 (c)	Closest identified relative (GenBank Accession no.)	% Identity	Phylum	Source of isolation of closest identified relative
OTU_02	**OTU_11**	-	*Sagenomella* sp. (FJ357782)	99	Ascomycota	Deep sea sediment of CIB
-	**OTU_21**	-	*Dothideomycete* sp. (EU680535)	99		Moth exoskeleton
-	**OTU_15**	**OTU_23**	*Aspergillus penicillioides* (DQ985959)	**97**		Lacaziosis patient
-	-	**OTU_24**	*Aspergillus restrictus* (AB008407)	99		Soil
OTU_01	**OTU_10**	-----	*Hortaea* sp. F 47 (FJ755827)	**95**		Marine sponge
OTU_04	**OTU_13**	-	*Stenella musicola* (EU514294)	99		*Musa acuminata*
OTU_05	**OTU_14**	-	*Candida* sp. F15 (FJ755821)	100		Coastal waters of china
-	**OTU_16**	**OTU_27**	*Aspergillus restrictus* (AB002079)	**94**		Not known
-	**OTU_19***	-	*Debaryomyces yamadae* (AB054266)	98		Not known
OTU_06*	-	-	*Pichia jadinii* (EU568927)	99		Pilot-scale Municipal compost
OTU_07*	-	-	*Nodulisporium* sp. (EF600033)	98		Stroma of *Epichloe typhina*
-	-	**OTU_25**	*Aspergillus niger* (GQ228449)	**96**		Soil from Kalabagh iron ore deposit
-	-	**OTU_28**	*Candida glucosophila* (AB013519)	**97**		Not known
-	-	**OTU_29***	*Aspergillus fumigatus* (GQ169424)	99		Fossa cheese
-	-	**OTU_30***	*Aspergillus sp.* (FJ864683)	99		Root of *Angelica sinensis*
-	-	**OTU_32**	*Phoma herbarum* (AY293777)	99		Not known

Table 3.4 contd.

OTUs with Fungal Specific ITS primers, ITS1F/ITS4 (a)	OTUs with Universal ITS primers, ITS1/ITS4 (b)	OTUs with Universal 18S rDNA primers, NS1/NS2 (c)	Closest identified relative (GenBank Accession no.)	% Identity	Phylum	Source of isolation of closest identified relative
-	-	**OTU_35**	*Aspergillus unguis* (EF067336)	99	Ascomycota	Culture collection
-	-	**OTU_37***	*Ulospora bilgramii* (DQ384071)	98		Dung
-	-	**OTU_39***	*Capnodium coffeae* (DQ247808)	**93**		Maize rhizosphere Soil
-	**OTU_22***	-	*Bionectriaceae* sp. (FJ821507)	**97**		Basidioma of *Phellinus gilvus*
OTU_08*	-	-	fungal sp. ARIZ L365 (FJ612791)	99		Tree seed
-	-	**OTU_26**	Uncultured fungal clone (GQ120138)	98		Arabian Sea sediment
-	-	**OTU_31***	Uncultured fungal clone (AB074653)	98		Arabian Sea sediment
-	-	**OTU_33**	Uncultured fungal clone (AJ515945)	**97**		Maize rhizosphere Soil
-	**OTU_20***	-	*Candida parapsilosis* (GQ395610)	99		Feeding production sample
-	**OTU_17**	-	*Malassezia pachydermatis* (DQ457640)	**96**	Basidiomycota	Not known
OTU_03	**OTU_12**	-	*Trichosporon asahii* (AB369919)	99		Skin of patient
-	-	OTU_34	*Wallemia sebi* (AY741380)	**97**		Hypersaline water of salterns
OTU_09*	-	-	*Rhodosporidium sphaerocarpum* (EF643587)	100		Deep-sea of pacific ocean
-		OTU_38	fungal sp. FCAS11 (GQ120154)	**97**		Arabian Sea sediment
-		OTU_36	Uncultured fungal clone (AJ515927)	99		Contaminated aquifer sediment
-	**OTU_18**	-	Uncultured fungal clone (FJ889114)	**94**		Hawaiian coastal waters

Singletons are represented with *

Table 3.5 Phylogenetic affiliations of the OTUs obtained with different primer sets for station SVBC-33 of the cruise #ABP26 (Sequences showing <97 % identity are represente in bold)

Fungal OTU obtained			Closest identified relative					% Identity
OTU no.	Primer set used	Accession no.	Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_01	ITS1F/ ITS4	HM572248	Unidentified isolate f4Fc56	Ascomycota	Dothideomycetes	EU680535	Filter dust	99
OTU_02		GU370751	Unidentified fungal clone S3	Basidiomycota	Wallemiomycetes	FJ820491	Air sample	**79**
OTU_03		GU370752	Uncultured basidiomycete clone	Basidiomycota	Exobasidiomycetes	AM901800	House dust	99
OTU_04*		GU370753	Uncultured *Wallemia* isolate	Basidiomycota	Wallemiomycetes	GU931736	House dust	99
OTU_05*		GU370754	*Aphyllophorales* sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_06*		GU370755	*Nectria mauritiicola*	Ascomycota	Sordariomycetes	AJ557830	Not known	99
OTU_07*		GU370756	*Rhodotorula calyptogenae*	Basidiomycota	Cystobasidiomycetes	EU669878	Sea water	99
OTU_08		HM572249	*Trichosporon asahii*	Basidiomycota	Tremellomycetes	AB369919	Not known	99
OTU_09	NS1/ NS2	HM572252	Uncultured *Malassezia* clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	99
OTU_10		HM572251	Uncultured *Aspergillus* clone	Ascomycota	Eurotiomycetes	FJ393417	Gut of HBV infected patients	99
OTU_11	18S-42F/ Univ1492 RE	GU370757	*Fungal* sp. NIOCC F15	Ascomycota	Sordariomycetes	FJ357782	Deep-sea sediments	99
OTU_12		GU370758	Unidentified isolate f3Fc79	Basidiomycota	Wallemiomycetes	GU721564	Filter dust	99
OTU_13*		GU370759	Unidentified isolate f4Fc56	Ascomycota	Dothideomycetes	EU680535	Filter dust	99
OTU_14*		GU370760	*Aphyllophorales* sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_15*		GU370761	Unidentified fungal clone S3	Basidiomycota	Wallemiomycetes	FJ820491	Air sample	**79**

Table 3.5 contd.

Fungal OTU obtained			Closest identified relative					% Identity
OTU no.	**Primer set used**	**Accession no.**	**Taxon**	**Phylum**	**Class**	**Accession no.**	**Source of isolation**	
OTU_16*	18S-42F/ Univ1492RE	GU370762	*Nectria mauritiicola*	Ascomycota	Sordariomycetes	AJ557830	Not known	99
OTU_17		GU370763	*Wallemia* sp. F53	Basidiomycota	Wallemiomycetes	FJ755832	Marine sponge	**80**
OTU_18*		GU370765	Unidentified basidiomycete clone	Basidiomycota	Exobasidiomycetes	AM901883	House dust	99
OTU_19*		GU370766	*Rhodotorula* sp. SY-74	Basidiomycota	Microbotryomycetes	AB025984	Deep-sea	99
OTU_20*		GU370767	Dothideomycete sp.	Ascomycota	Dothideomycetes	EU680530	Leaf	**94**
OTU_21	EK555F/ EK1269R	GU370768	Uncultured *Malassezia* clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	**96**
OTU_22		GU370769	Uncultured *Aspergillus* clone	Ascomycota	Eurotiomycetes	FJ393417	Gut of HBV infected patients	**97**
OTU_23		HM587247	*Aspergillus restrictus*	Ascomycota	Eurotiomycetes	AB008407	Not known	99
OTU_24*		GU370771	*Wallemia sebi*	Basidiomycota	Wallemiomycetes	AY741380	Hypersaline water of saltern	98
OTU_25*		GU370772	*Candida orthopsilosis*	Ascomycota	Saccharomycetes	FN812686	Not known	99
OTU_26*		GU370773	*Aspergillus penicillioides*	Ascomycota	Eurotiomycetes	AB002077	Not known	**91**
OTU_27		GU370774	Uncultured *Malassezia* clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	99

Singletons are represented with *

The other 511 clones (~66.5%) were bacterial, eukaryotic, or chimeric in nature and thus were excluded from the study. These 39 fungal OTUs comprised 32 distinct fungal taxa (Table 3.4).

For the station SVBC-33, a total of 48 and 144 clones from ITS and 18S primer pairs respectively were sequenced from the environmental libraries. This resulted in 27 fungal OTUs, eight with ITS and nineteen with 18S rDNA primer pairs after clustering on a basis of 98% sequence identity criterion. A total of 20 distinct fungal species were obtained from these 27 OTUs. The results are presented as **OTU_01 to OTU_08** obtained with fungal-specific ITS primer pair, **OTU_9 and OTU_10** with universal 18S rDNA primer pairs, NS1/NS2, **OTU_11 to OTU_20** with 18S rDNA primer pair Euk18S-42F/ Euk18S-1492RE and **OTU_21 to OTU_27** with 18S rDNA primer pairs Euk18S-555F/ Euk18S-1269R (Table 3.5).

None of the primers amplified sequences affiliating with eukaryotes, other than fungi. Out of 48 and 144 total clones obtained from ITS and 18S rDNA primer sets, 23 (47.9 %) and 39 (27.1 %) respectively were bacterial or chimeric in nature and thus were excluded from the analysis. The fungal OTUs obtained, mostly belonged to the phyla Ascomycota and Basidiomycota (Table 3.5). The number of singletons i.e. detected only once were four and ten respectively for the ITS and 18S rDNA primer sets (marked with an *asterisk in Table 3.5). The remaining OTUs were present ≥2 times in different libraries.

During the cruise #ABP38, a total of 192 clones were sequenced for each primer pair. Out of these clones, 67 (~ 35%) and 55 clones (~ 29%) were found to be chimeric with ITS and 18S rDNA primer sets respectively and were removed from the subsequent analysis. A total of 18 and 28 OTUs were obtained from the resultant clones obtained with 18S rDNA and ITS primer sets (Table 3.6). The results are presented as **OTU_01 to OTU_18** obtained with universal 18S rDNA primer pairs, NS1/NS2 and **OTU_19 to OTU_46** with fungal -specific ITS primer pair (Table 3.6). All the 46 OTUs were found to be affiliating with distinct fungal species.

Table 3.6 Phylogenetic affiliations of the OTUs obtained with different primer sets from environmental libraries prepared with the samples during cruise #ABP38 (Sequences showing <97 % identity are represented in bold)

OTU no.	Primer set used	Closest identified relative					% Identity
		Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_01	NS1/ NS2	Uncultured soil ascomycete	Ascomycota	Eurotiomycetes	AJ515945	Soil	**97**
OTU_02*		Uncultured fungus clone	Ascomycota	Eurotiomycetes	GU370739	Marine sediment	**95**
OTU_03		Uncultured *Aspergillus* clone	Ascomycota	Eurotiomycetes	FJ393417	Gut	99
OTU_04		*Phialosimplex caninus*	Ascomycota	Eurotiomycetes	GQ169310	Culture Collection	**97**
OTU_05		Uncultured marine eukaryote clone	Alveolata	Dinophyceae	EF526792	Marine environment	**86**
OTU_06		Eimeriidae environmental clone	Alveolata	Apicomplexa	EF024655	Rhizosphere	**87**
OTU_07		*Angelica gigas*	Viridiplantae	Apiaceae	DQ647697	Culture Collection	99
OTU_08		*Saccharomyces* sp.	Ascomycota	Saccharomycetes	DQ345287	Culture Collection	99
OTU_09		Uncultured marine fungus clone	Ascomycota	Saccharomycetes	GQ120138	Arabian sea sediment	99
OTU_10		Uncultured soil ascomycete	Ascomycota	Sordariomycetes	AJ515930	Soil	100
OTU_11		Uncultured fungus clone	Ascomycota	Eurotiomycetes	GU370729	Marine sediment	**95**
OTU_12		*Pycnoporus* sp.	Basidiomycota	Agaricomycetes	GU182936	Kraurotic chump	100
OTU_13		*Nicotiana tabacum*	Viridiplantae	Solanaceae	AY079155	Culture Collection	99
OTU_14		Uncultured *Malassezia* clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut	99

Table 3.6 contd.

OTU no.	Primer set used	Closest identified relative					% Identity
		Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_15	NS1/ NS2	*Sterigmatomyces halophilus*	Basidiomycota	Agaricostilbomycetes	DQ092916	Culture Collection	100
OTU_16*		Uncultured soil ascomycete	Ascomycota	Dothideomycetes	AJ515936	Soil	99
OTU_17*		*Dothideomycete* sp.	Ascomycota	Dothideomycetes	AY275186	Culture Collection	99
OTU_18*		*Cerrena unicolor*	Basidiomycota	Agaricomycetes	AY850007	Culture Collection	98
OTU_19	ITS1F/ ITS4	*Sterigmatomyces* sp.	Basidiomycota	Agaricostilbomycetes	FJ755830	Marine sponge	99
OTU_20		*Gibberella moniliformis*	Ascomycota	Sordariomycetes	EU717682	Culture Collection	99
OTU_21		*Schizophyllum commune*	Basidiomycota	Agaricomycetes	EF155505	Decaying wood	99
OTU_22		Uncultured fungus clone	Ascomycota	Saccharomycetes	GU370744	Marine sediment	99
OTU_23		Uncultured fungus clone	Basidiomycota	Agaricomycetes	GU370760	Marine sediment	99
OTU_24*		*Aspergillus* sp.	Ascomycota	Eurotiomycetes	FJ755817	Marine sponge	97
OTU_25		*Trichosporon asahii*	Basidiomycota	Tremellomycetes	AB369919	Culture Collection	99
OTU_26		Uncultured fungus clone	Ascomycota	Dothideomycetes	GU721670	Filter dust	100
OTU_27		Uncultured fungus clone	Basidiomycota	Agaricomycetes	GQ999291	Air filter sample	97
OTU_28		*Rhodotorula slooffiae*	Basidiomycota	Cystobasidiomycetes	FJ515213	Sea surface microlayer	99
OTU_29		*Rhodotorula* sp.	Basidiomycota	Microbotryomycetes	AB025984	Deep-sea environments	99
OTU_30		Uncultured endophytic fungus clone	Basidiomycota	Wallemiomycetes	FJ524297	Root endophyte	99

Table 3.6 contd.

OTU no.	Primer set used	Closest identified relative					% Identity
		Taxon	Phylum	Class	Accession no.	Source of isolation	
OTU_31	ITS1F/ ITS4	*Debaryomyces hansenii*	Ascomycota	Saccharomycetes	GQ458025	Camembert cheese	99
OTU_32*		*Stenella musicola*	Ascomycota	Dothideomycetes	EU514294	Banana	99
OTU_33*		*Malassezia slooffiae*	Basidiomycota	Exobasidiomycetes	AY743633	Domestic animals	99
OTU_34*		*Gibberella moniliformis*	Ascomycota	Sordariomycetes	EU717682	Culture Collection	100
OTU_35		*Resinicium friabile*	Basidiomycota	Agaricomycetes	DQ826543	Hardwood	**97**
OTU_36*		*Aspergillus* sp.	Ascomycota	Eurotiomycetes	EU301661	Forest soil	99
OTU_37		*Rhodotorula mucilaginosa*	Basidiomycota	Microbotryomycetes	DQ386306	Culture Collection	99
OTU_38		*Malassezia restricta*	Basidiomycota	Exobasidiomycetes	EU400587	Rust pathogen	99
OTU_39		*Aspergillus penicillioides*	Ascomycota	Eurotiomycetes	GU017496	Seagrass Enhalus	**96**
OTU_40		*Aspergillus oryzae*	Ascomycota	Eurotiomycetes	EU301638	Forest soil	99
OTU_41*		*Neurospora* sp.	Ascomycota	Sordariomycetes	GU183173	Rice	99
OTU_42		*Penicillium* sp.	Ascomycota	Eurotiomycetes	GU566250	Rhizosphere	100
OTU_43*		Uncultured fungus clone	Ascomycota	Eurotiomycetes	FJ820795	Air sample	99
OTU_44*		*Hortaea* sp.	Ascomycota	Dothideomycetes	FJ755827	Marine sponge	98
OTU_45*		*Corynespora cassiicola*	Ascomycota	Dothideomycetes	AY238606	Cucumber	99
OTU_46*		*Aspergillus sp.*	Ascomycota	Eurotiomycetes	EU139858	Culture Collection	**95**

Singletons are represented with *

The fungal OTUs obtained belonged to Ascomycota and Basidiomycota phyla. In addition, the 18S rDNA primer set amplified four OTUs affiliating with eukaryotic sequences also (Table 3.6).

3.3.3 Analyses of Fungal Diversity Obtained with Different Primer Sets

For the stations A, B and C of cruise #ABP26, Out of the 39 fungal OTUs, four were exclusively amplified by primer set a, six with primer set b, and 15 by the primer set c (Table 3.4). Ten OTUs were simultaneously recovered with primer pairs a and b (Table 3.4) and four with b and c. The number of singletons, i.e., detected only once in libraries constructed with primer pairs a, b, and c were four, three, and five, respectively (marked with an asterisk in Table 3.4. Apart from these singletons, the remaining OTUs were represented ≥2 times in different libraries.

The fungal-specific primer set ITS1F/ITS4 (primer pair a) and universal fungal primers ITS1/ITS4 (primer pair b) amplified sequences exclusively from a diverse group of fungi belonging to Ascomycota and Basidiomycota (Figs. 3.2 and 3.3). Primer pair a and primer pair b recovered seven and ten OTUs respectively belonging to Ascomycota. Both the ITS primer sets detected Sordariomycetes, Dothideomycetes, and Saccharomycetes (Figs. 3.2 and 3.3). In addition, the primer set b amplified members of Eurotiomycetes as well., The primer pair a and the primer pair b amplified one (OTU_01) and two (OTU_10, OTU_16) new sequence types respectively having similarities less than 97% to the known fungal taxa belonging to ascomycota (Table 3.4). Primer pair a and primer pair b recovered two and three OTUs belonging to Basidiomycota, respectively. This group included members from Tremellomycetes, Microbotryomycetes, and Ustilaginomycetes. Out of these, only Tremellomycetes was the common clade shared by both the primer sets (Fig. 3.3).

Primer pair b amplified two new phylotypes of Basidiomycota (OTU_17, OTU_18). Out of the total 22 OTUs recovered with these two

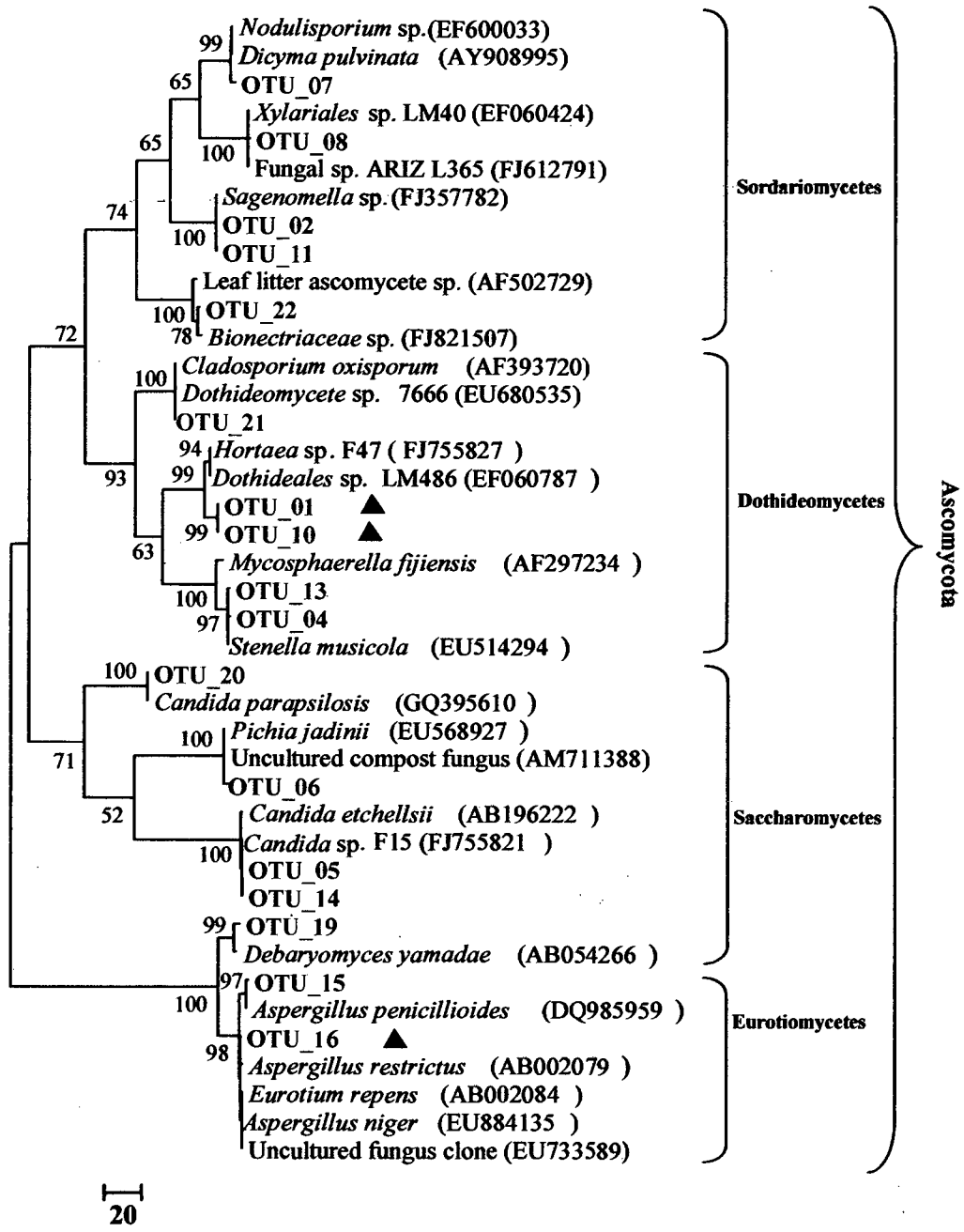


Fig 3.2 Phylogenetic tree based on fungal ITS gene sequences of phylum Ascomycota after amplification with primer set,ITS1F/ITS4 (fungal specific, marked with blue color) and ITS1/ITS4 (universal ITS, marked with green color).Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates.New sequence types are marked with triangle. Analysis was done for the stations A, B and C of the cruise #ABP26.

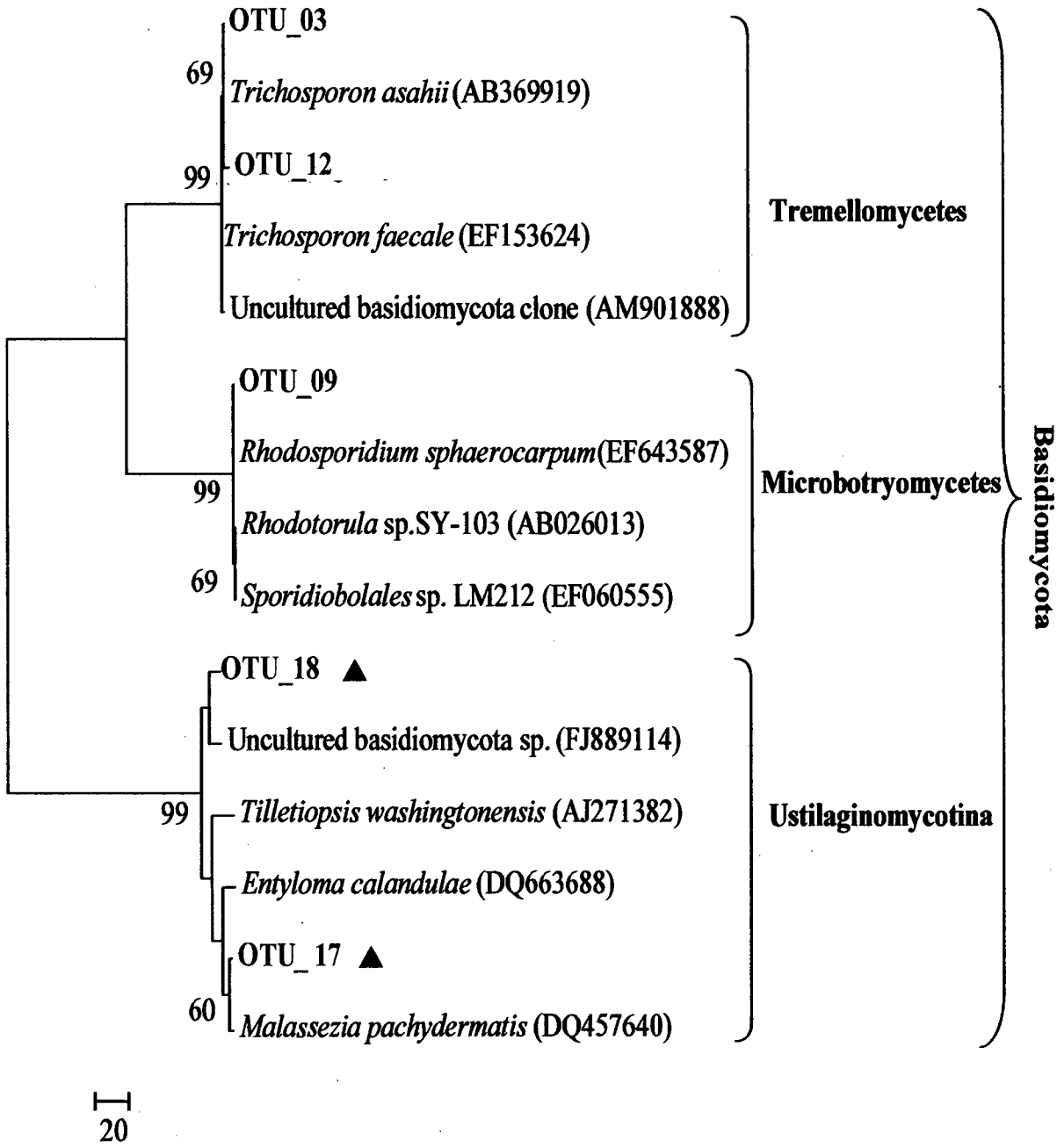


Fig 3.3 Phylogenetic tree based on fungal ITS gene sequences of phylum Basidiomycota after amplification with primer set,ITS1F/ITS4 (fungal specific, marked withblue color) and ITS1/ITS4 (universal ITS, marked with green color).Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates.New sequence types are marked with triangle. Analysis was done for the stations A, B and C of the cruise #ABP26.

primers sets, eight OTUs affiliated with sequences reported from marine environment (Table 3.4). Out of these eight OTUs, three were new phylotypes. Five fungal taxa were simultaneously recovered with universal and fungal specific ITS primer sets (Table 3.4). A major part of the sequences recovered with universal 18S rDNA primer set NS1/NS2 (primer pair c) belonged to Ascomycota (Fig. 3.4). The number of OTUs belonging to Ascomycota and Basidiomycota were 14 and 3, respectively. Out of these, three sequences showing less than 97% identity with the existing data base, OTU_25 and OTU_27 (with less than 50% bootstrap value) and OTU_39 (with bootstrap value of 91%) branched deeply within Eurotiomycetes (Fig. 3.4). OTU_34 belonging to the subclass Wallemiomycetes clustered with *Wallemia sebi* and OTU_38 clustered with an unclassified species of fungus with a 99% bootstrap value (Fig. 3.4). Only two fungal taxa namely *Aspergillus penicillioides* and *Aspergillus restrictus* were simultaneously amplified with universal ITS and 18S rDNA primers (Table 3.4).

For the Station SVBC-33, the fungal specific primer set ITS1F/ITS4 amplified eight OTUs belonging to seven different classes of Ascomycota and Basidiomycota. Majority of the sequences affiliated with the unidentified and uncultured fungal clones (Table 3.5). Wherein OTU_02 affiliated with an unidentified clone from the class Wallemiomycetes with 79% identity.

Universal 18S rDNA primer pair NS1/NS2 amplified sequences matching two phylotypes i.e. uncultured *Malassezia* and *Aspergillus* clone with 99% similarity belonging to the class Exobasidiomycetes and Eurotiomycetes respectively (Table 3.5).

The eukaryotic primer set 18S-42F/Univ1492RE, recovered sequences belonging to ten different phylotypes and these belonged to six different classes of the phyla Ascomycota and Basidiomycota (Table 3.5). Three OTUs (OTU_15, OTU_17 and OTU_20) showed very low similarity (79, 80 and 94 %) with their closest relative in NCBI database and thus may be novel phylotypes.

The other eukaryotic primer set EK555F/EK1269R, amplified seven fungal phylotypes belonging to four different classes. OTU_21 and OTU_22

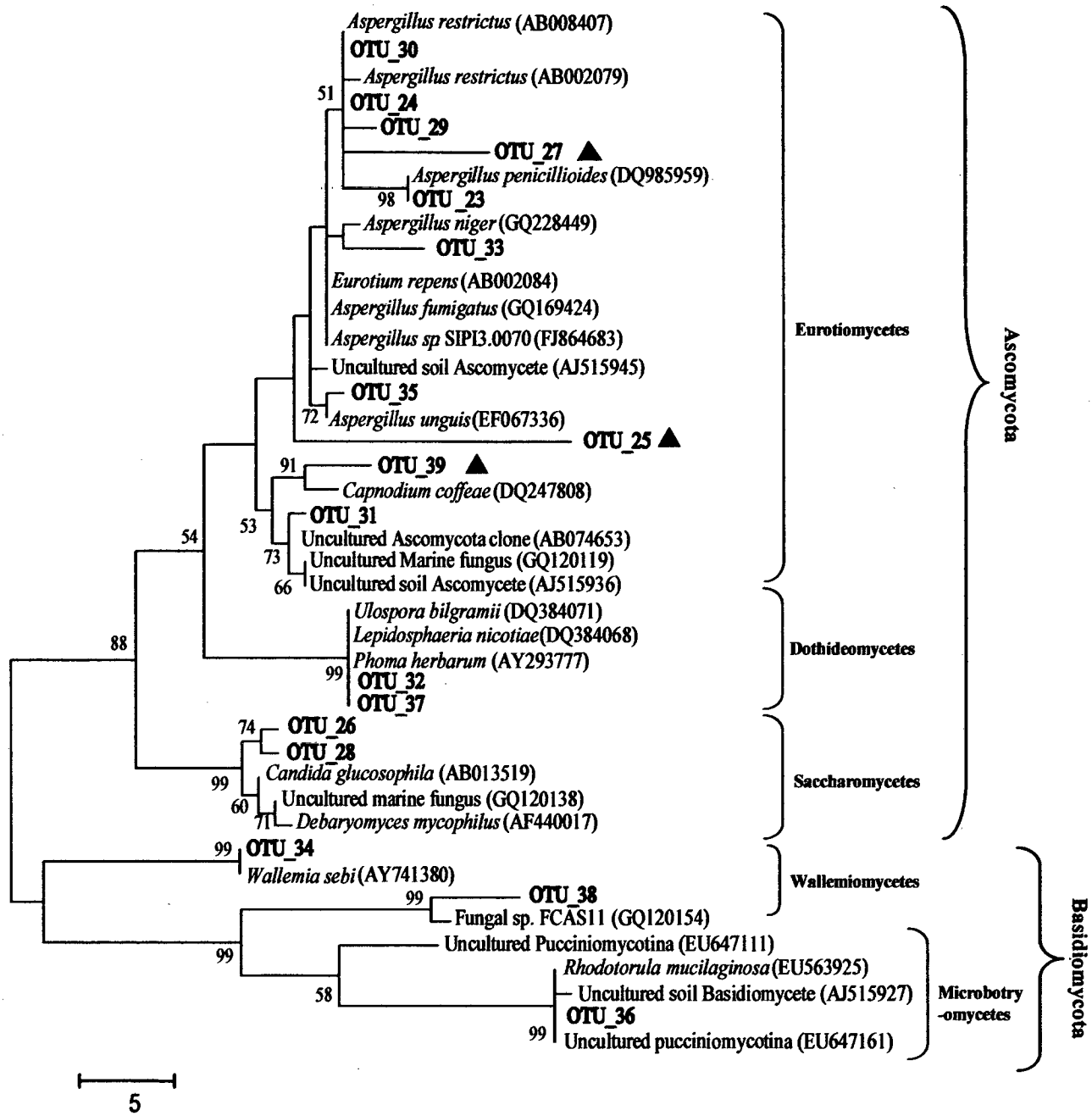


Fig 3.4 Phylogenetic tree based on fungal 18S rDNA gene sequences of phylum Ascomycota and Basidiomycota after amplification with universal 18S rDNA primer set, NS1/NS2 (Universal 18S rDNA, marked with Red color). Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle. Analysis was done for the stations A, B and C of the cruise #ABP26.

showing low similarity values of 96 and 97% respectively with the sequences existing in the data base. They affiliated with the sequences which were also amplified by NS1/NS2 primer set but with a similarity of 99% with sequences in the public database (Table 3.5). This eukaryotic primer set amplified a total of three sequences including OTU_26 besides the above two, showing < 97% identity with existing sequences in the database and thus may be new.

For the gene libraries prepared during **the cruise #ABP38,** both ITS and 18S rDNA primer sets amplified sequences affiliating with Ascomycota as majority (Table 3.6). Primer pair NS1/NS2 amplified two sequences clustering with Alveolata and 2 matching with Viridiplantae among eukaryotes (Fig 3.5). Among Ascomycetes, 10 different phylotypes were recovered belonging to three classes i.e. Eurotiomycetes, Saccharomycetes and Dothideomycetes. Four basidiomycetous phylotypes were found to show similarity with classes Agaricomycetes, Exobasidiomycetes and Agaricostilbomycetes. The basidiomycete phylotype OTU_18 belonged to *Cerrena unicolor* with a percentage identity of 98. Among other sequences most common forms were uncultured fungal clones, reported from marine environment in previous studies (Table 3.6). OTU_01, OTU_02, OTU_04 and OTU_11 showed ≤97% identity with their closest relative in the existing database.

The primer pair ITS1F/ITS4 also amplified sequences belonging to Ascomycota and Basidiomycota groups. Among Ascomycota, 16 fungal phylotypes were recovered affiliating with four different classes i.e. Sordariomycetes, Eurotiomycetes, Saccharomycetes and Dothideomycetes. Whereas among Basidiomycota, twelve phylotypes were amplified belonging to seven different classes as shown in Table 3.6. OTU_24, OTU_27, OTU_35, OTU_39 and OTU_46 were found to show ≤97% identity with their closest relative. One phylotype, OTU_44 clustered with the *Hortaea* sp. with a percentage identity of 98 (Fig 3.6).

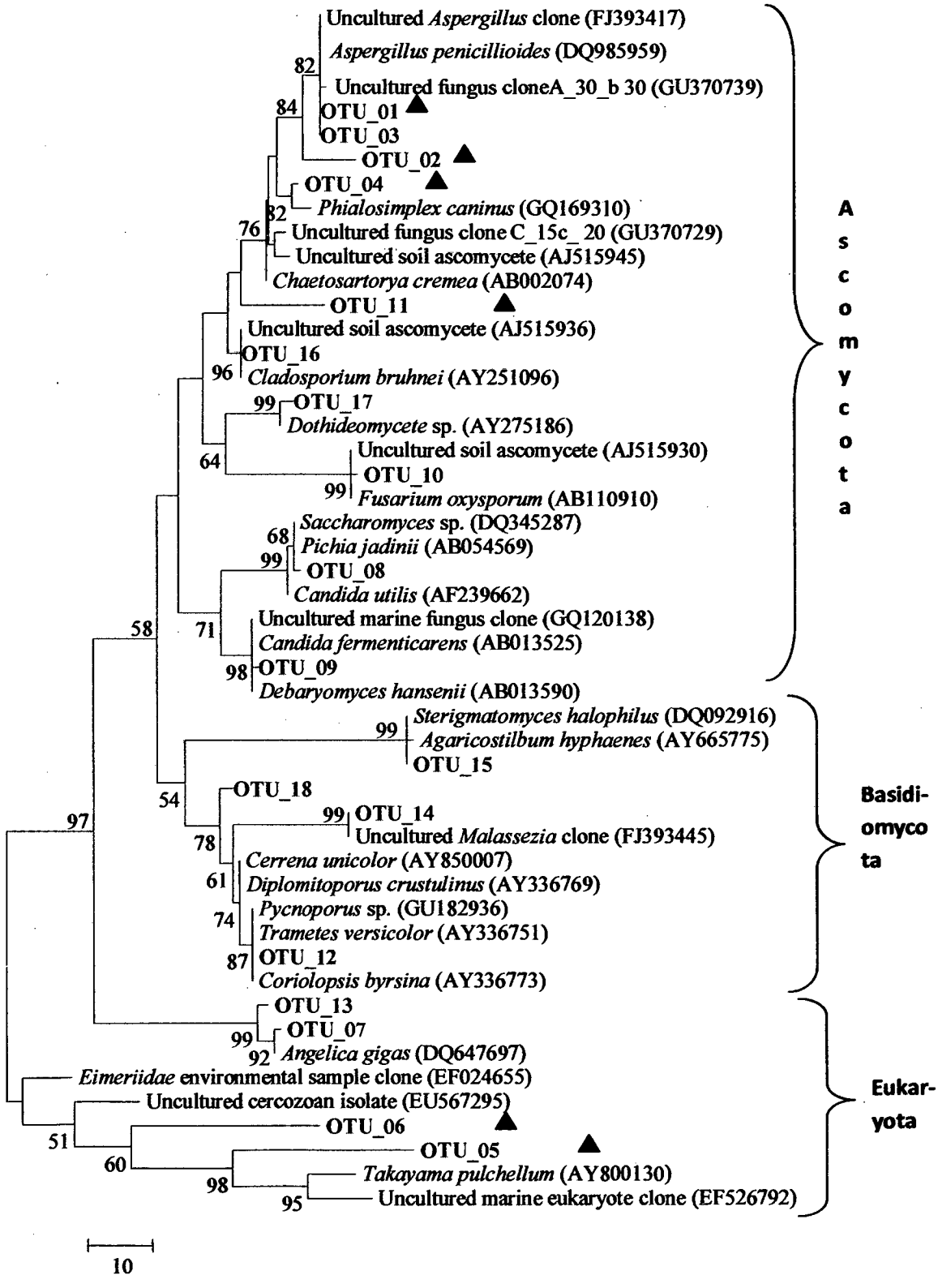


Fig. 3.5 Phylogenetic tree based on fungal 18S rDNA gene sequences of phylum Ascomycota and Basidiomycota after amplification with universal 18S rDNA primer set, NS1/NS2 (Universal 18S rDNA, marked with Red color) for stations of cruise #ABP38. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle.

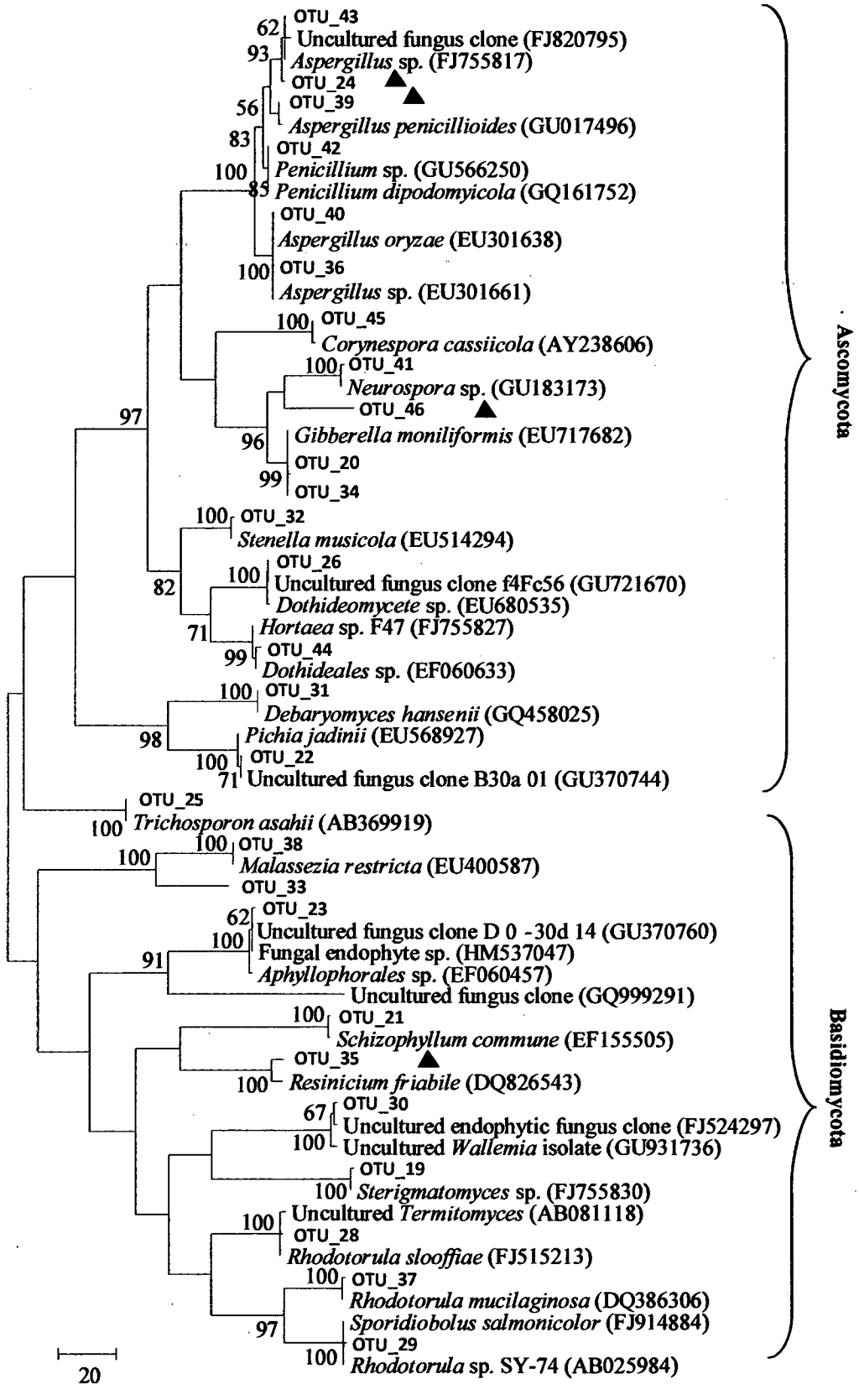


Fig. 3.6 Phylogenetic tree based on fungal ITS gene sequences of phylum Ascomycota and Basidiomycota after amplification with primer set, ITS1F/ITS4 (fungal specific, marked with blue color) for stations of cruise #ABP38. Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. New sequence types are marked with triangle. Ambiguously aligned sequences were removed from the analysis.

3.3.4 Fungal Diversity at Different Stations

Fungal taxa were more or less unevenly distributed at different stations (Table 3.7). Some of these OTUs were found exclusively in station A, B, or C of the cruise #ABP26. Distribution of fungal taxa in stations A, B, and C was 10, 21, and 16, respectively, indicating richness of diversity in station B. Species of *Sagenomella*, *A. penicillioides*, *Stenella musicola*, and *Candida* sp. occurred in high frequency (Table 3.7).

3.3.5 Distribution of Different Subclasses and Species Richness

Seven and five major subclasses of fungi were recovered with ITS and 18S rDNA primers, respectively from the stations A, B and C of the cruise #ABP26 (Fig. 3.7). Compositional difference was observed between stations and occasionally with different depths of the same station. In a few instances, individual primer sets amplified different subclasses from the environmental clone libraries. For example, Sordariomycetes, Tremellomycetes, and Ustilaginomycetes were amplified with ITS primer sets, whereas Wallemiomycetes was amplified only with 18S rDNA primer set (Figs. 3.2, 3.3 and 3.4). Members of Eurotiomycetes were present in all the stations. Members of Sordariomycetes and Dothideomycetes were found to be the major forms in stations A and C, whereas yeasts were dominant in station B. Sequences belonging to Wallemiomycetes were found only in station C. Shannon–Wiener diversity values (H) were 2.5, 3.0, and 4.5 for libraries constructed with universal ITS, fungal-specific ITS, and universal 18S rDNA primers, respectively. The species effort curves for libraries with all the primers were found to show positive slope, and thus no evidence of saturation was found.

The proportional distribution (shown in percentage) of fungal taxonomic groups varied in different clone libraries prepared for the station SVBC-33 (Figs. 3.8 and 3.9). *Trichosporon asahii* (32 %) formed a major portion of sequences amplified by the ITS primer set (Fig. 3.8).

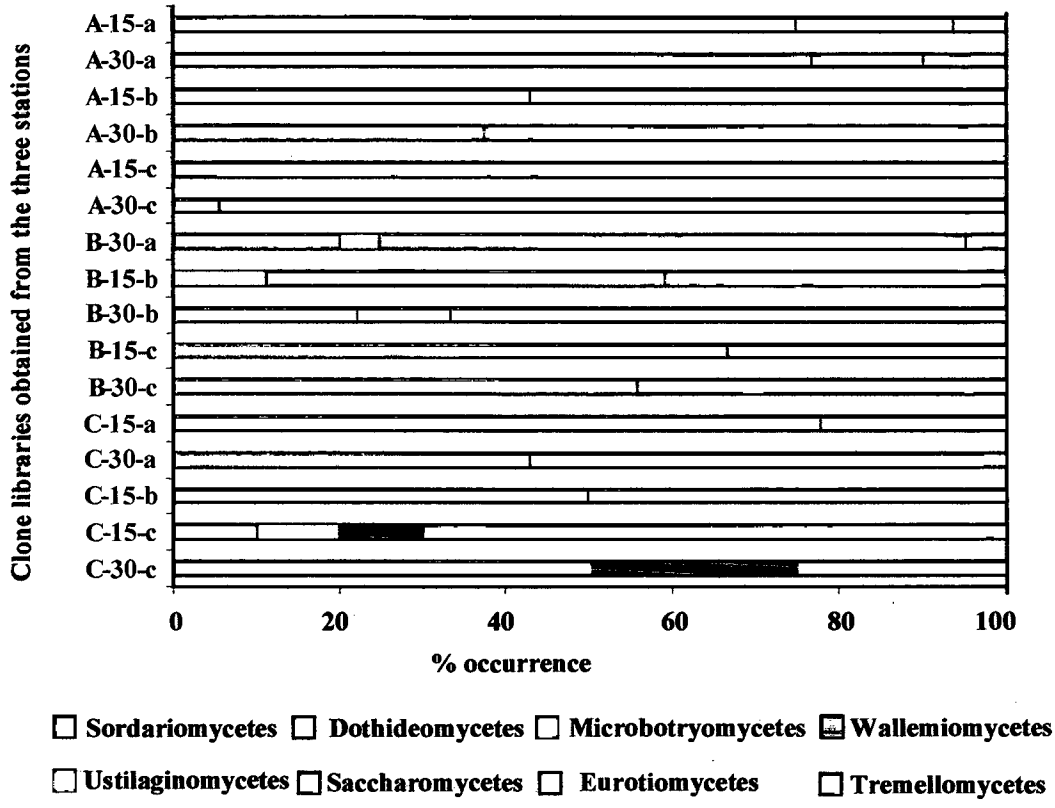
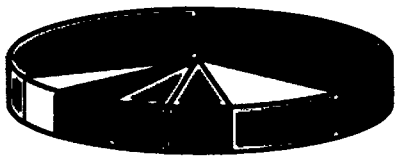


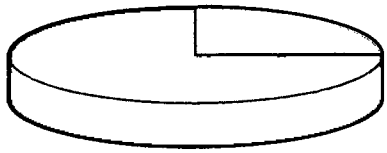
Fig 3.7 Proportional distribution of different taxonomic groups in various clone libraries for stations A, B and C of the cruise #ABP26. Percentage occurrence of sequences of each taxonomic group is indicated on x-axis. Clone libraries are represented on y-axis. The clone libraries are represented in bold, designated with Station number A(SVBC-04), B (SVBC-31) and C(SVBC-37) followed by depth of sediment sample (15 or 30 cm), primer pair used were a (ITS1F/ITS4), b(ITS1/ITS4) and c (NS1/NS2). The clone libraries B-15-a and C-30-b showed biased amplification of a single sequence type due to PCR artefact and were eliminated from the analyses.



- *Trichosporon asahii* (31.6%)
- Unidentified fungal clone S3 (21%)
- Unidentified isolate f4Fc56 (15.8%)
- Uncultured basidiomycete clone (10.5%)
- Uncultured *Wallemia* isolate (5.3%)
- *Aphyllophorales* sp. LM82 (5.3%)
- *Nectria mauritiicola* (5.3%)
- *Rhodotorula calyptogenae* (5.3%)

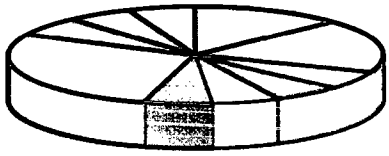
Fig 3.8 Proportional distribution of different fungal taxa in clone library constructed with primer set ITS1F/ITS4 for the station SVBC -33 of the cruise #ABP-26 (Percentage values are shown within brackets).

a



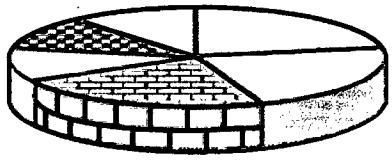
- Uncultured *Aspergillus* clone (75%)
- Uncultured *Malassezia* clone (25%)

b



- *Wallemia* sp. F53 (28%)
- Unidentified isolate f3Fc79 (17.6%)
- Fungal sp. NIOCC#F15 (13.2%)
- *Rhodotorula* sp. SY-74 (5.9%)
- Unidentified basidiomycete clone (5.9%)
- Unidentified isolate f4Fc56 (5.9%)
- *Aphyllophorales* sp. LM82 (5.9%)
- Unidentified fungal clone S3 (5.9%)
- *Nectria mauritiicola* (5.9%)
- Unidentified basidiomycete clone (5.9%)

c



- Uncultured *Malassezia* clone (22.2%)
- *Aspergillus restrictus* (22.2%)
- Uncultured *Aspergillus* clone (22.2%)
- *Wallemia sebi* (11.1%)
- *Candida orthopsilosis* (11.1%)
- *Aspergillus penicillioides* (11.1%)

Fig 3.9 Proportional distribution of different fungal taxa in clone libraries constructed with 18S rDNA primer sets for the station SVBC -33 of the cruise #ABP-26. a: primer set NS1/NS2; b: primer set Euk18S-42F/Euk18S-1492RE and c: Euk18S-555F/Euk18S-1269R Uncultured *Malassezia* clone (22.2%) includes OTU_21 (11.1%) and OTU_27 (11.1%) . (Percentage values are shown within brackets).

Table 3.7 Distribution of closest identified fungal taxa in different stations.

OTU no.	Closest identified relative (GenBank Accession no.)	Number of clones recovered			
		Station A	Station B	Station C	Total
OTU_02, OTU_11	*Sagenomella* sp.(FJ357782)	33	11	27	71
OTU_15, OTU_23	*Aspergillus penicillioides* (DQ985959)	21	20	1	42
OTU_05, OTU_14	*Candida* sp. F15 (FJ755821)	8	26	0	34
OTU_04, OTU_13	*Stenella musicola* (EU514294)	6	10	7	23
OTU_16, OTU_27	*Aspergillus restrictus* (AB002079)	4	6	4	14
OTU_25	*Aspergillus niger* (GQ228449)	3	6	4	13
OTU_01, OTU_10	*Hortaea* sp. F 47 (FJ755827)	2	0	4	6
OTU_28	*Candida glucosophila* (AB013519)	0	3	2	5
OTU_24	*Aspergillus restrictus* (AB008407)	3	2	0	5
OTU_03, OTU_12	*Trichosporon asahii* (AB369919)	3	1	0	4
OTU_26	Uncultured Ascomycota (GQ120138)	4	0	0	4
OTU_21	*Dothideomycete* sp. (EU680535)	0	3	0	3
OTU_18	Uncultured Basidiomycota sp. (FJ889114)	0	3	0	3
OTU_35	*Aspergillus unguis* (EF067336)	0	0	3	3
OTU_36	Uncultured soil basidiomycete (AJ515927)	0	0	3	3
OTU_38	fungal sp. FCAS11 (GQ120154)	0	0	3	3
OTU_17	*Malassezia pachydermatis* (DQ457640)	0	3	0	3
OTU_32	*Phoma herbarum* (AY293777)	0	0	2	2

Table 3.7 contd.

OTU no.	Closest relative (GenBank Accession no.)	Number of clones recovered			
		Station A	Station B	Station C	Total
OTU_33	Uncultured soil Ascomycete (AJ515945)	0	0	2	2
OTU_34	*Wallemia sebi* (AY741380)	0	0	2	2
OTU_19	*Debaryomyces yamadae* (AB054266)	0	1	0	1
OTU_06	*Pichia jadinii* (EU568927)	0	1	0	1
OTU_07	*Nodulisporium* sp. (EF600033)	0	1	0	1
OTU_29	*Aspergillus fumigatus* (GQ169424)	0	1	0	1
OTU_30	*Aspergillus sp.* (FJ864683)	0	1	0	1
OTU_37	*Ulospora bilgramii* (DQ384071)	0	0	1	1
OTU_39	*Capnodium coffeae* (DQ247808)	0	0	1	1
OTU_22	*Bionectriaceae* sp. (FJ821507)	0	1	0	1
OTU_08	fungal sp. ARIZ L365 (FJ612791)	0	1	0	1
OTU_31	Uncultured fungal clone (AB074653)	0	0	1	1
OTU_20	*Candida parapsilosis* (GQ395610.1)	0	1	0	1
OTU_09	*Rhodosporidium sphaerocarpum* (EF643587)	0	1	0	1

Primer pairs are represented by different colors- Blue: ITS1F/ITS4 (Fungal specific), Green: ITS1/ITS4 (Universal ITS), Red: NS1/NS2 (Universal 18S rDNA)

Two sequences, namely uncultured clones of *Aspergillus* and *Malassezia* sp. amplified with 18S rDNA primers set NS1/NS2 showed distribution of 75 and 25% respectively (Fig. 3.9a). The four phylotypes, *Aphyllophorales* sp., *Nectria mauritiicola*, unidentified isolate f4Fc56 and unidentified fungal clone S3 amplified by the ITS1F/ITS4 primer set were amplified by18S-42F/Univ1492RE as well (Fig. 3.9b). Maximum number of distinct fungal sequences was obtained with eukaryotic primer set, 18S-42F/Univ1492RE, out of which *Wallemia* sp. (28%) contributed to the major proportion (Fig. 3.9b). A majority of the sequences amplified by these two primer sets belonged to Basidiomycota. The two sequences amplified by the primer pair NS1/NS2 were amplified by EK555F/EK1269R as well (Fig. 3.9a and 3.9c). There were no common OTUs shared by the primer sets 18S-42F/Univ1492RE and EK555F/EK1269R. The major phylotypes amplified by the primer set EK555F/EK1269R belonged to the class Eurotiomycetes of the phyla Ascomycota (Fig. 3.9c).

3.4 Discussion

3.4.1 Diversity with Multiple Primers

Primer sets for the amplification of two different segments of rRNA gene were chosen in the present study because 18S rDNA is supposed to be conserved in nature and thus discrimination between closely related species is difficult to be resolved. On the other hand, ITS region of rRNA gene is known to give better resolution of taxonomic species, but phylogenetic analyses of unknown sequences cannot be resolved due to its less conserved nature (O'Brien et al, 2005). Therefore, to overcome this problem and get a better diversity estimate, both of these segments were amplified from environmental DNA samples. The use of different primers enabled the recovery of larger and varied number of fungal OTUs from deep-sea sediments than would have been possible otherwise (O'Brien et al, 2005; Jebaraj et al, 2010).

3.4.2 Fungal diversity for the stations A, B and C of cruise #ABP26

A total of 22 and 17 OTUs were recovered exclusively with ITS and 18S rDNA primer sets, respectively. Primer sets for ITS and 18S rDNA region exclusively identified 15 fungal taxa each. Two fungal taxa were commonly recovered with ITS and 18S rDNA primer sets (Table 3.4). Jebaraj et al. (2010) have also recovered a large number of fungal OTUs from anoxic sediments using multiple primer approach. In contrast, yeasts were found to be dominating forms in deep oceans including distantly related forms in a study conducted by the amplification of SSU region with fungal-specific 18S rDNA primers (Bass et al, 2007).

In the previous chapter employing culture-dependent approach, only 12 filamentous fungi and 8 species of yeasts from a total of 188 sediment samples from 20 stations of CIB were isolated during cruise #ABP26. Therefore, diversity observed through culture-independent method (32 distinct species) with multiple primer sets was ~38% higher than that obtained through culture-dependent (20 fungal species) approach from the same geographical location. The need to use different primer sets is also highlighted by the fact that the phylogenetic affinities of OTUs recovered with different primer sets seemed to be different (Table 3.4). In the present study, Asco- and Basidiomycota were the most common phylogenetic sequences identified with ITS and 18S rDNA primers as observed by O'Brien et al. (2005) using the same primer sets. In contrast to our results, O'Brien et al. (2005) recovered sequences belonging to Chytridiomycota and Zygomycota with these primer pairs. Lai et al. (2007) also reported the presence of Asco- and Basidiomycota from methane hydrate seeps using only the fungal-specific ITS primer pair. Multiple sequence analysis approach resulted in identifying eight OTUs showing less than 97% similarity to the existing fungal taxa and thus appeared to be novel. Out of these, six were affiliated to Ascomycota and two to Basidiomycota. Thus, these new sequences add to the existing database for probable novel fungal signatures.

This study suggests that fungal diversity may be heterogeneous in deep-sea sediments as some of the subclasses were restricted to a few stations (Fig. 3.7). Biological communities in deep sea have shown dependence on variables such as substrate availability and type, biogeochemistry, nutrient input, productivity, and hydrological conditions on regional scale (Levin et al, 2001). Substrate heterogeneity was shown to play an important role in structuring nematode diversity in deep-sea sediments (Vanreusel et al, 2010). These authors reported nematode assemblages that varied in hard nodule substratum from soft sediments beneath the nodules. Such small-scale habitat heterogeneity may also impact fungal diversity in deep-sea sediments. Sequences belonging to Chytridiomycota and Zygomycota were recovered by using fungal-specific 18S rDNA primers from marine sources (Bass et al, 2007). Therefore, their absence in the present study suggests that their distribution may be site-specific. Microbial diversity has been shown to be impacted by temporal and spatial dimensions of deep-sea (Sogin et al, 2006).

Out of 39 OTUs, 12 OTUs showed affiliation with sequences already reported from marine sources (Table 3.4), thus adding support to the view that these were possibly typically marine. Out of these 12, two OTUs representing *Sagenomella* sp. and the yeast *Rhodosporidium* sp. were recovered in culture from the CIB in the earlier chapter using culture-dependent approach. This is the first report of occurrence of *Wallemia sebi* from deep-sea sediments. It is a halophilic and osmophilic fungus isolated from hypersaline water of salterns and is described to have successfully adapted to life in extremely saline environments (Kuncič et al, 2010). Its sequence was detected in a marine sponge from Hawaii (Gao et al, 2008) and was also isolated in culturable form from different species of marine sponges of the South China Sea (Liu et al, 2010), suggesting its wide-spread occurrence in marine environment. Cantrell et al. (2006) identified a series of halotolerant fungi, including *Aspergillus, Penicillium, Cladosporium,* and *Hortaea* sp. from solar salterns using ITS1/ITS4 primer set. Using the same primer set, some of these were recovered in the present study also. We are reporting here for the first time on the recovery of sequences showing affiliation with Microbotryomycetes and

Tremellomycetes from deep-sea sediments. The percentage abundance of these subclasses was very low in comparison with other groups, suggesting their low diversity under deep-sea conditions. The class Microbotryomycetes (represented by *Rhodotorula, Rhodosporidium*) includes mycoparasites, phytopathogens, and putative saprotrophs with a diversity of micromorphological characters. Many species contain organelles, termed colacosomes, which are associated with mycoparasitism (Bauer and Oberwinkler, 1991). The recovery of other pathogenic yeasts like *Candida* and *Debaryomyces*, suggests that deep-sea sediments may act as reservoir of such forms. Pathogenic black yeast in mussel and other animal parasitic forms have been reported from hydrothermal vents (Moreira and Lopez-Garcia, 2003; Van Dover et al, 2007). These results are in accordance with earlier observation that oceans are incubators and conveyor belt for pathogenic microorganisms (Harvell et al, 2002).

A large proportion (~ 69%) of fungal sequences recovered in high frequency (Table 3.7) matched with terrestrial taxa, supporting the earlier hypothesis (Raghukumar et al, 2004; Damare et al, 2006, 2008) that they are transported into the deep sea and subsequently adapt to the extreme conditions. One of the most common terrestrial fungus *Aspergillus* has been recovered both by culture-dependent (Damare et al, 2006; Burgaud et al, 2009) and culture-independent methods from various marine habitats (Bass et al, 2007; Lai et al, 2007; Jebaraj et al, 2010). Therefore, the ecological role and adaptations of these ubiquitous terrestrial forms in such extreme conditions need to be further investigated. Takishita et al. (2006) and Shao and Sun et al. (2007) have emphasized the role of sedimentation in accumulating facultative marine fungi in deep-sea sediments. Irrespective of their mode of transport, these facultative marine fungi offer an excellent tool to study physiology of deep-sea-adapted fungi. Fungi-like *Sagenomella* which is reported for the first time from deep-sea by culture-dependent as well as culture-independent approach (Table 3.4) from the same site can be one such model organism. Several of these terrestrial taxa were isolated from marine sponges too, and they exhibited in vitro antifungal properties (Paz et al, 2010)

and anti-tumor and anti-bacterial activities (Liu et al, 2010), suggesting that terrestrial taxa found in the marine environment could be a rich source of new biologically active natural products (Liu et al, 2010).

3.4.2 Fungal diversity for the station SVBC-33 of cruise #ABP26

The diversity study was carried out using a single core of deep-sea sediment from the Central Indian Basin by using four different sets of primers. Previous studies on fungal diversity from the same area in three distantly located (Stations A, B and C) stations did not show a rich fungal diversity and was perhaps due to primer bias and limited number of primers used. Therefore, an attempt was made to study fungal diversity from a single core using four primer sets which included three 18S rDNA primers besides one fungal-specific ITS primer set. The diversity obtained from one sediment core of the station SVBC-33 in the present study with four primer pairs was higher with a total of 8 and 19 fungal OTUs recovered from 48 and 144 clones obtained with ITS and 18S primer sets (relative frequency, 17 and 13 % respectively) than in the previous study. A total of 20 distinct fungal sequences were obtained. In the previous study from the same site (Stations A, B and C), using three primer pairs from three sediment cores and two depths, a total of 9 and 17 fungal OTUs from 240 and 288 clones (relative frequency= 4 and 6 % respectively) were obtained with fungal specific ITS and universal 18S rDNA primer pairs respectively from 16 environmental libraries. Therefore, a multiple-primer approach appears to be better for assessment of fungal diversity. The primers used in the present study have been reported to amplify diverse forms of eukaryotic lineages from mixed environmental samples from various habitats (Lopez-García et al, 2003; Takishita et al, 2005; Stoeck et al, 2006; Edgcomb et al, 2010). However, the absence of eukaryotic sequences other than fungi in the present study was intriguing. All the recovered sequences showed affiliation either with Ascomycota or Basidiomycota phyla whereas, the same fungal specific primers have been known to amplify fungal sequences from Zygomycota and Chytridiomycota also (O'Brien et al, 2005;

Lai et al, 2007; Nagano et al, 2010). Non-detection of these two groups either by culture-dependent or by culture-independent approach suggests their absence or low abundance in sediments of the sampling sites. As suggested by Gao et al. (2010) the fungal primers used may have low specificity towards amplification of 18S rDNA sequences of Zygomycota and Chytridiomycota from mixed environmental samples. Out of the eight fungal OTUs obtained with ITS primer set, 4 were singletons and out of the nineteen OTUs obtained with 18S rDNA primer sets 10 were singletons. Thus, ~ 50 % of the sequences were singletons indicating low abundance of these phylotypes. It also points out that diversity analysis is far from saturation in the present study and a greater number of sampling of clone libraries is required.

Fungal specific primer set ITS1F/ITS4 amplified a sequence (OTU_04), showing high similarity and one novel sequence, showing low similarity with uncultured or unidentified form of the class Wallemiomycetes. One sequence (OTU_24) amplified with EK555F/EK1269R showed high similarity with *Wallemia sebi* which has been isolated from hypersaline waters of saltern and is known to be very efficient halophilic and osmophilic species (Kuncic et al, 2010). This species was also isolated from marine sponges (Gao et al, 2008; Liu et al, 2010). These findings suggest presence of salt tolerant species under such extreme conditions. The sequences recovered with ITS1F/ITS4 primer set showed high number of phylotypes belonging to Basidiomycota which is concordant with the previous studies where basidiomycetous yeasts have been found to be dominating in various deep-sea environments (Takishita et al, 2006; Bass et al, 2007).

One of the noticeable points in the present study was amplification of only two major phylotypes of unidentified *Malassezia* and *Aspergillus* species by the 18S rDNA primer set NS1/NS2. However, PCR artefact or primer bias cannot be attributed to this because these two phylotypes contributed to a large share in the phylotypes amplified by EK555F/EK1269R primer pair too (Fig. 3.9c). These species have been reported as potential pathogenic forms of animals (Lai et al, 2007). In addition, *Aspergillus* species has been reported to play important role in denitrification process from anaerobic marine sediments

off Goa (Jebaraj et al, 2010). This further suggests the versatile role of fungi in major ecological processes in the deep-sea.

The eukaryotic primer sets, 18S-42F/Univ1492RE and EK555F/EK1269R amplified a total of 17 fungal OTUs. Among these 5 sequences (OTU_11, OTU_14, OTU_17, OTU_19 and OTU_24) affiliated with the sequences reported from marine habitat. Out of these, OTU_11 affiliated with the fungal sp. NIOCC#F15, which was obtained in culture from the CIB sediments and was identified as *Sagenomella* species by amplification of its 18S rDNA gene as reported in the previous chapter. Apart from this, OTU_07 and OTU_19 (*Rhodotorula* sp.) and OTU_23 and OTU_26 (*Aspergillus* sp.) were also obtained in culture. There was no overlapping sequence amplified by these two eukaryotic primer sets which suggests that each primer pair amplified 18S rRNA gene sequences not recoverable with other primer pairs.

None of the fungal phylotypes obtained from the sampling site SVBC-33 matched with the phylotypes obtained from the other three cores from the CIB during the same sampling period reported in the earlier study (Stations A, B and C) except *Trichosporon asahii* amplified with ITS primer pair, suggesting a spatial variation in distribution of fungal phylotypes even in not so dynamic habitat such as deep-sea at ~5,000 m depth.

The sequences belonging to Exobasidiomycetes amplified by all the four primer sets used in the present study is being reported for the first time from the deep-sea environment. Its presence in anoxic sediments off Goa (Jebaraj et al, 2010) and in coastal Hawaiian waters (Gao et al, 2010) has been reported and is known to consist of species which are plant pathogen (Begerow et al, 2006). In addition, *Rhodotorula* sp. from Cystobasidiomycetes subphylum is reported as mycoparasite (Bauer and Oberwinkler, 1991).

Proportional distribution of fungal sequences recovered varied for different primer sets (Figs. 3.8 and 3.9). Diverse types of sequences were recovered with eukaryotic primer set 18S-42F/Univ1492RE. This suggests the efficiency of this primer set to amplify diverse forms which is in contrast to the previous studies (López-García et al, 2003; Stoeck et al, 2006) where only a

few fungal OTUs were obtained. In addition, there was hardly any overlap of sequences between different primer sets resulting in detection of higher diversity of fungal forms by multiple-primer approach (Fig 3.9).

A total of seven OTUs affiliating with a percentage similarity of <97% with the existing sequences reported in the database were obtained in the present study. This indicates the efficient nature of the primers used to amplify such novel forms present in deep-sea sediments. These novel forms comprised of mostly the sequences matching with *Aspergillus*, *Malassezia* and unidentified fungal clones suggesting their possibility of being novel marine variants of the existing species. Jebaraj et al. (2010) also reported recovery of novel fungal sequences from suboxic sediments of the Arabian Sea, off Goa by using multiple primer approach.

3.4.2 Fungal diversity observed during the cruise #ABP38

A total of 18 and 28 fungal OTUs recovered from 192 clones each obtained with 18S rDNA and ITS primer sets (relative frequency, 9.3 and 14.5 % respectively). These results are comparable to two studies with the samples, 1. Stations A, B, C and 2. SVBC-33 (Table 3.1) of the cruise #ABP26. The OTUs from the samples of the cruise #ABP38 resulted in a total of 46 distinct fungal species which is quite high in spite of few number of clones screened. This may be due to the substrate, spatial or temporal heterogeneity existing in the deep-sea environment affecting diversity patterns (Sogin et al, 2006; Vanreusel et al, 2010). The singletons obtained during this study were 4 and 10 with 18S rDNA and ITS primer sets respectively, reflecting a lack of saturation level in sampling for clones.

Amplification of four sequences belonging to eukaryotic phylotypes by 18S rDNA primer set was a striking feature of the present study (Table 3.6). Two of them belonged to Alveolata whereas other two affiliated with Viridiplantae. These sequences were not recovered from the sample of the station SVBC-33 of cruise #ABP26 even after amplification with eukaryotic primers. The sites selected for analyses during cruise #ABP26 i.e. 1. Stations

A, B, C and 2. SVBC-33 (Table 3.1) had different geographical locations than the sites during cruise #ABP38 (Table 3.2). Therefore, the recovery of eukaryotic sequences during cruise #ABP38 and their absence in the samples of #ABP26 may be the attributed to the spatial variation in distribution patterns. Edgcomb et al. (2010) reported amplification of similar eukaryotic sequences from subsurface marine environments by using eukaryotic primers. Several other studies have also reported recovery of these eukaryotic phylotypes from different habitats such as Cariaco Basin (Stoeck et al, 2003) the Mid-Atlantic Ridge (Lopez-Garcia et al, 2003) the Guaymas Basin (Edgcomb et al, 2002) and the coastal and equatorial Pacific Ocean (Moonvander Staay et al, 2001). In addition, Lopez-Garcia et al. (2001) reported the amazing variety of alveolates to be the dominating forms among deep-sea plankton. One of the phylotypes in the present study, OTU_05, was detected to cluster with uncultured marine eukaryote clone (Behnke et al, 2010), which was recovered from an O(2)/H(2)S gradient in a Norwegian fjord. The percentage similarity was found to be 86 (Table 3.6), suggesting it to be probable novel species of such alveolate. The recovery of such marine eukaryotic signatures may provide insight into the ecological evolutionary history of protists (Cavalier-Smith, 1998; Moreira and Lopez-Garcia, 2002; Steenkamp et al, 2006).

In addition, the amplification of eukaryotic sequences using 18S rDNA primer set NS1/NS2 has been reported by O'Brien et al. (2005). This primer set amplified eukaryotic sequences from the stations of cruise #ABP38 in the present study also suggesting their efficiency towards amplification of eukaryotic sequences from mixed environmental samples. However, among fungi only Dikaryotic forms could be recovered in the present study whereas O'Brien et al. (2005) reported amplification of other phylotypes of fungi also using NS1/NS2 primer set. The absence of other forms like Zygomycetes and Chytridiomycetes from all the studies conducted with the samples of the CIB suggests their non abundant nature in these environments.

Among other OTUs, most of them clustered with sequences of uncultured fungal clones (Table 3.6). These sequences were also detected in the environmental libraries prepared with samples of stations A, B and C of cruise #ABP26. In spite of sampling from a different site during cruise #ABP38, the recovery of the similar sequences reveals the abundant nature of these fungal signatures in the sediments of CIB. Further a total of four and five OTUs obtained with 18S rDNA and ITS primer sets respectively showed <97% similarity with sequences of existing database suggesting their probability of being novel (Table 3.6). The Ascomycete phylotypes, OTU_24, OTU_39 and OTU_46 showed affiliation with the *Aspergillus* sp. with a percentage similarity of ≤97. This species has also been isolated from marine sponge by other workers (Wang et al, 2008; Liu et al, 2010). The phylotype, OTU_46, showing 95% similarity with *Aspergillus* sp. was also isolated in culture form from the same site, as mentioned in the previous chapter (NIOCC#55). Such high divergence of this phylotype from the existing terrestrial species may be employed in understanding its adaptation and evolution strategies under extreme conditions of deep-sea. Likewise, the recovery of few other phylotypes such as OTU_18 and OTU_44, affiliating with *Cerrena* and *Hortaea* sp., which have also been isolated in culture form, from samples of cruise #ABP38 as reported in the previous chapter, strongly support their possibility to be the native forms of deep-sea. *Cerrena* species has been proved to be a potent source for production of lignin degrading enzymes (Michniewicz et al, 2006; D'Souza-Ticlo et al, 2009) in the terrestrial ecosystems. *Hortaea* species is reported to be a halotolerant and halophilic marine fungi in the earlier studies (Kogej et al, 2005).

One Ascomycete phylotype, OTU_08 belonged to *Saccharomyces* species with a percentage similarity of 99 (Table 3.6). The recovery of this species has been reported for the first time from this area in the present study. Several studies have used this species as a model organism for high pressure stress effects at physiological, genomic and proteomic level (Guerzoni et al, 1999; Abe and Horikoshi, 2000; Iwahashi et al, 2001; Arao et al, 2005; Fernandes, 2005; Domitrovic et al, 2006; Sheehan et al, 2007). The

isolation of this species in culture form in future studies from such extreme environments can help in stress related studies for identification of novel genes responsible for adaptation mechanism.

Other phylotypes recovered during this cruise were also detected in previous studies from station SVBC-33 of CIB during cruise #ABP26. For example phylotype belonging to *Malassezia* sp., was amplified from samples of both the cruises (Table 3.8).The sequences affiliating with *Malassezia* were also recovered from methane hydrate bearing deep-sea sediments (Lai et al, 2007). The Basidiomycete phylotypes, OTU_19 and OTU_15, amplified with both the primer sets were closely related to *Sterigmatomyces* sp., a yeast isolated from the marine areas at Biscayne Bay, Florida, USA (Fell, 1966).

Remaining subgroups amplified from the samples of this cruise were same (Table 3.6) as obtained from two analyses i.e. 1. Stations A, B, C and 2. SVBC-33 of cruise #ABP26 (Tables 3.1, 3.4 and 3.5). From these observations, it is suggested that fungal sequences belonging to some of the classes such as Eurotiomycetes, Sordariomycetes, Dothideomycetes, Agaricomycetes, Saccharomycetes and Wallemiomycetes are abundant in deep-sea sediments of CIB. Also, recovery of marine fungal sequences from this environment suggests that they may not be anthropogenic contaminant and have an ecological role in the deep-sea ecosystems of the CIB. However most of the phylotypes matching with terrestrial species such as *Resinicium, Gibberella, Stenella, Neurospora* and *Corynespora* sp. were also detected suggesting their transport and gradual adaptation under extreme conditions of deep-sea. Attempts can be made to cultivate these terrestrial fungi identified in the clone libraries to determine their adaptive strategies under such extreme conditions of pressure and temperature.

Table 3.8 Fungal phylotypes represented with closest identified relatives recovered during the cruises, #ABP26 and #ABP38 using culture-dependent and culture-independent approaches based on 18S rDNA sequences (Fungal isolate/clone obtained only once i.e. singleton is marked with *)

#ABP26		#ABP38	
Culture-dependent approach	**Culture-independent approach**	**Culture-dependent approach**	**Culture-independent approach**
*Penicillium phialosporum**	*Aspergillus penicillioides* (23 clones)	*Nigrospora oryzae**	Uncultured soil ascomycete (3 clones)
Pezizomycotina sp. (2 isolates)	*Aspergillus restrictus* (15 clones)	*Cladosporium* sp. (2 isolates)	Uncultured fungus clone (2 clones)
Cladosporium sp.*	*Aspergillus niger* (13 clones)	*Trametes versicolor**	Uncultured *Aspergillus* clone*
Sagenomella sclerotialis (3 isolates)	*Candida glucosophila* (5 clones)	*Chaetomium elatum**	*Phialosimplex caninus**
Ascomycete sp. MV_26C (2 isolates)	Uncultured Ascomycota clone (6 clones)	*Aspergillus versicolor* (2 isolates)	*Saccharomyces* sp.*
Tilletiopsis albescens (2 isolates)	*Aspergillus unguis* (3 clones)	*Ascotricha lusitanica**	Uncultured marine fungus clone*
Ascomycete sp. MV_25C*	Uncultured soil basidiomycete (3 clones)	*Pleospora herbarum**	*Pycnoporus* sp.*
*Aspergillus restrictus**	fungal sp. FCAS11 (3 clones)	*Eurotium herbariorum**	Uncultured *Malassezia* clone*
*Capronia coronata**	*Phoma herbarum* (2 clones)	*Cerrena sp.* NIOCC#2a (4 isolates)	*Sterigmatomyces halophilus**
Acremonium sp.*	*Wallemia sebi* (2 clones)	*Penicillium griseofulvum* (2 isolates)	*Dothideomycete* sp.*
Rhodotorula cassiicola, (2 isolates)	*Aspergillus fumigates**	*Sagenomella sp.**	*Cerrena unicolor**
Sporidiobolus johnsonii (4 isolates)	*Aspergillus sp.**	*Hortaea werneckii* (2 isolates)	---
*Rhodosporidium toruloides**	*Ulospora bilgramii**	---	---
*Coniosporium perforans**	*Capnodium coffeae**	---	---
*Graphiola cylindricl**	Uncultured fungus clone*	---	---
*Rhodotorula mucilaginosa**	Uncultured *Malassezia* clone*	---	---
Cryptococcus vishniacii (2 isolates)	Uncultured *Aspergillus* clone*	---	---

In conclusion, the use of multiple primer approach enabled the recovery of diverse forms of fungal sequences from mixed environmental sediment samples from different locations of the Central Indian Basin. The higher diversity obtained in spite of less number of clones screened for station SVBC-33 of cruise #ABP26 and two stations of cruise #ABP38, suggested the abundance of fungal forms under such extreme conditions. Some of the fungal sequences obtained in the present work have been earlier reported from marine environment. This supports their presence and ecological role in various biological processes in this environment. The presence of halophilic forms like *Wallemia* sp. from culture-independent study should be proved by culture-dependent approach using various culturing techniques which may enhance our understanding of salt tolerance genes in deep-sea conditions. Fungi such as *Aspergillus, Penicillium and Cerrena,* species obtained by culture-dependent as well as culture-independent approach (Table 3.8) could be used as a model organism to understand pressure tolerance in fungi. The lack of saturation level with all the primer sets used indicates that the actual diversity of fungi must be greater than detected here. Therefore, exhaustive sampling to obtain near accurate estimation of fungal diversity in deep-sea is recommended. In addition, designing more fungal-specific primers to identify novel species obtained by culture-independent approach is required. It is not appropriate to speculate that all the fungal diversity obtained by using DNA amplification represent viable fungi because low temperature conditions of deep-sea environment will favor preservation of DNA. Thus, it is possible that only a fraction of the uncultured fungi will be viable. Culture-independent approach provides valuable indication of the presence of the organism/community, and in turn information gained could be utilized for their recovery in culturable form using targeted culturing methods.

The methods to isolate the yet-to-be-cultured fungi from deep-sea sediments such as improvement of isolation media, incubation techniques should be evolved so that rare and slow growing forms could be obtained. Biological diversity in this extreme ecosystem may have enormous potential in the development of new products such as pharmaceuticals,

molecular probes, enzymes, cosmetics, nutritional supplements, and agrichemicals (Synnes, 2007). A recently described antibiotic-resistance enzyme from the deep-sea bacterium *Oceanobacillus iheyensis* isolated from 1,050 m depth in the Pacific Ocean (Toth et al, 2010) and a novel λ-carrageenase with no similarity to any reported protein, obtained from a deep-sea bacterium isolated from Suruga Bay, Japan, at a depth of 2,409 m (Ohta and Hatada, 2006) are some of the examples. Deep-sea sediments may harbor new fungal taxa that are tolerant to low temperature and elevated hydrostatic pressure which may lead to discovery of new metabolites or enzymes that are useful for novel biotransformation processes.

Chapter 4

Effect of simulated deep-sea conditions on growth and protein patterns of a few select cultures

4.1 Introduction

Oceans contribute towards major proportion of biosphere with an average depth of ~ 3800 m. Therefore various marine organisms are subjected to such elevated hydrostatic pressure i.e. 38 MPa and low temperature conditions. Since the majority of the biosphere is composed of elevated pressure environments, the effect of such extreme conditions on biological systems needs to be studied. There is a growing interest in understanding microbes and potential applications of them in these extreme environments (Yayanos, 1995; Abe et al, 1999; Abe and Horikoshi, 2001; Bartlett, 2002; Simonato et al, 2006). Evidence of elevated pressure adaptation has been discovered in deep-sea organisms belonging to the lower Eukarya (Atkins, 2000), invertebrates and fishes (Kelly et al, 1999), and even in deep diving marine mammals (Castellini et al, 2002). In general, elevated pressure either inhibits or favors those biochemical processes which occur with an expansion or reduction in system volume, respectively (Somero, 1992). Likewise, mechanisms associated with low-temperature growth of microorganisms involve alterations in cellular membrane fluidity, uptake or synthesis of compatible solutes, structures of macromolecules such as ribosomes and protein synthesis mechanisms (Wemekamp-Kamphuis et al, 2002). Therefore, understanding the connections between high pressure and low temperature can provide valuable insight about the particular signals generated by these stress conditions (Bartlett, 2002).

4.1.1 Effect of elevated pressure and low temperature on microbial cells

Pressures and temperatures of different magnitudes exert different effects on organisms. In general, elevated hydrostatic pressure in the range of several dozen MPa, have been found to be nonlethal, but may affect adversely the organisms which are adapted to atmospheric pressure (Abe et al, 1999; Bartlett, 2002; Abe, 2004). The growth of mesophilic microorganisms have been shown to be inhibited at an elevated pressure of 40-50 MPa. The

cessation of growth is accompanied by morphological changes such as formation of filaments in *Escherichia coli* (ZoBell, 1970) and cell chains or pseudomycelia in the marine yeast *Rhodosporidium sphaerocarpum* (Lorenz and Molitoris, 1992; Lorenz, 1993). Much higher pressures, in the range of 100 MPa may also be used for sterilization purposes (Sonoike et al, 1992; Takahashi, 1992). These effects depend not only on the magnitude but also on the duration of pressure applied in combination with temperature, pH, oxygen supply and composition of culture media (Abe, 2007a). Both low temperature and elevated pressure inhibit an early step of translation (Schwarz and Landau, 1972; Broeze et al, 1978). The cold shock response has been suggested to be an adaptive response to facilitate the expression of genes involved in translation initiation (Jones et al, 1987). Also, elevated pressure, like low temperature incubation, results in the continued synthesis of stringently controlled proteins, involved in transcription and translation despite the growth rate decrease; this behavior also suggests decreased translation capacity (Welch et al, 1993). Finally, both low temperature and elevated pressure decrease membrane fluidity (Chong et al, 1981; MacDonald, 1984), which perturbs a variety of membrane associated processes, including trans-membrane ion and nutrient flux, and DNA replication.

To study the effect of elevated hydrostatic pressure and low temperature some model organisms such as *E. coli*, *Bacillus subtilis*, and the budding yeast *Saccharomyces cerevisiae,* whose complete genomes have been sequenced, are used as powerful genetic tools by several workers (Welch et al, 1993; Abe and Horikoshi, 2000). These microbes can be classified either as piezophilic i.e. high-pressure loving or piezo-tolerant i.e. capable of tolerating elevated pressure but showing better growth at atmospheric pressure. The first pure culture of a piezophilic bacterial isolate, strain CNPT-3 was reported in 1979 (Yayanos et al, 1979). This spirillum-like bacterium showed efficient doubling rate at elevated pressure i.e. 50 MPa, but no growth could be observed at atmospheric pressure conditions even after incubation for several weeks. Numerous piezophilic and piezotolerant bacteria have since been isolated and characterized by the DEEPSTAR group at JAMSTEC, from deep-

sea sediments at depths ranging from 2,500 m-11,000 m (Kato et al, 1995, 1996, 1998). Most of the strains reported were found to be not only piezophilic, but also psychrophilic i.e. low temperature loving, showing no growth at temperatures above 20 °C. In addition, high-pressure induction of heat-shock proteins has been confirmed in other studies with *E. coli* (Haskin et al, 1993; Groβ et al, 1994). Heat-shock proteins may also be induced in piezophiles upon decompression. A stress protein showing similarity to a heat-shock protein was found to be induced in the deep-sea piezophilic hyperthermophilic bacteria *Thermococcus barophilus* subsequent to shift to atmospheric pressure (Marteinsson et al, 1999). Since high pressure and low temperature are known to show similar effects on protein synthesis and membrane structure, the induction of both pressure-shock and cold-shock proteins may represent an attempt by some bacterial isolates to ameliorate the damaging effects of elevated pressure on membrane integrity, translation processes, and the stability of macromolecules.

4.1.2 Effect of simulated deep-sea conditions on growth and protein patterns of fungi

In comparison to bacteria, only a few studies have reported the effect of elevated hydrostatic pressure and low temperature conditions on growth patterns of fungi. Raghukumar et al. (2004), reported germination and growth of spores of A. Sydowii, isolated from deep-sea sediments of Chagos Trench under an elevated hydrostatic pressure and low temperature conditions. In addition, mycelial inocula of fungi isolated from deep-sea sediments of the Central Indian Basin produced substantial growth at both 30° and 5°C under elevated hydrostatic pressure (Damare et al, 2006). Among yeasts, *Saccharomyces cerevisiae* is a facultative anaerobe and has been used in various studies for analyzing the effects of elevated hydrostatic pressure and low temperature. Being a facultative anaerobe, it has been proved as most suitable organism to cope up with the closed pressure vessel condition where the oxygen concentration is very low during incubations (Abe, 2007a). The

genome of this yeast encodes approximately 6000 genes out of which more than 4800 are non essential ones. Recent large scale phenotypic screening of the *Saccharomyces cerevisiae* gene-deletion library has revealed numerous unexpected genes and metabolic pathways that are involved in tolerance of environmental stresses (Martin and Drubin, 2003; Chasse and Dohlman, 2004). Analyses of genome-wide gene expression profiles of this yeast were done showing no growth at elevated pressure and low temperature conditions. However, after being recovered from the stress conditions the genes responsible for transcription of energy metabolism, cell defense and protein metabolism have been found to be highly upregulated (Iwahashi et al, 2003). Also, the survival of *Saccharomyces cerevisiae* at elevated pressures has been shown to be enhanced by preceding heat-shock treatment (Iwahashi et al, 1991). The heat shock treatments confer enhanced synthesis of heat shock proteins (Hsps) and metabolism of trehalose in this yeast (Singer and Lindquist, 1998). Among many Hsps, Hsp104 plays an essential role in acquired tolerance by unfolding denatured intracellular proteins in an ATP-dependent manner (Sanchez and Lindquist, 1990). In addition, in *S. cerevisiae*, the rate of synthesis of several other proteins, e.g., ubiquitin, some glycolytic enzymes, and a plasma membrane protein, is strongly enhanced upon exposure of cells to stress. A plasmid carrying the *TAT2* gene, which encodes a high-affinity tryptophan permease, enabled the cells of *S. cerevisiae* to grow under conditions of pressure in the range of 15 to 25 MPa. Additionally, cells expressing the Tat2 protein at high levels became endowed with the ability to grow under low-temperature conditions at 10 or 15°C as well as at high pressure (Abe and Horikoshi, 2000). Abe and Horikoshi. (1995) found that hydrostatic pressures of 40–60 MPa promoted acidification of the vacuoles in yeast cells and that expression of the tryptophan permease i.e. TAT2 gene can be the rate limiting factor affecting the growth of tryptophan requiring yeast cells.

Apart from *S. cerevisiae,* there are very few other fungal isolates which have been used as model organisms to study the effect of elevated hydrostatic pressure and low temperature stresses. By applying high throughput

techniques these stress conditions need to be applied for understanding their effects on filamentous and unicellular marine fungi too.

Objective

The objective of this chapter is to analyze the effect of elevated hydrostatic pressure and low temperature conditions on spore germination, growth properties and protein patterns of deep-sea fungi isolated from sediments of the Central Indian Basin. The hydrostatic pressure and temperature at the sampling site of the Central Indian Basin at a depth of ~5000 m is ~50 MPa and 3°C respectively.

4.2 Materials and methods

4.2.1 Growth of mycelial fungi and yeasts under simulated deep-sea conditions

Growth and production of biomass of deep-sea filamentous fungi and yeast was compared under simulated conditions of elevated hydrostatic pressure and low temperature. For raising mycelial inoculum, cultures of 16 deep-sea filamentous fungi isolated during the cruise #ABP26 (Table 2.5) were grown in MEB for 3 days at 0.1 MPa and 30°C. Vegetative mycelium prior to the onset of sporulation was homogenized with sterile glass beads. A known weight of finely broken mycelial suspension was inoculated in 5 ml of MEB in pouches made with sterilized gas permeable polypropylene sheets and sealed without trapping any air bubbles. The pouches were suspended in a deep-sea culture vessel (Tsurumi & Seiki Co., Japan) (Fig. 4.1) and incubated at 0.1 MPa/30°C, 20 MPa/30°C, 0.1 MPa/5°C and 20 MPa/5°C for comparing the effect of different pressure and temperature conditions on growth. After 20 days, the contents of the pouches were filtered over pre-weighed filter papers, dried to a constant dry weight and the difference between the initial and final biomass determined as mycelial dry weight (Raghukumar and Raghukumar, 1998). Three terrestrial filamentous fungi were also grown and incubated

under different conditions of elevated hydrostatic pressure and low temperature as mentioned above and were compared with deep-sea isolates. The yeasts isolated during the cruise #ABP26 (Table 2.5) were similarly grown in YPD broth and the biomass was lyophilized and weighed. The biomass of mycelial fungi and yeasts were also compared in media made with sea water (35 ppt) and distilled water.

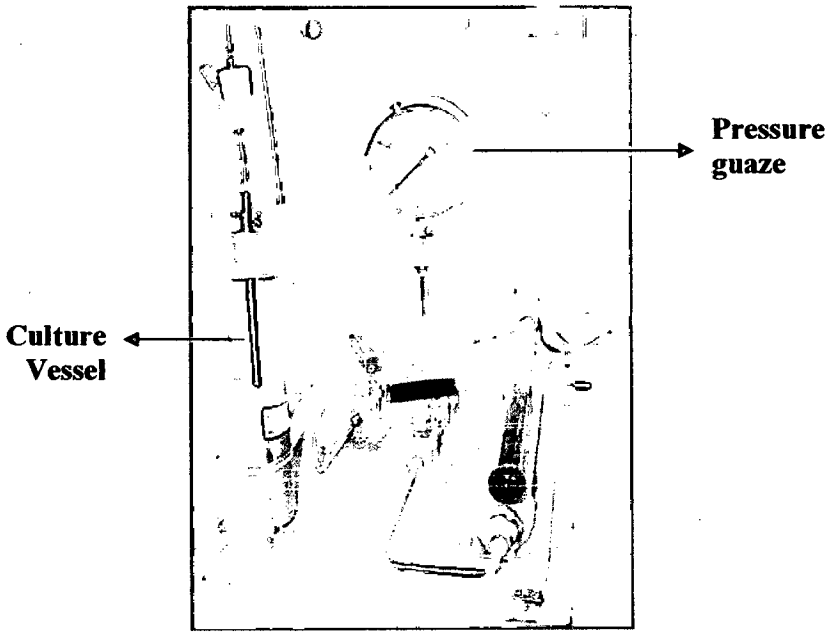


Fig. 4.1 Deep-sea culture vessel.

4.2.2 Comparison of growth and oxido-reductase production of deep-sea fungi with their counterparts from mangroves

Two of the filamentous fungi i.e. NIOCC#F53 and #F61 isolated during cruise #ABP38 (Table 2.6), showed affiliation with *Cerrena* and *Trametes* species respectively. These two species have also been isolated previously from degrading wood samples of mangroves in the lab and are known as efficient producer of oxido-reductase enzymes (D'Souza-Ticlo et al, 2006; Verma et al, 2010). To compare the growth of deep-sea isolates with their counterparts from mangroves all the four cultures i.e. mangrove isolates: NIOCC#2a, NIOCC#DV2, and deep-sea isolates: NIOCC#F53, NIOCC#F61, were grown on MEA plates prepared with 100% seawter. All these four cultures were also

grown on Boyd and Kohlmeyer (B&K) agar medium prepared with 50% seawater (Appendix 6.1h) containing 4 mM guaiacol, a substrate for oxido-reductase enzyme. The production of an intense brown color under and around the fungal colony in guaiacol-supplemented medium indicated presence of laccase activity (D'Souza-Ticlo et al, 2006). To compare the growth under simulated deep-sea conditions, the two deep-sea and two mangrove fungi were grown in MEB for 3 days at 0.1 MPa and 30°C. Vegetative mycelium prior to the onset of sporulation was homogenized with sterile glass beads. A known weight of finely broken mycelial suspension was inoculated in 5 ml of MEB in pouches made with sterilized gas permeable polypropylene sheets and sealed without trapping any air bubbles. The pouches were suspended in a deep-sea culture vessel and incubated at 0.1 MPa/30°C, 20 MPa/30°C, 0.1 MPa/5°C and 20 MPa/5°C for comparing the effect of different pressure and temperature conditions on growth. After 20 days, the contents of the pouches were filtered over pre-weighed filter papers, dried to a constant dry weight and the difference between the initial and final biomass determined as mycelial dry weight (Raghukumar and Raghukumar, 1998).

4.2.3 Spore germination under simulated deep-sea conditions

Four sporulating deep-sea fungi i.e. NIOCC#F2, #F5, #F6 and #F13, two terrestrial fungi i.e. MTCC#IM2, #IM3 and one fungal isolate, NIOCC#85 from Lakshadweep sample were compared for their spore germination properties at elevated hydrostatic pressure and low temperature conditions. All these fungi were grown on MEA agar plates at 0.1 MPa pressure and 30°C, and the spores were collected by gently flooding the plates with sterile seawater. The spore suspension was appropriately diluted with sea water containing 2% Tween 80 and vortexed for 5 min. One ml of this spore suspension was added to five ml of the MEB medium (Damare and Raghukumar, 2008) prepared with seawater and packed in polypropylene pouches. These pouches were suspended in a deep-sea culture vessel and

incubated at 0.1 MPa/30°C, 20 MPa/30°C, 30 MPa/30°C, 0.1 MPa/5°C, 20 MPa/5°C and 30 MPa/5°C. The percentage of germination was calculated by counting germinated spores in 20 microscope fields.

4.2.4 Synchronous effect of different hydrostatic pressures and salinities on spore germination of four sporulating fungi

Four sporulating deep-sea fungi i.e. NIOCC#F2, #F5, #F6 and #F13 were grown on MEA agar plates at 0.1 MPa pressure and 30°C, and the spores were collected by gently flooding the plates with sterile seawater. The spore suspension was appropriately diluted with sea water containing 2% Tween 80 and vortexed for 5 min. One ml of this spore suspension was added to five ml of the sediment extract medium (Damare and Raghukumar, 2008) prepared with three different salinities, 1.7, 17 and 34 ppt and packed in pouches. These pouches were incubated at 20, 30, 40 and 50 MPa pressure at 30°C for 15 days. The germination percentage was calculated by counting germinated spores in 20 microscope fields.

4.2.5 Growth and viability studies of Psychrotolerant and Mesophilic yeast isolates

Among twelve yeast cultures isolated during cruise #ABP26, four were selected for growth studies under elevated hydrostatic pressure and low temperature conditions. Out of these four, two psychro-tolerant yeasts, NIOCC#PY12 and #PY13 identified to be *Cryptococcus* sp. and two mesophilic yeasts, NIOCC#Y1 and #Y3 were *Rhodotorula cassiicola* and *Rhodosporidium toruloides* respectively (Table 2.7). These yeast isolates were maintained on MEA plates by repeated sub-culturing every 20 days. Psychrotolerant yeasts were maintained at 5°C. Yeast cultures were inoculated into 20 ml YPD medium and incubated at 28°C overnight at 150 rpm on a Rotary Shaker Incubator. One ml from the above grown culture was inoculated in fresh twenty ml YPD medium and incubated as described above.

Fresh YPD medium was inoculated with the above grown culture to get an initial O.D. of 0.1 at a wavelength of 600 nm. Cultures were diluted and O.D. was taken at 12 h intervals at 600 nm. Growth curves were constructed for these yeast isolates at three temperatures i.e. 5, 15 and 28°C.

To check the viability under elevated pressure and low temperature conditions, all these four deep-sea yeast and one terrestrial yeast i.e. MTCC#1716, were grown in the 20 ml YPD broth in an incubator shaker at 150 rpm for a period of four days as initial inoculum. One ml of the above grown culture was further inoculated into hundred ml of the YPD medium and incubated for another four days until the absorbance at 600 nm reached a value of 1.0. These cultures were packed in sterile plastic bags and were sealed at both the ends with sealing machine. The packed bags were placed in a pressure vessel and it was filled completely with distilled water. The pressure vessels were incubated at following temperature and pressure conditions for a period of 24 h: 1) 0.1 MPa/30°C, 2) 0.1 MPa/5°C, 3) 30 MPa/30°C, 4) 30 MPa/5°C, 5) 50 MPa/30°C, 6) 50 MPa/5°C. The pressure vessels were opened after the 24 h shock and the cultures were diluted 100 times i.e. 10^{-2} dilution with sterile seawater. Twenty five µl of the above diluted cultures was spread plated on MEA plates in duplicates. The plates were further incubated at room temperature for 48 h until colonies appeared. The viability was determined by calculation of colony forming units (CFU) as follows:

CFU/ml = No. of colonies * volume plated / Dilution factor

4.2.6 Growth curve of NIOCC#PY13 at different conditions of temperature, pressure and nutrient concentrations

Based on growth and viability studies as mentioned above, NIOCC#PY13 was selected for further studies Growth curve was constructed for the psychro-tolerant yeast #PY13 by estimating its growth at different conditions of temperatures, 5, 15 and 28°C, pressures, 20 and 30 MPA and YPD concentrations, 5, 50 and 100%. This isolate was grown to get the starting O.D. of 0.1 at 600 nm as described above. Four ml of this medium was packed

in sterile polypropylene plastic bags and the ends were sealed with a sealing machine (Quickseal, Sevana, India). These bags were placed in four pressure vessels in triplicates. Absorbance at a wavelength of 600 nm was measured at 48 h interval to monitor the growth. Each time one pressure vessel was used.

4.2.7 Intracellular Protein profile of NIOCC#PY13 after giving shocks of different elevated pressure and low temperatures

The psychro-tolerant yeast NIOCC #PY13 was grown in YPD medium at 0.1 MPa/30°C in an incubator shaker at 150 rpm for 48 h. One ml of the above grown culture was inoculated to fresh YPD medium to get an absorbance of 0.1 at a wavelength of 600 nm. Ten ml of the above diluted culture was packed in sterile plastic bags and incubated at the following pressure and temperature conditions for 24 h in the pressure vessels:

1. 5°C
2. 30°C
3. 20 MPa/5°C
4. 20 MPa/30°C
5. 30 MPa/5°C
6. 30 MPa/30°C
7. 40 MPa/5°C
8. 40 MPa/30°C
9. 50 MPa/5°C
10. 50 MPa/30°C

After 24 h shock, the pressure vessels were depressurized and the biomass harvested, washed with sterile distilled water and centrifuged at 3000 g for 5 min. The pellet obtained was used for preparing the total cell protein extracts. Protein extraction was done by Yeast buster protein extraction reagent protocol (Merck, Novagen, U.S.A) (Appendix 6.2a). Protein concentration was estimated spectrophotometrically using Bradford reagent (Sigma, St. Louis, Montana, U.S.A), (Appendix 6.2b). Ten µl protein of each extract (containing equal concentration of protein) was loaded on to 12% resolving gel for electrophoresis (Appendix 6.2c). Electrophoresis was carried out at a constant voltage of 80V. Thereafter the gels were stained by silver staining method (Chevallet et al, 2006) (Appendix 6.2d). The stained gels were photographed using Gel documentation system (AlphaImager 2200, U.S.A).

4.2.8 Statistical analyses

The statistical analyses were carried out in STATISTICA 5.0 for the preparation of 3D scatter plots and Microsoft Excel program software (1-way ANOVA) for growth curves experiments.

4.3 Results

4.3.1 Biomass production and spore germination of fungi under simulated deep-sea conditions

All of the deep-sea fungi, filamentous as well as unicellular yeasts showed growth under 20 MPa pressure and 5°C temperature (Figs. 4.2 and 4.3). In contrast, terrestrial isolates did not show considerable growth at elevated pressure and low temperature conditions (Fig 4.4). One-way analysis of variance (ANOVA) revealed that biomass produced was significantly different at different hydrostatic pressure and temperature conditions for deep-sea fungi (see the legend for Figs. 4.2 and 4.3). Also the deep-sea isolates showed better growth in media prepared with sea water than with distilled water (Figs. 4.5 and 4.6). A one-way ANOVA clearly demonstrated a distinct difference in growth of fungi in seawater versus distilled water (see the legend for Figs. 4.5 and 4.6). The results of ANOVA were significant at 0.01%. However, none of the cultures showed absolute requirement of seawater for growth. All of the mycelial fungi showed sporulation in seawater medium.

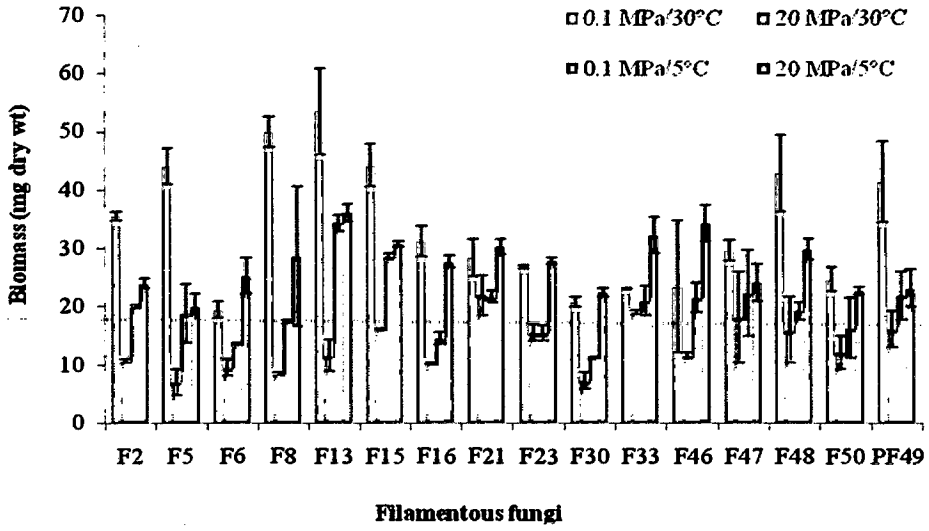


Fig. 4.2 Biomass (mg dry wt/20 ml) of mycelial fungi after 20 days incubation in malt extract broth (MEB) under different pressure and temperature conditions (SD values<10%)

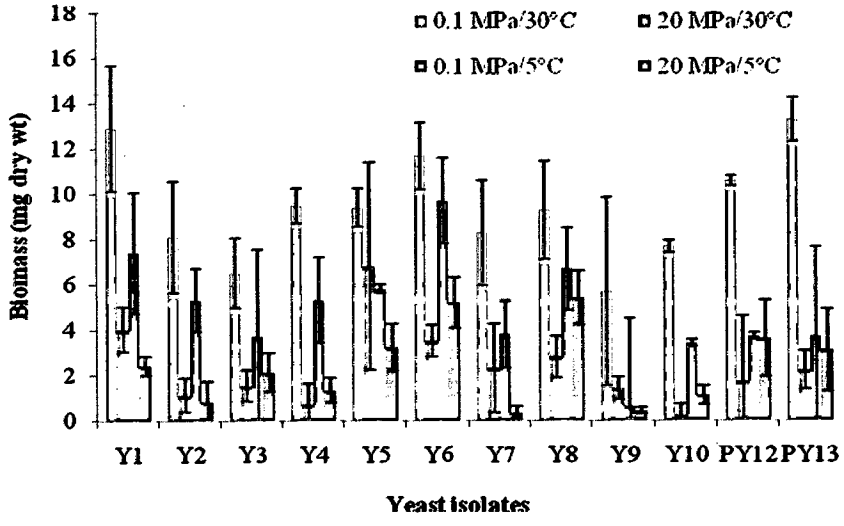


Fig. 4.3 Biomass (mg dry wt/20 ml) of yeasts after 20 days incubation in yeast extract, peptone, dextrose (YPD) broth under different pressure and temperature conditions (SD values<10%)

Results of 1-way ANOVA performed for significance of different growth conditions on production of biomass (Fig. 4.2 & 4.3):

	Fungi	**Yeast**
F critical	2.8	2.8
F value	25.5	30.5
P value	9.06-E-11***	7.83E-11***
Degrees of freedom	63	47

*** significant at>0.01 %. F value greater than F-critical value indicates significant difference

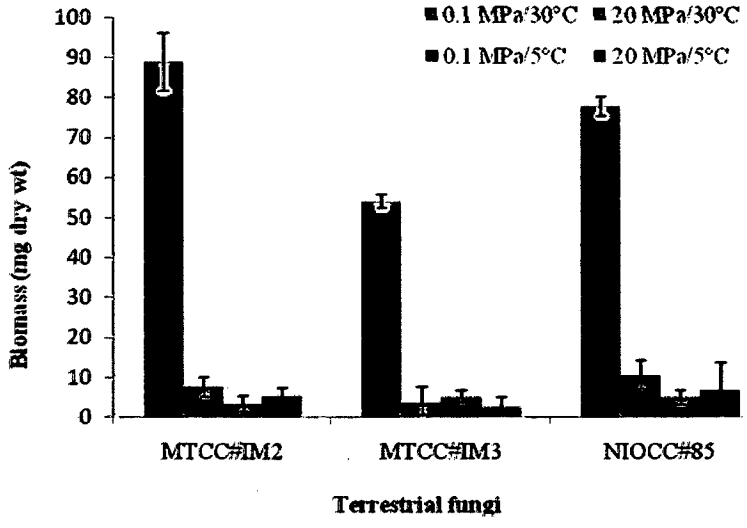


Fig. 4.4 Biomass (mg dry wt/20 ml) of terrestrial fungi after 20 days incubation in MEB under different pressure and temperature conditions (SD values<10%)

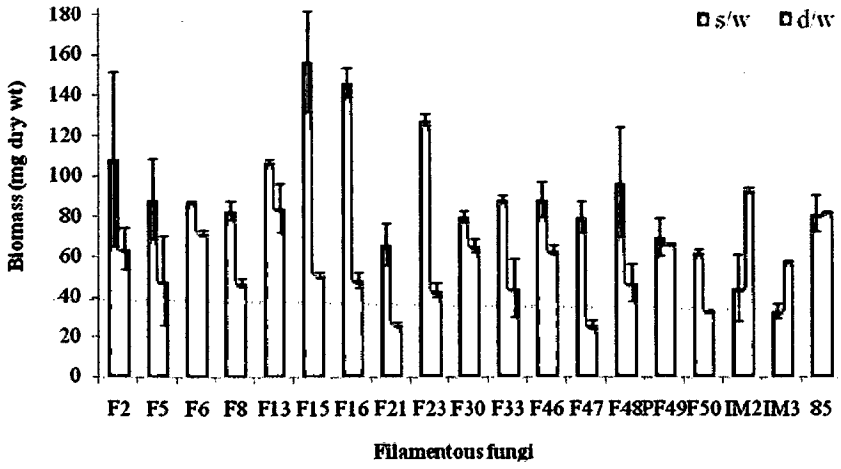


Fig. 4.5 Biomass (mg dry wt/10 ml) of mycelial fungi after 6 days incubation in MEB prepared in sea water (SW) and distilled water (DW) (SD values<10%)

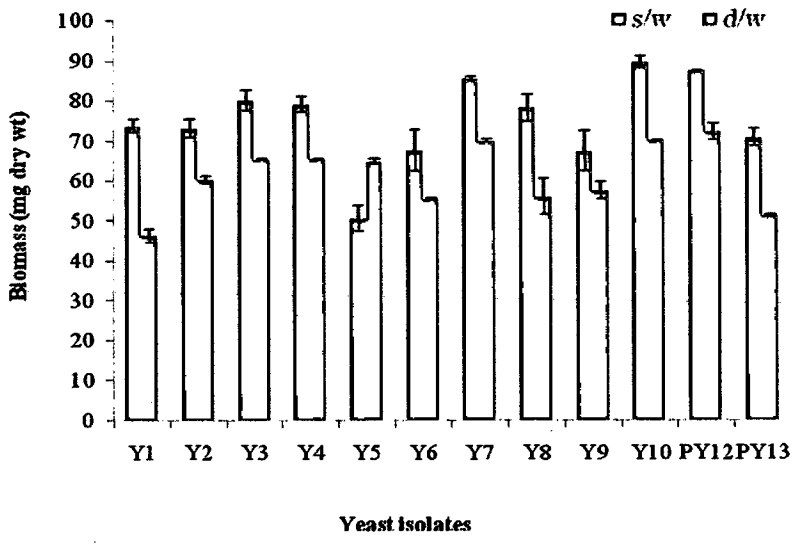


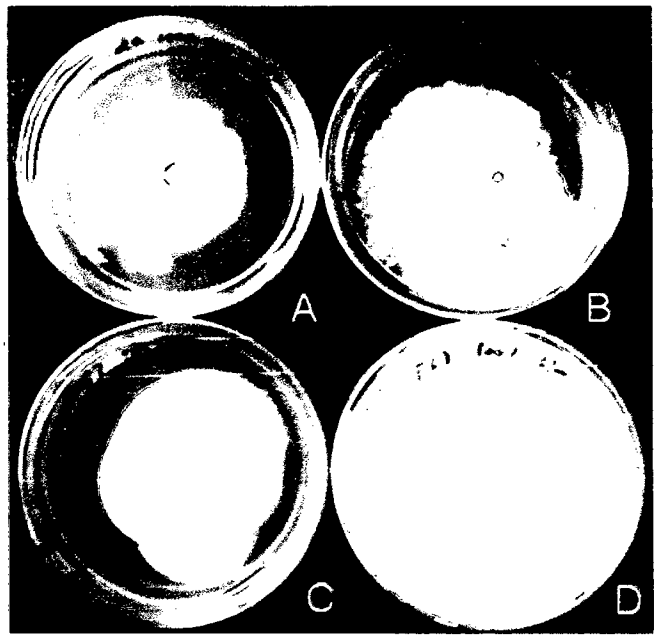
Fig. 4.6 Biomass (mg dry wt/10 ml) of yeasts after 6 days incubation in yeast extract peptone and dextrose (YPD) broth prepared in sea water (SW) and distilled water (DW) (SD values<10%)

Results of 1-Way analysis of variance (ANOVA) performed for the sigtnificance of salinity effect on filamentous fungi and yeasts (data in Fig. 4.5 & 4.6)

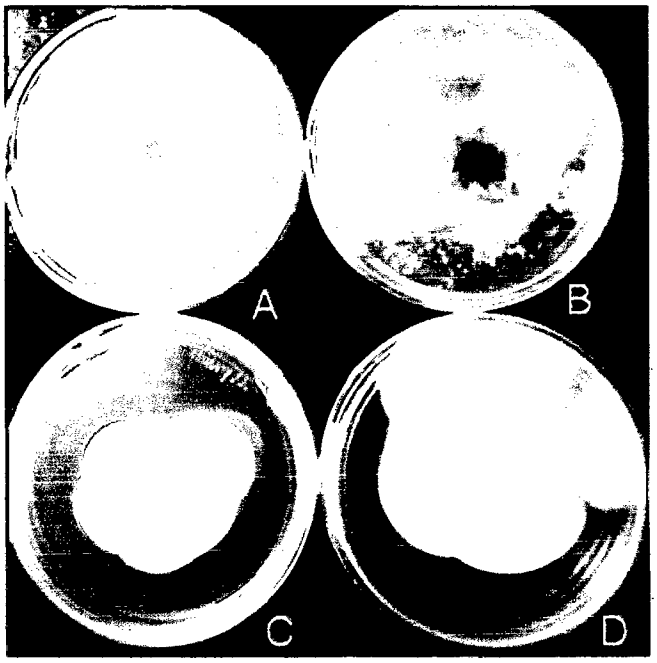
	Fungi	**Yeast**
F critical	4.2	4.3
F value	30.8	13.1
P value	4.9E-06***	0.002***
Degrees of freedom	31	23

*** significant at >0.01 %. F value greater than F-critical value indicates significant difference

Two deep-sea isolates i.e. *Cerrena* and *Trametes* sp. showed better growth on MEA plates prepared with 100% seawater than their counterpart isolates from mangroves (Fig 4.7a). Also, these deep-sea isolates did not show production of oxido-reductase enzyme i.e. laccase on the media plates supplemented with guaiacol whereas mangrove isolates showed brown color, indicating laccase production (Fig 4.7b). Under simulated deep-sea conditions of elevated hydrostatic pressure and low temperature (20 MPa/5°C) deep-sea isolates showed better production of biomass than the mangrove isolates (Fig 4.8).



4.7 a) Growth in media with 100% seawater



4.7 b) Laccase production

Fig. 4.7 Growth characteristics (a) and oxido-reductase production (b) of the deep-sea cultures in comparison to their counterparts, isolated from mangroves.
A. *Cerrena* sp., B. *Trametes* sp. (Mangroves)
C. *Cerrena* sp., D. *Trametes* sp. (Deep-sea)

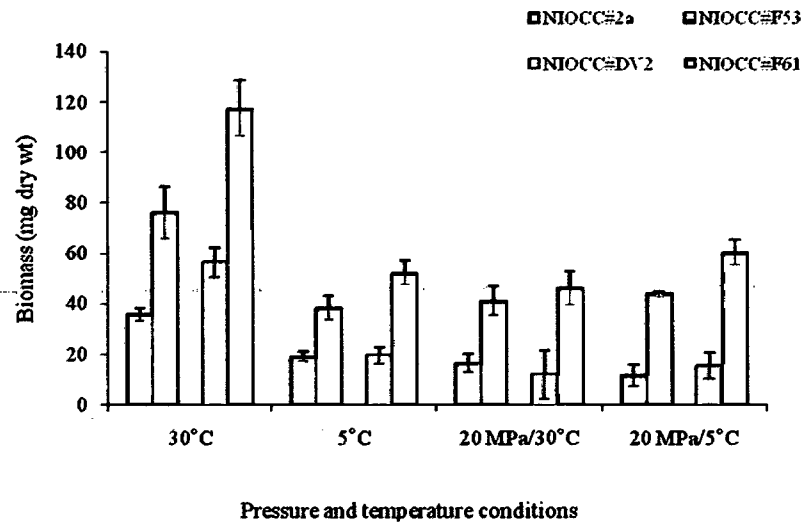


Fig. 4.8 Biomass (mg dry wt/20 ml) of mycelial fungi after 20 days incubation in malt extract broth under different pressure and temperature conditions

NIOCC#2a and #DV2: Mangroves isolates

NIOCC#F53 and #F61: Deep-sea isolates

Spores of four fungi from deep-sea showed germination of spores (Fig. 4.9) under 20 MPa/30°C whereas only one isolate i.e. NIOCC#F13 could germinate at 30 MPa/30°C (Table 4.1). All the terrestrial isolates failed to germinate at elevated pressure even at room temperature. None of the fungi showed spore germination at 5°C both at atmospheric as well as elevated pressure conditions (Table 4.1). In addition, spores from these four sporulating deep-sea fungi showed germination of spores under 20-50 MPa pressure at 30°C and no dependence on salinity (Fig. 4.10). A one-way ANOVA showed statistically significant effect of hydrostatic pressure on spore germination but salinity did not show such effect (see the legend for Fig. 4.10). With increasing hydrostatic pressure, a decrease in spore germination was observed. The four fungi appeared to be euryhaline because the spores germinated from 1.7 to 34 ppt salinity.

Table 4.1 Percentage Germination of spores

Culture no.	0.1 MPa /30°C	20 MPa /30°C	30 MPa /30°C	0.1 MPa /5°C	30 MPa /5°C	20 MPa /5°C
NIOCC#F2	100	76.9	0	No germination		
NIOCC#F5	100	100	0			
NIOCC#F6	100	100	0			
NIOCC#F13	100	76.24	31.25			
MTCC#IM2	100	0	0			
MTCC#IM3	100	0	0			
NIOCC#85	100	0	0			

NIOCC#F2, #F5, #F6, #F13: Deep-sea isolates
MTCC#IM2, #IM3: Terrestrial isolates
NIOCC#85: Isolate from shallow water from a lagoon in the lakshadweep island

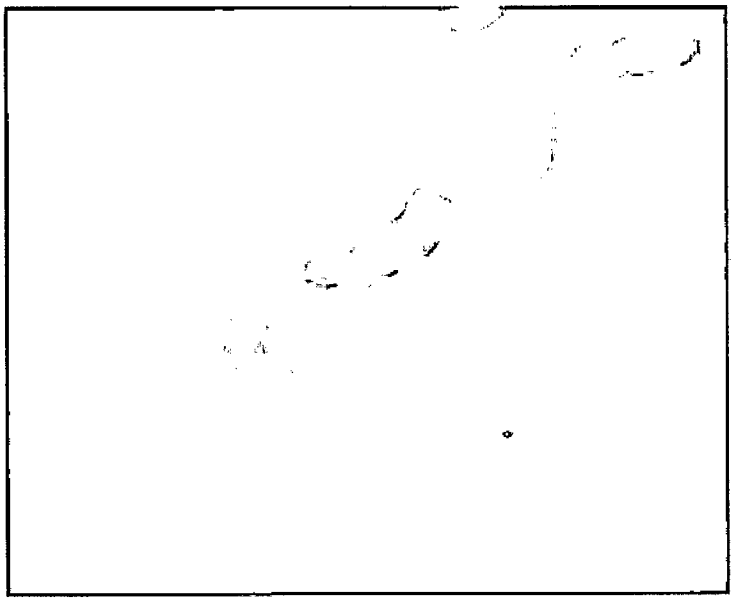
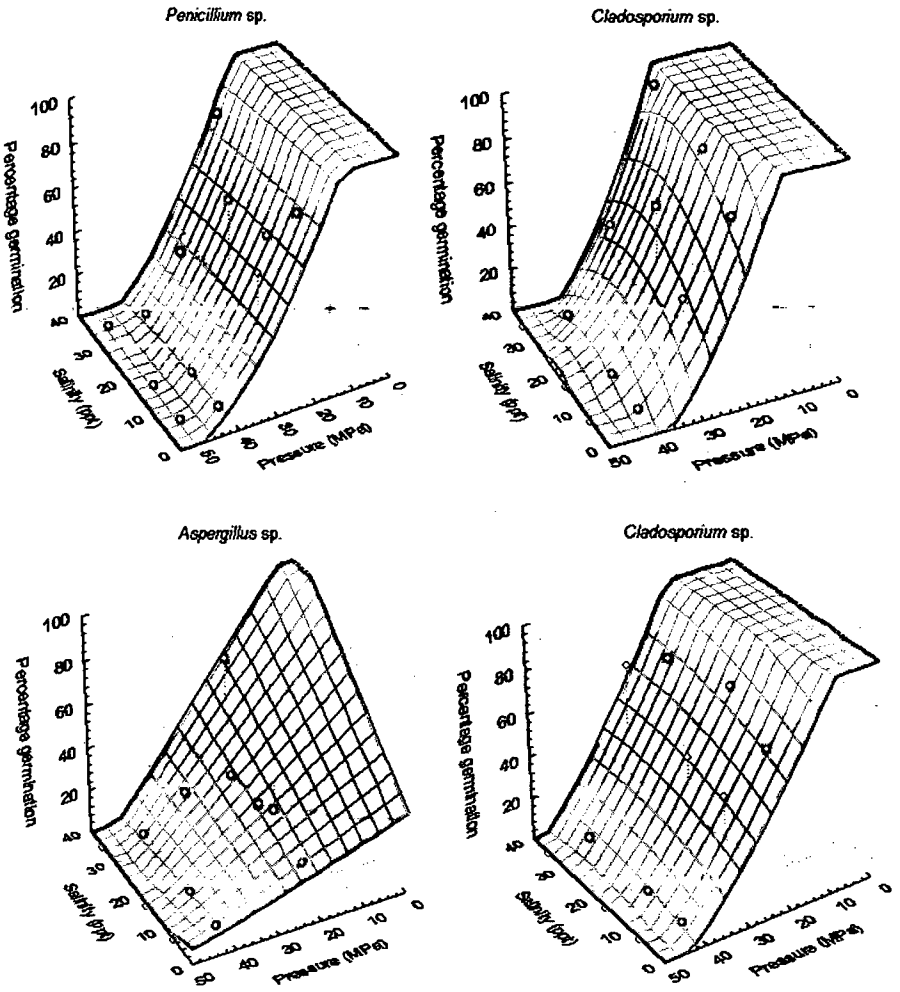


Fig 4.9 Spores of NIOCC#F6 (*Aspergillus sp.*) germinated under 20 MPa pressure for 15 days and stained with calcofluor



Values representing different colors indicate percentage germination of spores

Fig. 4.10 Synchronous effect of different hydrostatic pressure (20, 30, 40 and 50 MPa) and salinity (1.7, 17 and 34 ppt) on spore germination of four fungi after incubation in Malt Extrat Broth for 15 days (SD values<10%).

Results of 1-Way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F2

	Pressure/% germination	**Salinity/% germination**
F critical	4.3	4.3
F value	82.6	1.3
P value	6.7E-09***	0.3
Degrees of freedom	23	23

*** significant at >0.01 %. F value greater than F-critical value indicates significant difference

Results of 1-way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F5

	Pressure vs% germination	**Salinity vs% germination**
F critical	4.3	4.3
F value	78.3	1.9
P value	1.06E-08***	0.2
Degrees of freedom	23	23

*** significant at >0.01 %. F value greater than F-critical value indicates significant difference

Results of 1-way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F6

	Pressure vs% germination	**Salinity vs% germination**
F critical	4.3	4.3
F value	92.2	0.02
P value	2.5E-09***	0.9
Degrees of freedom	23	23

*** significant at >0.01 %. F value greater than F-critical value indicates significant difference

Results of 1-Way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F13

	Pressure vs% germination	**Salinity vs% germination**
F critical	4.3	4.3
F value	80.5	2.0
P value	8.4E-09***	0.2
Degrees of freedom	23	23

*** significant at >0.01 %. F value greater than F-critical value indicates significant difference

4.3.2 Growth and viability patterns of yeast isolates under simulated deep-sea conditions

All the four yeast isolates showed growth at 15 and 28°C. Out of these only two yeasts i.e. NIOCC#Y1 and #PY13 showed some growth at 5°C (Fig. 4.11). The psychrotolerant yeast #PY13 showed higher viability at 50 MPa/5°C than 30°C (Fig. 4.12). Other three deep-sea isolates were also found to be viable under simulated deep-sea conditions. The terrestrial yeast MTCC#1716 showed very less viability under all the elevated pressure and low temperature conditions (Fig. 4.12).

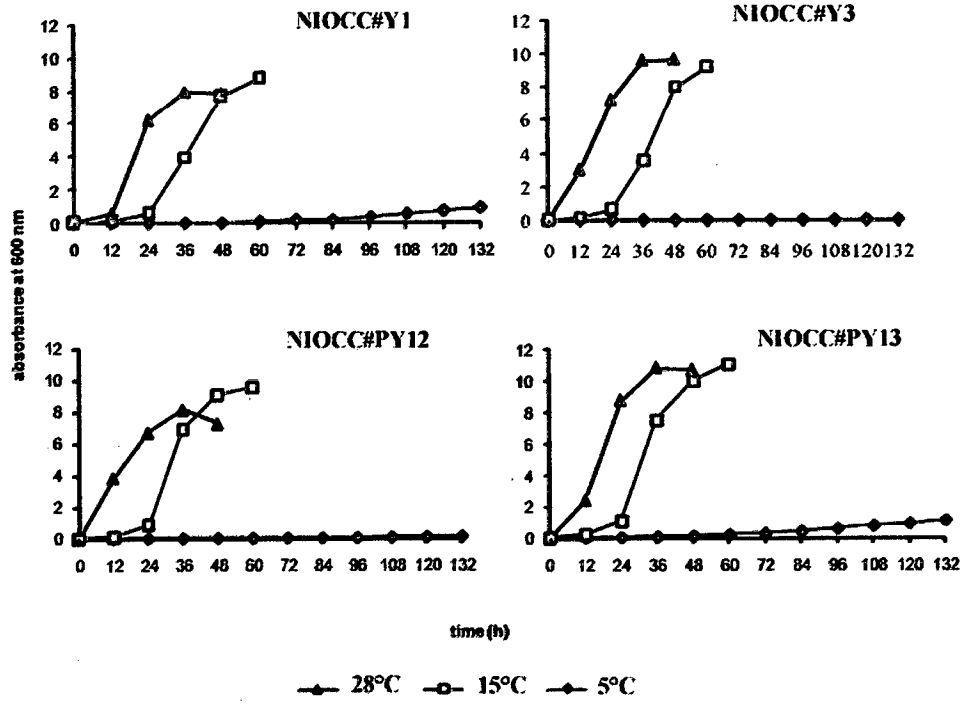


Fig. 4.11 Growth curves of four yeast cultures at different temperatures (SD values <10%)

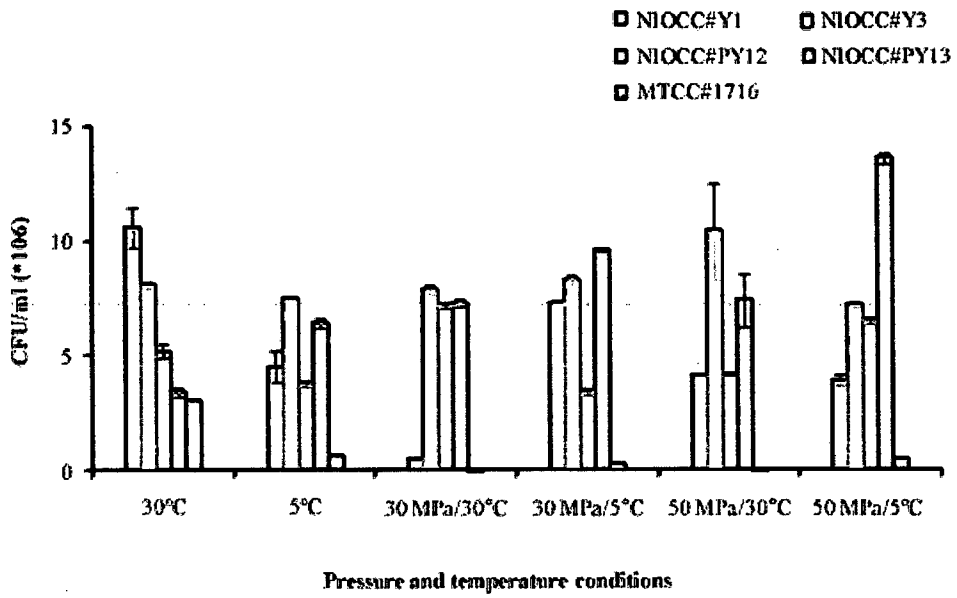
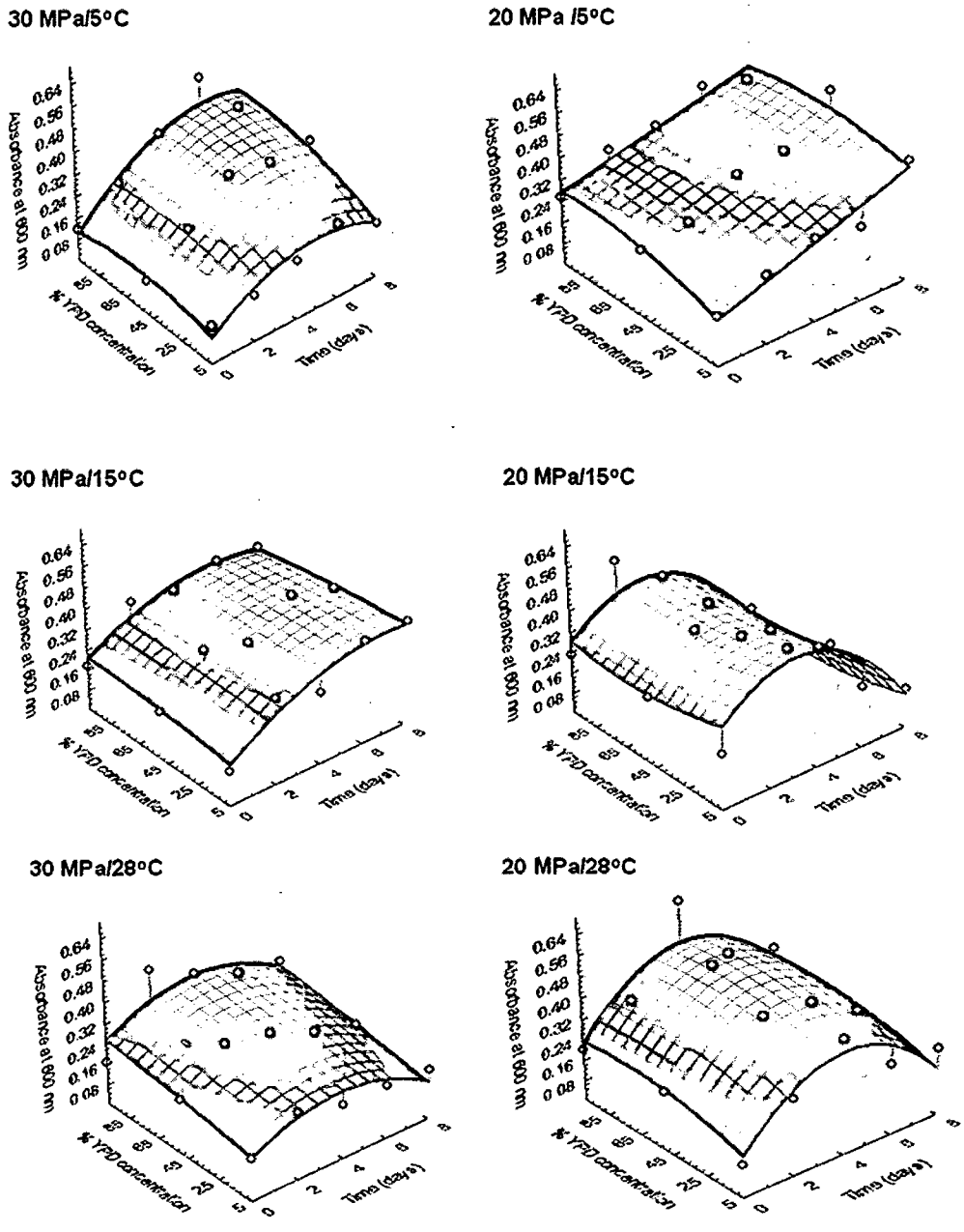


Fig. 4.12 Viability of yeast cultures subjected to different pressure and temperature shocks for a period of 24 h

NIOCC#Y1; NIOCC#Y3; NIOCC#PY12; NIOCC#PY13: Deep-sea Yeasts and MTCC#1716: a terrestrial yeast

4.3.3 Effect of different pressure, temperature and nutrient concentrations on growth of NIOCC#PY13

Low temperature was found to be the major factor affecting the growth of the psychro-tolerant yeast #PY13 than the elevated pressure. The yeast showed better growth at higher nutrient concentration when grown at 5°C (Fig. 4.13). However at 15 and 28°C, the culture was found to grow to the same degree with all the nutrient concentrations. Time taken to achieve maximum growth was highest at 5°C i.e. ~ 8^{th} day whereas at higher temperatures the growth was maximum on 4^{th} day (Fig. 4.13). Best growth was observed at 15°C under all the pressure and nutrient concentrations.



Values representing different colors indicate absorbance at 600 nm.

Fig. 4.13 Surface plot graph showing effect of varying nutrient concentrations, temperature and pressure on growth of NIOCC#PY13

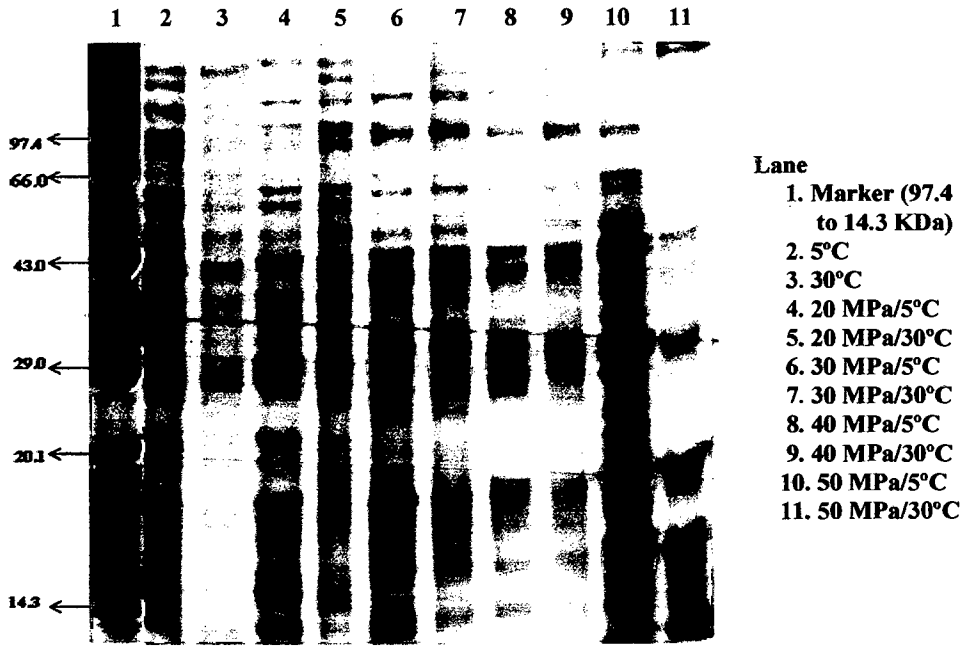


Fig. 4.14 SDS-PAGE for total cellular protein of the yeast NIOCC#PY13 after subjecting them to shocks of different temperature and pressure conditions for a period of 24 h

4.3.4 Protein profiles at different pressure and temperature conditions

Protein profile was found to be different under different pressure and temperature conditions. More number of protein bands were observed at 5°C than 30°C (Fig. 4.14). The protein concentration was also high at lower temperatures. A combination of 50 MPa and 5°C was found to produce maximum number of protein bands. Elevated hydrostatic and low temperature shocks induced production of more number of low molecular weight protein molecules (Fig. 4.14)

4.4 Discussion

4.4.1 Effect of elevated hydrostatic pressure and low temperature on growth and spore germination of fungi

Although most of the fungi isolated from deep-sea sediments of the CIB were terrestrial species, they seem to have adapted to growth in the deep-sea and evolved into distinct physiological species. This was inferred from the fact that all the cultures showed growth at 20 MPa pressure and 5°C. All the filamentous fungi and a few yeasts showed better biomass production at 20 MPa/5°C than at 20 MPa/30°C (Figs. 4.2 and 4.3). These results were concordant with the earlier reports by Damare et al. (2006), where deep-sea fungi belonging to terrestrial sporulating species also showed growth at 20 MPa/5°C. However no correlation has been reported between isolation depths and optimal pressure for growth of these deep-sea fungi (Jannasch, 1987; Yayanos & Delong, 1987). In contrast, three terrestrial sporulating fungi obtained from culture collection belonging to *Aspergillus* sp. did not show increase in biomass at elevated hydrostatic pressure and low temperature conditions (Fig. 4.4) suggesting the adaptation of deep-sea isolates for growth under such extreme conditions. This study supports the hypothesis put forth by Raghukumar and Raghukumar. (1998) and Damare et al. (2006), that terrestrial fungi blown to the sea surface and sinking to the deep-sea sediments have adapted to the alien environmental conditions. These forms may take some time to adapt to the extreme conditions existing in the deep-sea resulting in the adaptation of most sturdy forms whereas other forms either die or remain in dormant but viable state (Raghukumar and Raghukumar, 1998).

Biomass production for deep-sea fungal isolates was much better in seawater medium than in medium prepared with distilled water in filamentous as well as unicellular fungi (Figs. 4.5 and 4.6). However, none of these fungi showed absolute requirement for sea water or NaCl for growth as has been shown for a few deep-sea actinomycetes (Jensen et al, 2005). The fact that the culturable fungi from the deep-sea sediments showed homology to ITS and

18S sequences to those in GenBank does not necessarily imply that they are identical species. It is possible that they are physiologically distinct as was reported for *Aspergillus sydowii*, the fungal pathogen of seafan (Alker et al, 2001). Although being a terrestrial fungus, it was metabolically distinct from nonpathogenic terrestrial strains. This hypothesis is further supported by the comparative studies between two deep-sea isolates i.e. *Cerrena* and *Trametes* sp. and their counterparts from mangroves. These two white rot fungi have been isolated earlier from various terrestrial environments and reported as an efficient source of lignin modifying enzymes (Kim et al, 2002; Elisashvili et al, 2002; Michniewicz et al, 2006). Among these lignin modifying enzymes, laccase is an extracellular enzyme belonging to the copper-containing polyphenol oxidases capable of oxidizing phenols and aromatic amines (Thurston, 1994). Laccase has been found to be produced by many white-rot fungi but also occurs in molds and yeasts as well as in plants and bacteria (Thurston, 1994). Fungal laccases have been reported to take part in the formation of fruit bodies and rhizomorphs, in pigmentation of spores, in sex differentiation and as virulent factors of plant pathogenic fungi. Laccases of soil saprophytic fungi are also involved in the humification of organic residues (Eggert et al, 1996; Gianfreda et al, 1999; Zavarzina et al, 2004). The laccases of ligninoliytic basidiomycetes, the white-rot fungi, where laccases are an essential component of ligninolytic complex, are the most abundant and the most extensively studied (Leonowicz et al, 2001; Rabinovich et al, 2004).

The deep-sea fungi belonging to *Cerrena* and *Trametes* sp. showed better growth on the MEA media prepared with 100% seawater than the mangrove isolates (Fig. 4.7a). Also these two deep-sea isolates did not show any production of laccase enzyme when grown on specific substrates whereas mangrove isolates showed good production of laccase (Fig.4.7b). The adaptation strategies of these fungi under extreme conditions of pressure and temperature are further supported by their better growth under these conditions compared to their counterparts isolated from mangrove (Fig. 4.8). The absence of lignin modifying enzyme production and better growth under simulated deep-sea conditions of these fungi suggests their different physiological

properties than their terrestrial and mangrove counterparts. The gene responsible for the transcription of the above enzyme may be present in non-functional form in deep-sea isolates which can be further supported by their analysis by using molecular approaches such as PCR amplification and sequencing. Recently more interest is growing in using marine fungi as efficient producers of bioactive compounds for bioremediation (Maneerat et al, 2006; Bhadury et al, 2006). Therefore, transformation and expression of such useful lignin modifying enzyme transcribing genes in marine fungi can prove to be beneficial for efficient bioremediation processes.

Spores of four deep-sea fungi i.e. NIOCC#F2, F5, F6 and F13 germinated under elevated pressure conditions whereas spores of other terrestrial fungi failed to germinate under elevated pressure even at 30°C. However spores from none of these fungi germinated at 5°C under elevated hydrostatic pressure or even at 0.1 MPa at this temperature. It has been demonstrated that low temperature and not the elevated hydrostatic pressure is the limiting factor for spore germination of deep-sea adapted fungi (Damare et al, 2006, 2008). The probable reason for no germination at 5°C may be the reduced metabolic activities and cellular processes at low temperature (Wirsen and Jannasch, 1975). Three terrestrial cultures (obtained from a culture collection centre) showed slight mycelial growth under 20 MPa pressure at 5°C as well as at 30°C (data not shown) but their spores failed completely to germinate under these conditions (Table 4.1). Therefore, as reported by Damare et al. (2008) the effect of hydrostatic pressure and low temperature is different on mycelial fragments and fungal spores. Further there was no significant effect of different salinities on spore germination of the above four deep-sea fungi when incubated under a range of 20-50 MPa pressure at 30°C.
In contrast, an elevated salinity was previously shown to increase the maximum hydrostatic pressure for growth in the psychrophiles *Moritella marina* (Palmer and Albright, 1970; Urakawa et al, 1998) and *Streptococcus faecalis,* reclassified as *Enterococcus faecalis* (Schleifer and Kilpper-Balz, 1984; Marquis and ZoBell, 1971) and to decrease the piezo-sensitivity of *Escherichia coli* to an extremely high hydrostatic pressure of 270 MPa

(Hauben et al, 1998). However, the results obtained in the present study support the hypothesis proposed by earlier workers that "feast and famine" condition of deep-sea environment does not seem to have effect on spore germination (Hawker and Madelin, 1976; Damare, 2006). The spores having lower metabolic rates than the mature mycelium under elevated hydrostatic pressure and low temperature do not germinate even after supply of higher concentration of nutrients.

4.4.2 Effect of simulated deep-sea conditions on growth, viability and protein pattern of psychro-tolerant yeast NIOCC#PY13

The yeast NIOCC#PY13 showed comparatively higher growth at 5°C than other three yeast isolates suggesting the psychro-tolerant nature of #PY13 and therefore better adaptation to grow at lower temperatures compared to their mesophilic counterparts. In general, cold adaptability of microorganisms requires structural flexibility, which favors greater complementarity at low energy cost. It provides a rational explanation of the high specific activity of some cold-adapted enzymes (Gerday et al, 2000). Isolation and characterization of DNA-dependent RNA polymerase (Uma et al, 1999), ribonuclease (Reddy et al, 1994) and alkaline phosphatase (Chattopadhyay et al, 1995) from different Antarctic strains have been reported earlier. Other cold-active enzymes have been reported from different cold-tolerant bacteria, including protein-tyrosine phosphatase (Tsuruta et al, 2004), α-amylase, β-galactosidase (Groudieva et al, 2004) and aminopeptidase (Huston et al, 2004). Following a downshift of temperature from 37°C to 18°C, several genes were shown to be transcriptionally upregulated in a strain of *Bacillus subtilis*, using DNA microarray analysis. Among them were the genes, which encode enzymes involved in the degradation of the branched-chain amino acids (Kaan et al, 2002).

Also the viability of the yeast #PY13 was high when subjected to shock at 50 MPa/5°C than at 30°C and ambient pressure conditions (Fig. 4.12). However viability of other mesophilic and terrestrial yeasts was found

to decrease under extreme conditions of deep-sea. In addition, the temperature was found to be the major factor affecting growth of #PY13 when grown at different concentrations of nutrients and elevated pressure. The growth was maximum at 15°C both under 20 and 30 MPa pressure and was not found to be affected by change in nutrient concentrations. However, at 5°C the maximum growth was attained at a higher concentration of nutrients (Fig. 4.13). These results suggest this yeast are adapted to the elevated hydrostatic pressure and low temperature conditions of deep-sea, provided there are sufficient nutrients to increase its metabolic rates. The degree of microbial inactivation by pressure has been reported to depend on the type of microorganism, pH, composition of the media, as well as on the parameters of the process (Patterson et al, 1995; Kalchayanand et al, 1998; Alpas et al, 2000). The viability and growth of microorganisms have been shown by numerous studies to depend strongly on the osmotic pressure (Scott, 1957; Esener et al, 1981) and temperature (Walton and Pringle, 1980; Hottiger et al, 1987; Suutari et al, 1990) of the growth medium. Beney et al. (2001) reported increase of survival of *S. cerevisiae* cells by exposure to an osmotic stress of 100 MPa by a factor of 2.5 after shifting the cells from 25 to 11°C. Same results were obtained in the present study also where a combination of 50 MPa pressure and 5°C enhanced the viability of #PY13 than atmospheric pressure and 30°C.

Protein profile of the yeast #PY13 obtained under different pressure and temperature shock indicated more number of proteins produced at elevated pressure and low temperature conditions (Fig. 4.14). Likewise, a subset of the DAN/TIR cell wall mannoprotein genes, which were well-documented to be anaerobic and cold-inducible genes (Abramova et al, 2001a, 2001b), was dramatically up-regulated by high pressure and low temperature (Abe, 2007b). The moderately barophilie deep-sea bacterium *Photobacterium* SS9 modulates the abundance of several outer membrane proteins in response to hydrostatic pressure (Chi and Bartlett, 1993). One outer membrane porin protein, designated OmpH, which facilitates uptake of larger substrates, has been found to be induced 10-100-fold in mid-log cells of SS9, grown at the

pressure optima of 28 MPa, as compared with mid-log cells grown at atmospheric pressure (Bartlett et al, 1996). Elevated pressure have also been reported to up-regulate a heat shock protein, *HSP12* (Fernandes et al, 2004) which codes for a protein related to cell wall flexibility (Motshwene et al, 2004), suggesting that pressure directly affects cell wall integrity and the cell responds by production of Hsp12p. It has been confirmed that the cell wall protein Hsp12p is highly hydrophilic and acts on the cell wall as a plasticizer in plastic polymers, interrupting the hydrogen bonding and ionic interactions between adjacent polysaccharide polymers that otherwise result in a stable inflexible structure (Sales et al, 2000; Motshwene et al, 2004).

Also, the maintenance of appropriate membrane fluidity is thought to be one of the key factors for survival and growth under elevated pressure conditions, as suggested by the results of studies with prokaryotes (DeLong and Yayanos, 1985; Abe et al, 1999; Allen et al, 1999). Abramova et al. (2001a) speculated that cells exhibited reduced membrane fluidity under hypoxia as a possible outcome of anaerobiosis and at low temperature as a result of reduced lateral diffusion and increased microviscosity. In addition, yeast cells expressing a substantial level of Tat2 i.e. tryptophan permease, became endowed with the ability to grow at low temperatures as well as at high hydrostatic pressures (Abe and Horikoshi, 2000). The induction of more proteins at elevated hydrostatic pressure and low temperature in the present study suggest enhanced expression of some of the genes transcribing essential proteins required for growth under such extreme conditions. These proteins need to be identified using advanced level techniques such as 2D-Mass spectrometry in order to understand their contribution towards survival of cells under deep-sea conditions. Mass spectrometry is an important emerging method for the characterization of proteins after running on 1D or 2D PAGE (Poly acrylamide gel electrophoresis). The protein to be analyzed is ionized first. The two primary methods for ionization of whole proteins are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). MALDI-TOF MS is a relatively novel technique in which a co-precipitate of an UV-light absorbing matrix and a biomolecule is irradiated by

a nanosecond laser pulse (Karas et al, 1987). Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the biomolecule. The ionized biomolecules are accelerated in an elctric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass to charge ratio and reach the detector at different times. In this way each molecule yields a distinct signal. The method is used for detection and characterization of other biomolecules also such as peptides, oligosaccharides and oligonucleotides, with molecular masses between 400 and 350,000 Da. It is a very sensitive method, which allows the detection of low i.e. 10^{-15} to 10^{-18} mole quantities of sample with an accuracy of 0.1 - 0.01 %.

In conclusion, elevated hydrostatic pressure and low temperature is generally assumed to have adverse effects on biological systems, but it can serve as a useful parameter to elucidate dynamic structural changes associated with any reaction. Elevated pressure changes the rate of reactions in a manner separable from those due to low temperature. Accordingly, combined analyses using pressure and temperature offer new insights into cellular functions such as the assembly of macromolecules, membrane trafficking, or membrane protein functions. Understanding the connections between different environmental conditions in living cells may contribute to information about the particular signals generated by stresses and may shed light on sensory transduction and biochemical adaptation in organisms.

Chapter 5

Summary

5.1 Summary

Deep-sea covers ~ 65% area of the total earth surface (Svendrup et al., 1942) and is usually characterized by the extreme conditions of temperature, hydrostatic pressure and nutrient conditions. There are several reports on diversity of bacteria from deep-sea (Li et al, 1999a; Kato and Qureshi, 1999; Schauer et al, 2010) but fungi have not received much attention from such extreme environments. Fungi in marine ecosystem occupy an important position as they are ubiquitous and involved in the decomposition and mineralization of organic matter. Deep-sea fungi were first reported in shells collected from deep-sea waters of 4610 m depth (Hohnk, 1961 & 1969). Recently few studies have reported diversity of fungi from deep-sea environments using culture-dependent as well as culture-independent approaches (Lopez-Garcia et al, 2003; Malosso et al, 2006; Wang et al, 2008; Lai et al, 2007; Bass et al, 2007; Burgaud et al, 2010; Liu et al, 2010). 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). Diverse fungal forms have been reported from the deep-sea sediments of the Indian Ocean at a depth of ~ 5000 m (Damare et al, 2006). The cultures were identified by classical taxonomic methods in these studies. However, the non-sporulating forms could not be identified on the basis of morphological features by this method.

Therefore, the objectives of the present study were to isolate and identify fungi from deep-sea sediments of the Central Indian Basin (CIB) using culture-dependent as well as culture-independent approaches by molecular methods. Further, the effect of simulated deep-sea conditions was analyzed on the growth, spore germination and protein profiles of a few selected cultures in order to understand their adaptation strategies under such extreme conditions.

Sediment samples were collected from 20 and 2 stations of Central Indian Basin during two cruises i.e. #ABP26 and # ABP38 respectively at an average

depth of ~ 5000 m. A total of 28 and 19 fungi were isolated during cruises #ABP26 and # ABP38 respectively using four different isolation techniques and four culture media. Fungal isolates belonged mostly to Ascomycota and Basidiomycota with majority being the filamentous fungi. Pressure Incubation yielded maximum number of fungi (Tables 2.3 and 2.4). Fungi could be isolated from deeper depths i.e. 35-40 cm of sediment core also (Table 2.6). Best medium for isolation was found to be MEA during cruise #ABP26 whereas both CDA and PDA yielded maximum number of fungi during the cruise # ABP38 (Tables 2.5 and 2.6). The fungi were identified by classical taxonomic method as well as by amplification and sequencing of their 18S and ITS regions of SSU rDNA. During the cruise #ABP26, fungi belonging to the phylum Ascomycota were clustered into 7 subgroups, namely *Aspergillus* sp., *Sagenomella* sp. (Fig 2.4), *Exophiala* sp. (Fig 2.5), *Capronia* sp., *Cladosporium* sp., *Acremonium* sp. and *Tritirachium* sp. based on 18S sequences (Fig. 2.6). Based on ITS sequences they grouped into 6 clusters showing maximum similarity to *Aspergillus* sp., uncultured member of Hypocreaceae family, *Exophiala* sp., an uncultured soil fungus clone DQ682584.1, *Hypocreales* and *Trichothecium* sp. (Fig. 2.7). Species of filamentous fungi *Sagenomella*, *Exophiala*, *Capronia*, and *Tilletiopsis* are being reported for the first time from the deep-sea sediments. Majority of the yeast belonged to genera *Sporobolomyces* sp., *Sporidiobolus*, *Rhodosporidium*, *Rhodotorula* and *Cryptococcus* sp. of the phylum Basidiomycota. The cultures were deposited in a recognized microbial culture repository, Microbial Type Culture Collection (MTCC) at Chandigarh, India, under the accession numbers, MTCC9332-MTCC9334 and MTCC9336-MTCC9341.

During cruise #ABP38, a total of 19 fungi were isolated belonging to 12 distinct genera. Majority of the forms belonged to Ascomycota on the basis of 18S sequences and included members from *Aspergillus* sp., *Eurotium* sp., *Cladosporium* sp., *Pleospora* sp., *Chaetomium* sp., *Ascotricha* sp., *Penicillium* sp., *Sagenomella* sp. among filamentous fungi (Fig. 2.10). Two isolates matched

with a black yeast i.e. *Hortaea* sp. Among basidiomycota, two fungal genera belonging to *Cerrena* and *Trametes* sp. were identified (Table 2.8). . The isolation of *Cerrena* and *Tremetes* sp. is being reported for the first time from the deep-sea sediments of the Central Indian Basin. Some of the fungi such as *Aspergillus*, *Cladosporium*, *Penicillium* and *Sagenomella* sp. were isolated during both the cruises, #ABP26 and #ABP38. Diversity and species richness of the fungi was higher during the cruise #ABP26 than #ABP38 (Tables 2.9 and 2.10).

Diversity of fungi was also evaluated from deep-sea sediments of CIB collected during two cruises #ABP26 and #ABP38 using culture-independent approach. Sediment DNA samples from the three stations (A, B and C) were amplified using fungal-specific ITS1F/ITS4, "primer pair a" as well as universal ITS1/ITS4, "primer pair b", and universal 18S rDNA NS1/NS2 primer set, "primer pair c". For the station SVBC-33of the cruise #ABP26, sediment DNA sample was amplified using fungal-specific primer pair, ITS1F/ITS4 as well as universal 18S rDNA primers, NS1/NS2, Euk18S-42F/Euk18S-1492RE and Euk18S-555F/Euk18S-1269R (Tables 3.1 and 3.3). Sediment DNA samples isolated during the cruise #ABP38 were amplified using fungal specific ITS1F/ITS4 and universal 18S rDNA NS1/NS2 primer set (Tables 3.2 and 3.3). A total of 768 clones were sequenced from the 16 environmental libraries for the 3 stations (A, B and C) of the cruise #ABP26. Of the resultant sequences, 257 sequences were found to be fungal, resulting in a total of 39 OTUs and 32 distinct taxa, after clustering on a basis of 98% sequence identity criterion. For the station SVBC-33, a total of 48 and 144 clones from ITS and 18S primer pairs respectively were sequenced from the environmental libraries. This resulted in 27 fungal OTUs and 20 distinct fungal species. During the cruise #ABP38, a total of 192 clones resulted in 46 OTUs found to be affiliating with distinct fungal species. All the OTUs obtained during these three studies belonged to Ascomycota and Basidiomycota.

Among Ascomycota the major classes amplified were Sordariomycetes, Dothideomycetes, Eurotiomycetes and Saccharomycetes. Basidiomycete phylotypes included members from Tremellomycetes, Agaricomycetes, Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Wallemiomycetes, Exobasidiomycetes and Ustilaginomycetes. During cruise #ABP38, two OTUs affiliating with eukaryotic sequences, other than fungi were also recovered (Table 3.6). Some of the OTUs during all the three studies matched at a % similarity of ≤ 97 to their closest relative suggesting them to be novel phylotypes (Tables 3.4, 3.5 and 3.6).This is the first report of occurrence of a halophilic and osmophilic fungus *Wallemia sebi* from deep-sea sediments. Phylotype belonging to *Malassezia* sp., was amplified from samples of both the cruises. Some of the classes such as Eurotiomycetes, Sordariomycetes, Dothideomycetes, Agaricomycetes, Saccharomycetes and Wallemiomycetes recovered during all the three studies (Tables 3.1 and 3.2) suggesting them to be abundant in deep-sea sediments of CIB. Fungal OTUs affiliating with *Aspergillus, Penicillium and Cerrena* species obtained by culture-dependent as well as culture-independent approach (Table 3.8). Higher diversity of fungi was observed by using multiple primer approach with the station SVBC-33 in spite of screening less number of clones (Table 3.5). Some of the OTUs recovered affiliated with sequences of fungi which have been reported previously from deep-sea environment (Tables 3.4, 3.5 and 3.6).

Effect of simulated deep-sea conditions on growth, spore germination and protein profiles of a few selected cultures was analyzed. All of the deep-sea fungi, mycelial as well as the yeasts isolated during the cruise #ABP26, showed growth under 20 MPa pressure and 5°C temperature suggesting their adaptation to grow under such extreme conditions (Figs. 4.1 and 4.2). Also these isolates grew better in media prepared with seawater than with distilled water. Two Basidiomycete fungi belonging to *Cerrena* and *Trametes* sp. were compared for their growth under simulated deep-sea conditions and production of oxido-reductase enzyme.

Deep-sea isolates grew better at elevated pressure and low temperature and did not show production of oxido-reductase enzyme in contrast with mangrove counterparts suggesting their different physiological characteristics (Fig. 4.6). Spores of four sporulating deep-sea fungi showed germination of spores under 20-50 MPa pressure at 30°C and no dependence on salinity (Fig. 4.9). Among yeast isolates, psychro-tolerant yeast NIOCC#PY13 showed considerable growth at 5°C. It also showed higher viability at elevated 50MPa/5°C than 30°C (Fig. 4.11) suggesting its adaptation to extreme conditions of deep-sea. This yeast grew better with higher nutrient conditions when grown at 5°C whereas the grwoth was not found to be afftected by nutrients at 15 or 28°C. The protein profiles observed were distinct under different pressure and temperature stresses suggesting the role of different stress proteins in survival of the yeast #PY13 under extreme conditions of deep-sea (Fig. 4.13). These proteins further need to be identified using advanced level techniques such as 2D-Mass Spectrometry in order to understand their contribution towards survival of cells under deep-sea conditions.

5.2 Conclusions

Major inferences that could be drawn from this study are:

- Diversity of culturable as well as uncultured fungi was found to be comparatively low in deep-sea sediments of the Central Indian Basin.
- Fungal isolates could be identified till genus or species level using molecular approaches for identification.
- Fungal communities detected in deep-sea sediments mostly belonged to phyla Ascomycota and Basidiomycota using both culture-dependent and culture-independent approaches. No other groups could be recovered.
- Culturable filamentous fungi are mostly from ascomycetes whereas, yeast belonged to basidiomycetes. Some of the species such as *Tilletiopsis, Sagenomella Exophiala and Cerrena* spp.are being reported for the first time from the deep-sea.

- ❖ Fungal OTUs belonging to marine sequences in existing database were detected in all the studies.
- ❖ The recovery of fungi such as *Aspergillus, Penicillium* and *Cerrena* species with both the culture-dependent as well as culture-independent approaches suggest them as the major component of fungal community in the deep-sea.
- ❖ Occurrence of similar types of phyla/subclasses in the environmental libraries from different stations of the CIB suggests their abundant nature in such extreme environments.
- ❖ Increase in biomass and spore germination of the cultures isolated from deep-sea sediments suggests their adaptation under such extreme conditions.
- ❖ Increased viability of one of the psychro-tolerant yeast at elevated hydrostatic pressure and low temperature conditions suggests its survival at such extreme conditions.
- ❖ The protein patterns observed for the above yeast at different pressure and temperature conditions were distinctly different.

The protein profiles observed under different elevated pressure and temperature shocks were distinct and need to be identified by using advanced techniques in order to understand the cellular mechanism of yeast under stress. The following analysis is being carried out as a continuation of the present study.

Suppression Subtractive hybridization (SSH) for identification of differentially expressed genes:

Suppression Subtractive hybridization (SSH) is a powerful technique that may be used to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. Although there are several different methods, the basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA, containing specific i.e. differentially expressed transcripts as **tester,** and the reference cDNA as **driver.** Tester and driver cDNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining un-hybridized cDNAs represent genes that are expressed in the tester yet absent from the driver mRNA. Sets of genes or proteins associated with salinity adaptation have been effectively identified in non-model organisms by the SSH, proteomics and cDNA microarray experiments that are based on normalized cDNA libraries from non-model species or on cross-species hybridization (Gracey et al, 2001; Podrabsky and Somero, 2004; Buckley et al, 2006). SSH used in conjunction with high throughput differential screening has been reported for rapid and easy identification of differentially expressed genes in yeast and mycelia forms of *Ophiostoma piceae* (Dogra and Breuil, 2004). Marques et al. (2004) reported identification of genes preferentially expressed in the pathogenic yeast phase of *Paracoccidioides brasiliensis*, using suppression subtraction hybridization and differential macroarray analysis.

Elevated pressure and low temperature are also known to induce expression of various genes in yeast enabling it to survive under such extreme conditions (Iwahashi et al, 2003; Domitrovic et al, 2006). In my study, I tried to isolate total RNA from yeast cells after giving shocks of elevated hydrostatic pressure and low temperature according to manufacturer protocol (Takara Clontech, U.S.A.) (Fig. 5.1A). It was followed by purification of total mRNA (Fig. 5.1B).

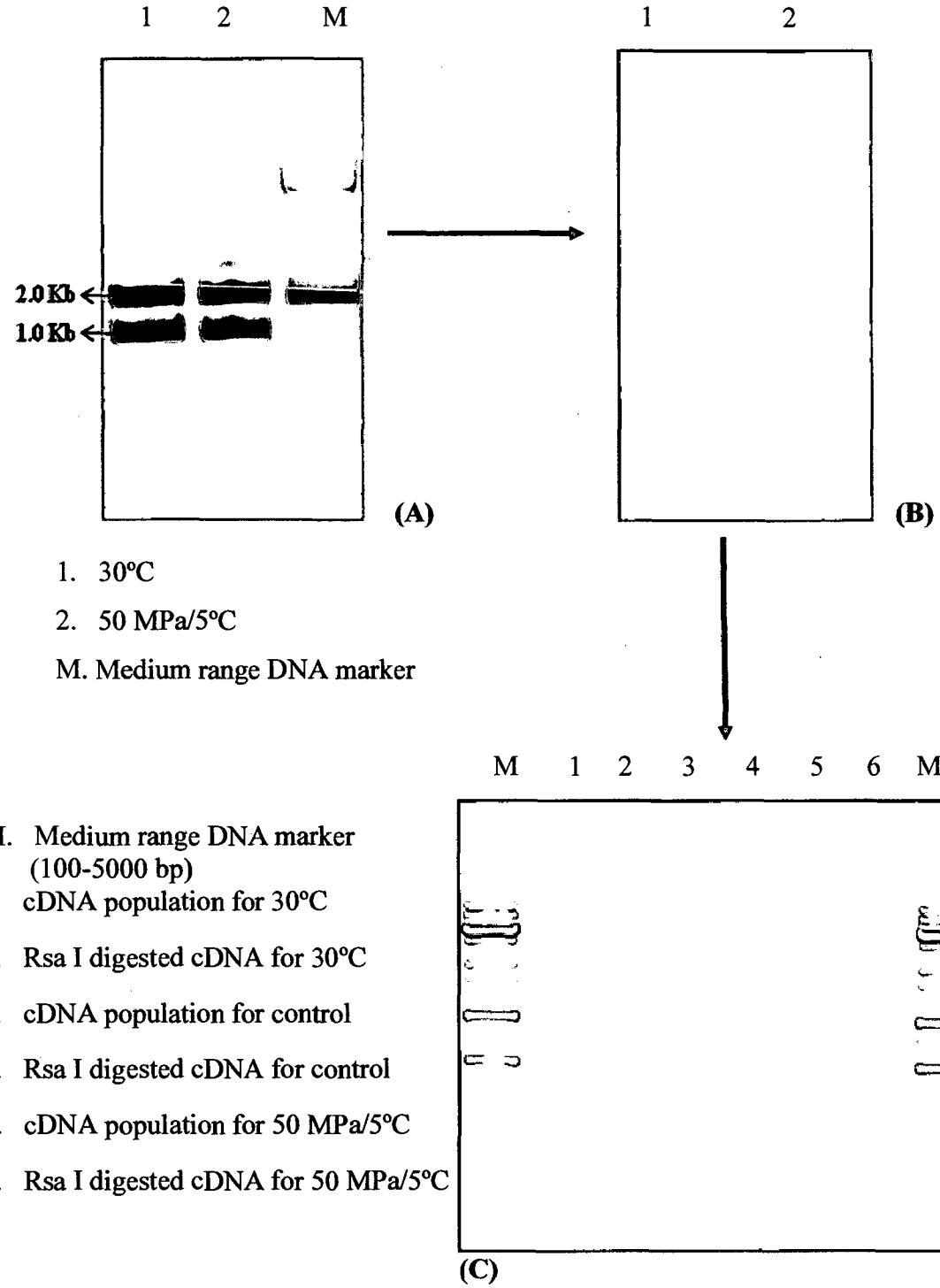


Fig 5.1 (A) Total RNA, (B) mRNA, (C) cDNA populations

Total cDNA populations were constructed with these mRNA for both the shock conditions along with control according to the steps mentioned in the kit (Fig. 5.1C).

The concentration of cDNA obtained was not enough in comparison with control to proceed for the next hybridization step. The possible reason for the above may be not enough biomass of yeast to get required concentration of cDNA. By standardizing the concentration of cDNA, subtractive hybridization may be performed for the yeast subjected to different pressure and temperature shocks. This may provide insight in to identification of differentially expressed genes under various pressures and temperature stresses and therefore better understanding of cellular mechanism of yeast adopted for survival.

5.3 Future directions

- Assessment of biological activity of fungi in the deep-sea sediments by the application of functional genomics.
- Improvise isolation techniques from deep-sea sediments to get yet uncultured fungi in culture.
- To study the ecological role of fungi in deep-sea sediments.
- To develop probes for identification of differentially expressed genes under elevated hydrostatic pressure and low temperature stresses.
- Analysis of fatty acid profiles of fungi subjected to different pressure and temperature shocks.
- To study the osmo-regulatory mechanism of fungi subjected to elevated hydrostatic pressure and low temperature stress using microscopic and biochemical methods.
- Screening of biologically active metabolites from these fungi.

APPENDIX

6.1 Media

a) Malt Extract Agar (1/5 strength)

Malt Extract Agar Base (MEA) powder	1 g
Agar powder	1.5 g
Seawater	100 ml

b) Czapek Dox Agar (1/5 strength)

Czapek Dox Agar (CDA) powder	1 g
Agar powder	1.5 g
Seawater	100 ml

c) Corn Meal Agar (1/5 strength)

Corn Meal Agar (CMA) powder	0.35 g
Agar powder	1.5 g
Seawater	100 ml

d) Potato Dextrose Agar (1/5 strength)

Potato Dextrose Agar (PDA) powder	0.75 g
Agar powder	1.5 g
Seawater	100 ml

e) Sabaraud Dextrose Agar (1/5 strength)

Sabaraud Dextrose Agar (SDA) powder	1.25 g
Agar powder	1.5 g
Seawater	100 ml

f) Malt Extract Broth

Malt Extract Broth base (MEB) powder	2.0 g
Seawater	100 ml

g) Yeast Extract, Peptone, Dextrose Broth

Yeast Extract powder	0.5 g
Peptone powder	1.0 g
Dextrose	1.0 g
Seawater	100 ml

h) Boyd and Kohlmeyer (B&K) agar medium

Yeast Extract powder	0.1 g
Peptone powder	0.2 g
Dextrose	1.0 g
Agar powder	1.5 g
Seawater/Distilled water	100 ml

6.2 Protein extraction and estimation methods

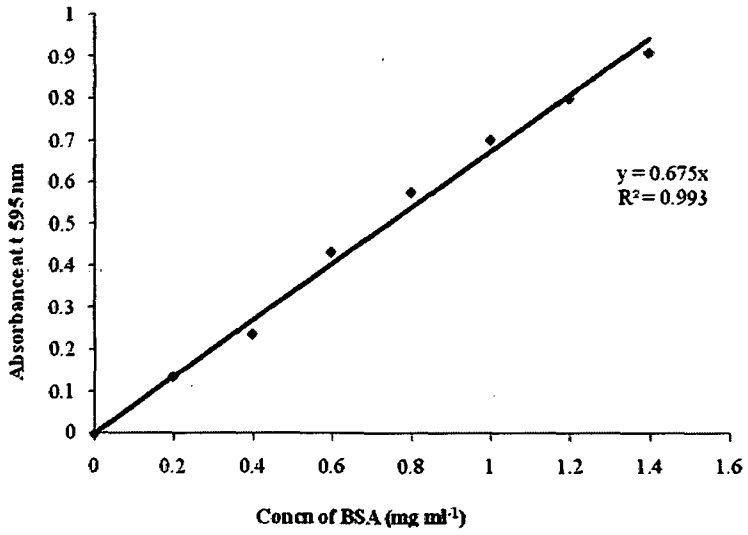
a) Merck Yeast buster protein extraction reagent (Cat. 71186) , protocol

One ml of overnight grown yeast cells were harvested by centrifugation and resuspended in 200 µl of yeast buster extraction buffer containing 2 µl of THP solution (provided with the kit). Cells were incubated at room temperature for 20 min with gentle shaking and harvested by centrifugation. Supernatant was collected and protein estimation was carried out.

b) Protein Estimation (Bradfords Method) (Bradford, 1976; Stoscheck, 1990)

Bovine serum albumin (BSA), 2 g was dissolved in 1 ml distilled water and used as stock for the preparation of protein standards for protein estimation by Bradfords method. Different dilutions were prepared with distilled water.

To 30 µl of each protein dilution, 900 µl of Bradfors reagent was added and incubated at room temperature for 30 min. The absorbance was recorded spectrophotometrically at 595 nm and a standard graph was plotted.



Calculations: Protein (mg ml^{-1}) = (A_{595}/0.675)

Where A_{595} = Absorbance at 595 nm.

Protein was expressed as mg ml^{-1}.

c) SDS-PAGE Composition and Reagents (Sambrook and Russell, 2001)

Components	**12% Resolving gel (ml)**	**5% stacking gel (ml)**
Distilled water	3.4	5.7
Monomer solution	4.0	1.7
Gel buffer	2.5	2.5
SDS (10%)	0.1	0.1
APS (10%), freshly prepared	0.05	0.05
TEMED	0.02	0.01

Resolving gel buffer:

Tris-HCl	1.5 M
pH	8.8 (adjusted with 6N HCl)

Stacking gel buffer:

Tris-HCl	0.5 M
pH	6.8 (adjusted with 6N HCl)

Monomer solution: (Acrylamide-bisacrylamide solution, 30%):

Acrylamide	29.2 g
N'N'-bis-methylene-acrylamide	0.8 g
Distilled water	100 ml

Solution was filter sterilized through 0.22 μm filter and stored at 4°C.

SDS-PAGE sample loading buffer (6x):

Tris-HCl	300 mM (pH 6.8)
β-mercaptoethanol	7.5%
SDS	10%
Glycerol	60%
Bromophenol blue	0.6%

SDS-PAGE running buffer (pH 8.3, 10x)

Tris base	30.2 g
Glycine	144 g
SDS (10%)	100 ml

Final volume made to 1000 ml with distilled water.

d) Silver staining protocol (Chevallet et al, 2006):

After electrophoresis, the gel was fixed in 30% ethanol, 10% acetic acid for at least 30 min. The gel was rinsed twice in 20% ethanol, and then twice in water, for 10 min each wash. Sensitization was done by soaking the gel for one min in 0.02 % $Na_2S_2O_3$ solution. The gel was rinsed twice with water for 1 min each wash. Gel was impregnated with 12 mM $AgNO_3$ for minimum 20 min. After staining the $AgNO_3$ was rinsed off quickly with de-ionized water. The gel was shifted with gloved hands to a box containing developer solution and shaken gently till protein bands appeared. The reaction was stopped by placing the gel in

stop solution. The gel was then placed in water for complete rehydration and the image was then documented using the gel documentation system.

Developer (prepared freshly):

Na_2CO_3	3 g
$Na_2S_2O_3$ solution (0.02%)	1 ml
Formalin	25 µl
Distilled Water	49 ml

Stop Solution:

Tris-HCl	4 g
Acetic acid	2 ml
Distilled water	100 ml

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Publications

List of Publications:

1. **Purnima Singh**, Chandralata Raghukumar, Pankaj Verma and Yogesh Shouche (2010) Phylogenetic diversity of culturable fungi from the deep-sea sediments of the Central Indian Basin and their growth characteristics. Fungal Diversity **40**:89-102.

2. Chandralata Raghukumar, Samir R Damare and **Purnima Singh** (2010) A review on deep-sea fungi: Occurrence, Diversity and Adaptations. Botanica Marina. **53**: 479-492.

3. **Purnima Singh**, Chandralata Raghukumar, Pankaj Verma and Yogesh Shouche (2011a) Fungal community analysis in the deep-sea sediments of the Central Indian Basin by culture-independent approach. Microbial Ecology **61:** 507-517.

4. **Purnima Singh**, Chandralata Raghukumar, Pankaj Verma and Yogesh Shouche (2011b). Assessment of fungal diversity in deep-sea sediments by multiple primer approach. World Journal of Microbiology and Biotechnology **DOI 10.1007/s11274-011-0859-3.**

Presentations at Conferences:

- Presented a paper titled "**Diversity of fungi from deep-sea sediments of the Central Indian Basin**" at 5th International conference on High Pressure Bioscience and Biotechnology, held at San Diego, California, USA, **September, 2008**.

- Presented a paper titled "**Fungal community analysis in the deep-sea sediments of the Central Indian Basin by culture-independent approach**" at 6th International conference on High Pressure Bioscience and Biotechnology, held at Freising , Germany , **August 2010**.

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Phylogenetic diversity of culturable fungi from the deep-sea sediments of the Central Indian Basin and their growth characteristics

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Abstract Very few studies have addressed the diversity of culturable fungi from deep-sea sediments. We are reporting here the diversity of culturable fungi in deep-sea sediments of the Central Indian Basin obtained from a depth of ~5,000 m. A total of 16 filamentous fungi and 12 yeasts isolated from 20 sediment cores of ~35 cm length were identified by 18S and ITS sequencing of SSU rDNA. Most of the filamentous fungi were Ascomycota, while two were Basidiomycota. Microscopic identification of sporulating cultures mostly matched either with 18S or ITS sequences but seldom with both the sequences. Phylogenetic analysis of ascomycetes using 18S sequence data grouped them into 7 clusters belonging to *Aspergillus* sp., *Sagenomella* sp., *Exophiala* sp., *Capronia* sp., *Cladosporium* sp., *Acremonium* sp. and *Tritirachium* sp. ITS sequence data grouped isolates into 6 clusters belonging to *Aspergillus* sp., uncultured member of Hypocreaceae, *Exophiala* sp., uncultured soil fungus, Hypocreales and *Trichothecium* sp. The two basidiomycete isolates were a *Tilletiopsis* sp. evident from 18S as well as ITS sequence data. In contrast, most of the yeast isolates belonged to Basidiomycota and only one isolate belonged to the phylum Ascomycota. Sequences of 18S as well as ITS gave matching identification of most of the yeasts. Filamentous fungi as well as the yeasts grew at 200 bar/5°C indicating their adaptations to deep-sea conditions. This is the first report on isolation of *Sagenomella, Exophiala, Capronia* and *Tilletiopsis* spp. from deep-sea sediments. This study reports on the presence of terrestrial fungi as a component of culturable fungi in deep-sea sediments.

Keywords Deep-sea sediments · Fungi · 18S · ITS sequences · Hydrostatic pressure · Central Indian Basin

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Introduction

Although only about 1% of the photosynthetically produced organic carbon reaches the deep-sea floor, the deep sea is considered the largest sink for organic carbon on the earth. A substantial part of the surface carbon dioxide from the euphotic zone is sequestered into the deep sea by biological-mediated processes. Carbon reaching the deep sea is both organic as well as inorganic, the former being contributed by dead plankton, faecal material and other organic waste, the latter being largely calcium carbonate (Raven and Falkowski 1999). Bacteria and fungi play an important role in decomposition of such organic matter, under elevated hydrostatic pressure and low temperatures of deep-sea habitat. These heterotrophs use organic carbon to build their biomass and respiration, releasing a part of the organic carbon as dissolved CO_2.

Several studies have shown high bacterial diversity and population, both cultured and yet-uncultivated, in relatively low organic carbon-containing deep-sea sediments and water column with its extreme environmental conditions (Takami et al. 1997; Li et al. 1999; Eilers et al. 2000). Diversity of culturable actinomycetes from tropical Pacific Ocean sediments up to a depth of 570 m was reported recently (Jensen et al. 2005). The presence of fungi was reported from deep-sea water columns (Roth et al. 1964),

calcareous sediments (Raghukumar and Raghukumar 1998), sediments from Mariana Trench at 11,500 m depth (Takami et al. 1997), Chagos Trench at a depth of 5,500 m (Raghukumar et al. 2004) and the Central Indian Basin at ~5,000 m depth (Damare et al. 2006). These developments clearly illustrate the increasing attention being paid to fungal abundance and diversity in deep-sea environments. In all of these reports culturable fungi have been identified by classical microscopic methods. As several of the cultured fungi did not show sporulating structures, these were grouped under non-sporulating fungi (Raghukumar et al. 1992, 2004; Damare et al. 2006). Identification of culturable filamentous and unicellular fungi from deep-sea sediments using molecular methods has not been reported, although ample evidence of fungal signatures in the form of 18S-rDNA genes have been reported from deep-sea sediments (Bass et al. 2007; López-Garcia et al. 2007). The aim of the present study was to isolate culturable fungi using different culturing techniques and culture media from different sections of deep-sea sediment cores obtained at a depth of ~5,500 m from the Central India Basin. Further, we aimed to identify them by classical microscopic and molecular methods. Small subunit (SSU) rDNA region was selected for identification by amplification and sequencing since it has been reported to be conserved in nature (Edgecomb et al. 2002). Amplification and sequencing was done for 18S and ITS regions using universal fungal primers (Smit et al. 1999) to identify the cultures up to species level. The deep-sea fungi were further tested for their growth and spore germination under simulated deep-sea conditions of elevated hydrostatic pressure, low temperature and varying salinities.

Materials and methods

Collection of sediment samples

Sediment samples were collected from depths of 4,000 to 5,700 m in the Central Indian Basin (10–16.5°S and 72–77°E) on board the Russian research vessel Akademic Boris Petrov (Cruise # ABP26) in December 2006 (Table 1). A total of 20 sediment cores were collected with an USNEL-type box corer of 50 cm^3 size. Sediments thus collected were mostly undisturbed and compact. Moderate brown sediments dominated the core tops followed by yellow-brown clayish sediments. Intercalations and mottling were seen in most of the sediment cores. Majority of the sediment cores were siliceous in texture. One core (BC-37) was fully calcareous in composition. Sub-cores of sediments were collected from a box corer using an alcohol-sterilized PVC cylinder of 5 cm inner diameter. Subsections of 2 cm down to a depth of 10 cm and thereafter every 5 cm length up to 35–40 cm depth were cut

Table 1 Details of sediment cores collected from the Central Indian Basin

Sr. no.	Box core (BC) no.	Touch Positions		Water depth (m)
		Lat (°S)	Long (°E)	
1	BC-03	12 00.000	76 29.900	5,373
2	BC-04	13 00.000	76 29.950	5,377
3	BC-13	14 59.919	75 29.849	4,894
4	BC-18	12 59.747	74 30.150	5,122
5	BC-19	13 00.070	75 29.900	5,104
6	BC-20	11 59.900	75 29.900	5,223
7	BC-25	11 00.000	75 29.800	5,303
8	BC-26	09 59.848	75 30.085	5,339
9	BC-27	11 30.130	75 59.600	5,274
10	BC-28	11 30.000	75 30.000	5,263
11	BC-29	12 29.900	74 59.900	5,174
12	BC-30	12 30.056	75 29.983	5,189
13	BC-31	12 00.000	74 59.900	5,153
14	BC-32	12 29.853	75 59.950	5,309
15	BC-33	12 59.980	74 59.960	5,265
16	BC-34	13 30.033	75 29.845	5,219
17	BC-35	14 30.081	75 29.792	5,211
18	BC-36	16 01.887	75 28.770	5,042
19	BC-37	16 06.891	75 24.828	3,992
20	BC-38	15 57.590	75 31.950	5,642

from the above sediment and directly introduced into sterile plastic bags to avoid any aerial contamination. The bags were closed and carried to the microbiology laboratory on board for further processing.

Isolation of fungi

An aliquot of sediment from the central part of the subsection was removed with a sterile spatula and placed in sterile vials for isolation (Raghukumar et al. 2004). Four different media were used for isolation of fungi and they were 1) Malt Extract Agar (MEA), 2) Czapek Dox Agar (CDA), 3) Corn Meal Agar (CMA) and 4) Malt Extract Broth (MEB) (Damare et al. 2006). The above media were used at 1/5 strength to simulate low nutrient conditions of deep-sea sediments. They were prepared in seawater and supplemented with Penicillin (40,000 units in 100 mL medium) and streptomycin (0.1 g in 100 mL medium) to inhibit bacterial growth. Isolation of fungi from sediments was done by the following methods: 1) Particle plating method of Bills and Polishook (1994); 2) Dilution plating 3) Pressure Incubation and 4) Enrichment method (Damare et al. 2006). The plates were incubated at 5°C for 30 days. Air-borne fungi contaminating the culture plates was monitored by exposing various media plates for 10 min on the deck of the research vessel where the cores were received, the microbiology laboratory on board the research vessel and the inoculation hood. This was repeated during every sampling station.

After isolation, fungal cultures were maintained on MEA slants at 5°C. The isolates were originally numbered as NIOCC (National Institute of Culture Collection). The sporulating fungi were deposited in a recognized microbial culture repository, Microbial Type Culture Collection (MTCC) at Chandigarh, India, under the accession number MTCC.

Isolation of DNA from the cultures

The fungi were grown in MEB for 4-5 days for DNA isolation. Yeasts were grown in yeast extract peptone and dextrose (YPD) medium and shaken at 170 rpm for 3-4 days. Mycelia and cells were harvested, lyophilized and crushed in a mortar and pestle to fine powder. Isolation of DNA was carried out following the modified form of standard procedure (Stoeck and Epstein 2003). The above samples were incubated at 65°C for 2 h in a high salt extraction buffer for fungal DNA (100 mM Tris-HCl buffer with 8 pH containing 100 mM Na_2EDTA, 100 mM $NaPO_4$, 1.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate, Proteinase K (100 μg mL^{-1} final concentration) A low salt extraction buffer containing 10 mM Tris-HCl with pH8, 10 mM Na_2EDTA pH8, 0.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate and Proteinase K (100 μg mL^{-1} final concentration) was used for isolating yeast DNA.

PCR amplification of 18S and ITS regions of rDNA

Partial region of SSU rDNA was PCR amplified by using universal fungal primers, NS1 (5'-GTAGTCATATGCTT GTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') of ~1,100 bp. Full length of ITS region of ~600 bp was amplified using the primers ITS1 (5'-TCCGTAGGTGAACC TGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The conditions for PCR included an initial hot start incubation (5 min at 94°C) followed by 34 cycles (denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min) and a final extension at 72°C for 5 min.

Sequencing and phylogenetic analyses

Fresh PCR products were purified by using gel extraction kit (Sigma, Genosys, USA) and sequenced at National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3730 DNA Analyzer, Applied Biosystems, USA). Sequence data were edited using Chromas Pro version 1.34. For tentative identification, fungal 18S and ITS rDNA sequences were compared with NCBI (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov) database. Fungal rDNA -18S and ITS sequences in this study and the matched sequences from GenBank were edited and aligned using CLUSTAL-X, version 1.81 (Thompson et al. 1997). The aligned sequences were imported into DAMBE 4.5.47 (Xia and Xie 2001). Neighbour-joining (NJ) trees were created using pairwise genetic distances using MEGA 3.1 (Sudhir kumar et al. 2008). The quality of the branching patterns for NJ was assessed by bootstrap resampling of the data sets with 1,000 replications.

Growth of mycelial fungi and yeasts under simulated deep-sea conditions

For raising mycelial inoculum, cultures of 16 deep-sea fungi were grown in MEB for 3 days at 1 bar and 30°C. Vegetative mycelium prior to the onset of sporulation was homogenized with sterile glass beads. A known weight of finely broken mycelial suspension was inoculated in 5 mL of MEB in pouches made with sterilized gas permeable polypropylene sheets and sealed without trapping any air bubbles. The pouches were suspended in a deep-sea culture vessel (Tsurumi & Seiki Co., Japan) and incubated at

Table 2 Number of fungi isolated by various isolation techniques

	Particle plating	Dilution plating	Pressure incubation at 300bar/5°C	Direct incubation in MEB at 5°C
Total no. of sediment samples used	116	188	39	153
No. of fungi isolated	7	12	6	3
% frequency of occurrence	6	6	15	2

MEB malt extract broth

30°C/1 bar, 30°C/200 bar, 5°C/1 bar and 5°C/200 bar for comparing the effect of different pressure and temperature conditions on growth. After 20 days, the contents of the pouches were filtered over pre-weighed filter papers, dried to a constant dry weight and the difference between the initial and final biomass determined as mycelial dry weight (Raghukumar and Raghukumar 1998). The yeasts were similarly grown in YPD broth and the biomass was lyophilized and weighed. The biomass of mycelial fungi and yeasts were also compared in media made with sea water (35 ppt) and distilled water.

Table 3 Identification of fungi by different methods and their accession numbers

Isolate no.	Identification			Accession number	
	Microscopic	18S	ITS	18S	ITS
NIOCC#F2	*Penicillium* sp.	*Penicillium* sp.	*Penicillium citreonigrum*	FJ357792	EU729705
NIOCC#F5	*Cladosporium* sp.	Pezizomycotina sp.	*Cladosporium cladosporioides*	EU723484	EU729711
NIOCC#F6 (MTCC9336)	*Aspergillus* sp.	Uncultured marine eukaryote clone U112H09	Aspergillus *sp.*	EU723485	EU729718
NIOCC#F8	*Cladosporium* sp.	Pezizomycotina sp.	*Cladosporium cladosporioides*	EU723486	EU729712
NIOCC#F13 (MTCC9333)	*Cladosporium* sp.	*Cladosporium* sp.	*Cladosporium* sp.	EU723487	EU729719
NIOCC#F15	*Sagenomella* sp.	*Sagenomella sclerotialis* sp.	Uncultured fungus clone	EU723488	FJ357782
NIOCC#F16 (MTCC9332)	*Rhinocladiella* sp.	Ascomycete MV_26C	*Exophiala spinifera*	EU723489	FJ357783
NIOCC#F21 (MTCC9339)	Sporulating, unidentified sp.	*Tilletiopsis albescens*	*Tilletiopsis albescens*	EU723490	FJ357784
NIOCC#F23	*Sagenomella* sp.	*Sagenomella sclerotialis* sp.	Uncultured fungus clone	EU723491	FJ357785
NIOCC#F30 (MTCC9337)	*Acremonium* sp.	Ascomycete MV_25C	Hypocreales sp.	EU723492	EU729707
NIOCC#F33	Sporulating, unidentified sp.	*Tilletiopsis albescens*	*Tilletiopsis albescens*	EU723493	EU729706
NIOCC#F46 (MTCC9334)	*Exophiala* sp.	Ascomycete MV_26C	*Exophiala xenobiotica*	EU723494	EU729708
NIOCC#F47	*Aspergillus* sp.	Aspergillus restrictus	*Aspergillus caesiellus*	EU723495	EU729709
NIOCC#F48 (MTCC9341)	*Sagenomella* sp.	*Sagenomella sclerotialis*	*Phialemonium dimorphosporum*	EU723496	FJ357786
NIOCC#F49 (MTCC9340)	*Acremonium* sp.	*Acremonium* sp.	*Trichothecium roseum*	EU723497	EU729713
NIOCC#F50	Non -sporulating sp.	*Capronia coronata*	*Exophiala dermatitidis*	EU723498	EU729710
NIOCC#Y1	Yeast sp.	*Rhodotorula cassiicola*	*Sporobolomyces* sp.	EU723499	FJ357787
NIOCC#Y2	Yeast sp.	*Sporidiobolus johnsonii*	*Sporidiobolus salmonicolor*	EU723500	FJ357788
NIOCC#Y3	Yeast sp.	*Rhodosporidium toruloides*	*Rhodosporidium toruloides*	EU723501	EU729715
NIOCC#Y4	Yeast sp.	*Sporidiobolus johnsonii*	*Sporobolomyces* sp.	EU723502	EU729716
NIOCC#Y5	Yeast sp.	*Sporidiobolus johnsonii*	*Rhodotorula* sp.	EU723503	FJ357793
NIOCC#Y6	Yeast sp.	*Sporidiobolus johnsonii*	*Sporidiobolus* sp.	EU723504	EU72972
NIOCC#Y7	Yeast sp.	*Rhodotorula cassiicola*	*Rhodotorula calyptogenae*	EU723505	FJ357789
NIOCC#Y8	Black Yeast sp.	*Coniosporium perforans*	*Sarcinomyces petricola*	EU723506	EU729717
NIOCC#Y9	Filamentous Yeast sp.	*Graphiola cylindrica*	*Tilletiopsis oryzicola*	EU723507	FJ357790
NIOCC#Y10	Yeast sp.	*Rhodotorula mucilaginosa*	*Cryptococcus albidosimilis*	EU723508	EU729721
NIOCC#PY12	Yeast sp.	*Cryptococcus vishniacii*	*Cryptococcus albidosimilis*	EU723509	FJ357791
NIOCC#PY13	Yeast sp.	*Cryptococcus vishniacii*	*Rhodotorula mucilaginosa*	EU723510	EU729714

MTCC Microbial Type Culture Collection, Chandigarh, *NIOCC* National Institute of Oceanography Culture Collection

Spore germination under simulated deep-sea conditions

Selected four sporulating fungi (NIOCC#F2, F5, F6 and F13) were grown in MEA plates at 1 bar pressure and 30°C, and the spores were collected by gently flooding the plates with sterile seawater. The spore suspension was appropriately diluted with sea water containing 2% Tween 80 and vortexed for 5 min. One mL of this spore suspension was added to 5 mL of the sediment extract medium (Damare and Raghukumar 2008) prepared with three different salinities, 1.7, 17 and 34 ppt and packed in pouches. These pouches were incubated at 200, 300, 400 and 500 bar pressure at 30°C for 15 days. The percentage of germination was calculated by counting germinated spores in 20 microscope fields.

Statistical analyses

The statistical analyses were carried out in Microsoft Excel program and PRIMER 5 software.

Results

Isolation and distribution

Pressure incubation of fungi prior to isolation yielded maximum recovery of culturable fungi (Table 2). A total of 16 filamentous fungi and 12 yeasts were isolated from deep-sea sediments during the cruise # ABP 26. No fungi were recovered below 35 cm depth of sediment cores, the maximum colony forming units were recovered at 4-6 cm depth. Species richness (2.8) and Shanon Index (1.8 log e) were accordingly highest at this depth. Species diversity was scanty in all the sections of cores, the number ranging from 1 to 4. Among the various media used for isolation of fungi, MEA appeared to be the best medium for maximum recovery of fungi. Dilution plating yielded the maximum diversity of fungi. The sporulating cultures were deposited in Microbial Type Culture Collection, Chandigarh, India under accession number MTCC. The basidiomycete *Tilletiopsis* sp. and the yeast *Sporidiobolus johnsonii* and one ascomycete

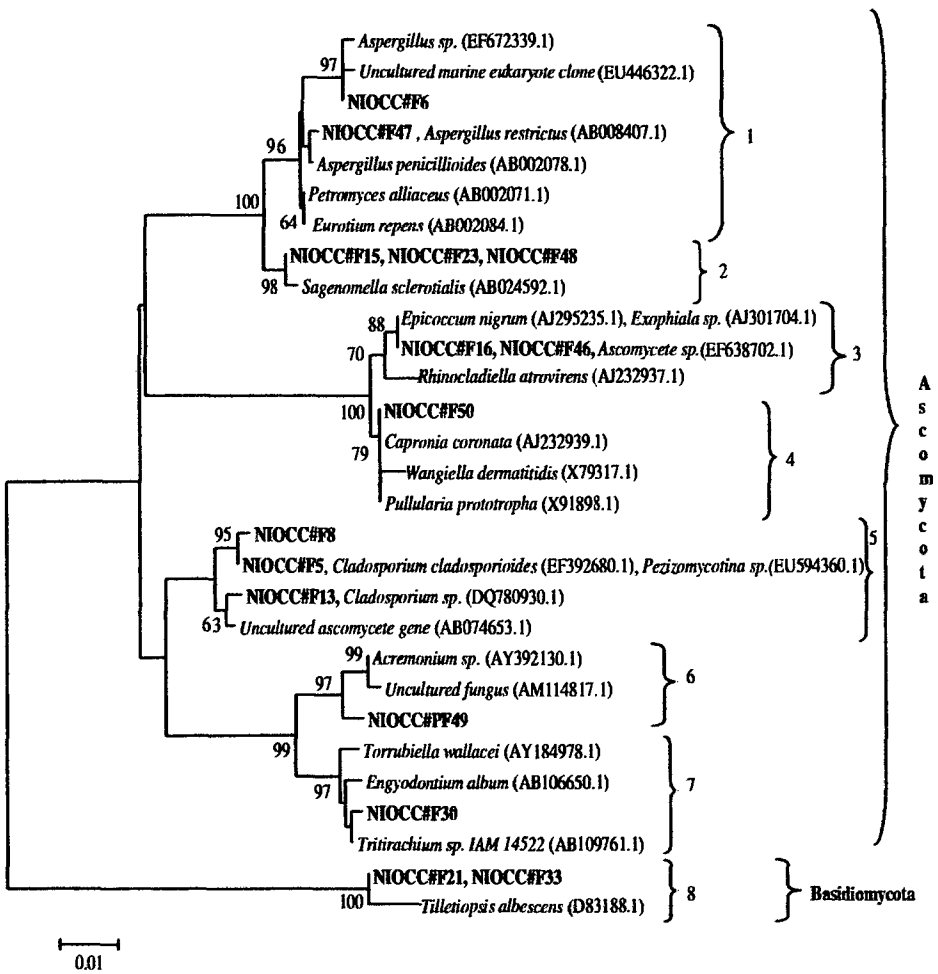


Fig. 1 Neighbor-joining phylogenetic tree for mycelial fungi based on rDNA-18S sequences (~1,100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1,000 replicates (values below 50% not shown)

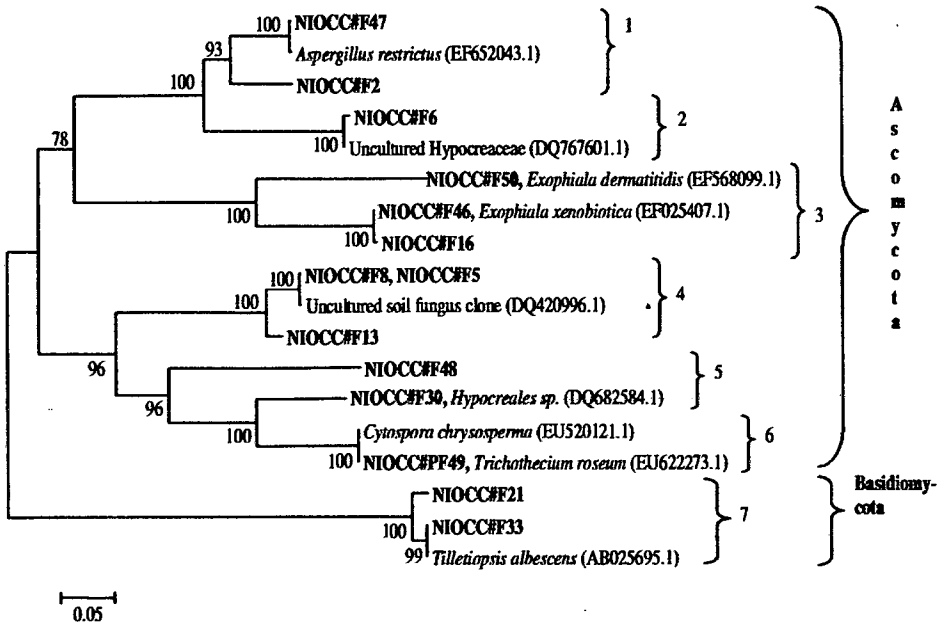


Fig. 2 Neighbor-joining phylogenetic tree for mycelial fungi based on rDNA-ITS sequences (~600 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1,000 replicates (values below 50% not shown)

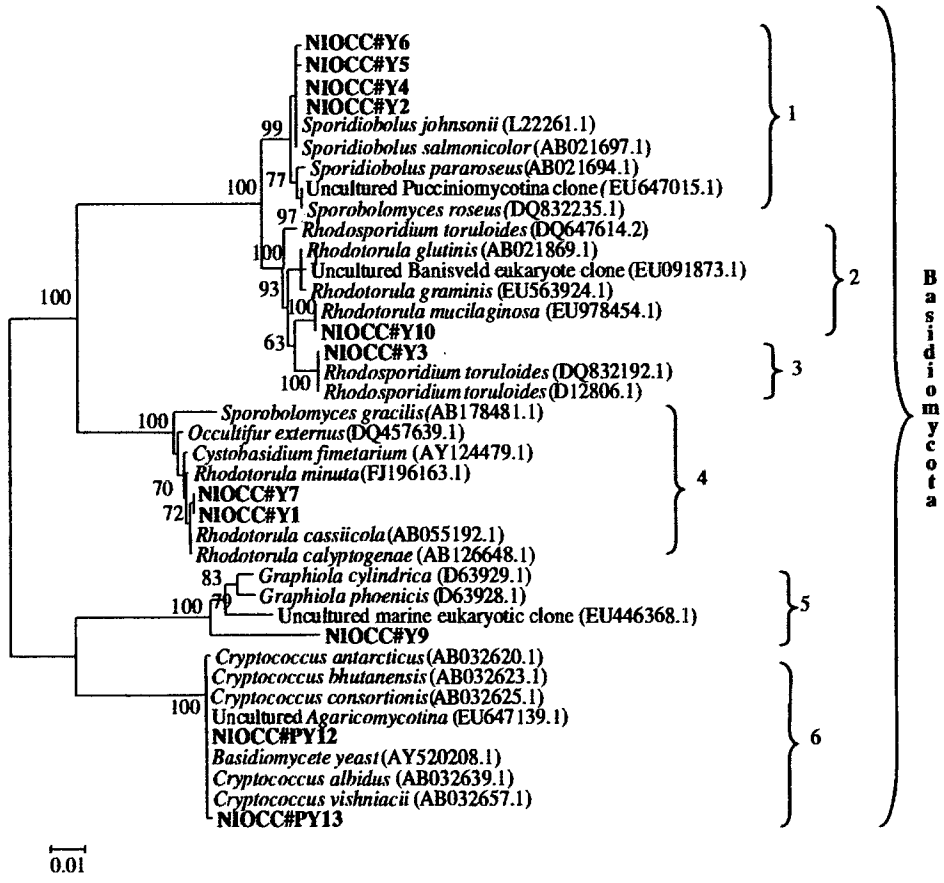


Fig. 3 Neighbor-joining phylogenetic tree for yeasts based on rDNA-18S sequences (~1,100 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1,000 replicates (values below 50% not shown)

Sagenomella sp. were the only three species recovered from the deepest part of the sediment core, namely 25–35 cm depth.

Diversity

The identification of sporulating cultures carried out by classical morphological taxonomy matched with molecular identification based on either 18S or ITS sequences and only seldom with both these sequences (Table 3). The 18S and ITS sequences of all the cultures were deposited in GenBank and accession numbers obtained (Table 3). Aligned sequences for the phylogenetic trees are submitted to TreeBASE (study accession No. S2215, journal peer reviewers Pin No. 166658). Most of the fungi belonged to the phylum Ascomycota and based on 18S sequences clustered into 7 subgroups, namely *Aspergillus* sp., *Sagenomella* sp., *Exophiala* sp., *Capronia* sp., *Cladosporium* sp., *Acremonium* sp. and *Tritirachium* sp. (Fig. 1). Based on ITS sequences they grouped into 6 clusters showing maximum similarity to *Aspergillus* sp., uncultured member of Hypocreaceae family, *Exophiala* sp., an uncultured soil fungus clone DQ682584.1, Hypocreales and *Trichothecium* sp. (Fig. 2). The two basidiomycetes (NIOCC#F21 and #F33) belonged to *Tilletiopsis* sp. both by 18S as well as ITS sequencing (Figs. 1, 2 and Table 3). Species of filamentous fungi *Sagenomella*, *Exophiala*, *Capronia*, and *Tilletiopsis* are being reported for the first time from the deep-sea sediments.

The majority of the yeast isolates belonged to the phylum Basidiomycota (Figs. 3 and 4) and only one isolate, NIOCC # Y8 belonged to Ascomycota identified both by 18S and ITS sequencing (Fig. 4 and Table 3). Sequences of 18S as well as ITS gave matching identification of yeasts (Table 3). The basidiomycetous yeast belonged to the genera *Sporobolomyces* sp., *Sporidiobolus*, *Rhodosporidium*, *Rhodotorula* and *Cryptococcus* sp. A 100% consensus in identification was seen between 18S and ITS sequences of the isolates NIOCC#Y2, Y3, Y6, Y7 and PY12 up to generic level (Table 3).

Growth characteristics

All of the deep-sea fungi, mycelial as well as the yeasts showed growth under 200 bar pressure and 5°C temperature (Fig. 5a and b). A one-way analysis of variance (ANOVA) revealed that biomass produced was significantly different at different hydrostatic pressure and temperature conditions (see the legend for Fig. 5a and b). These cultures showed better growth in media prepared

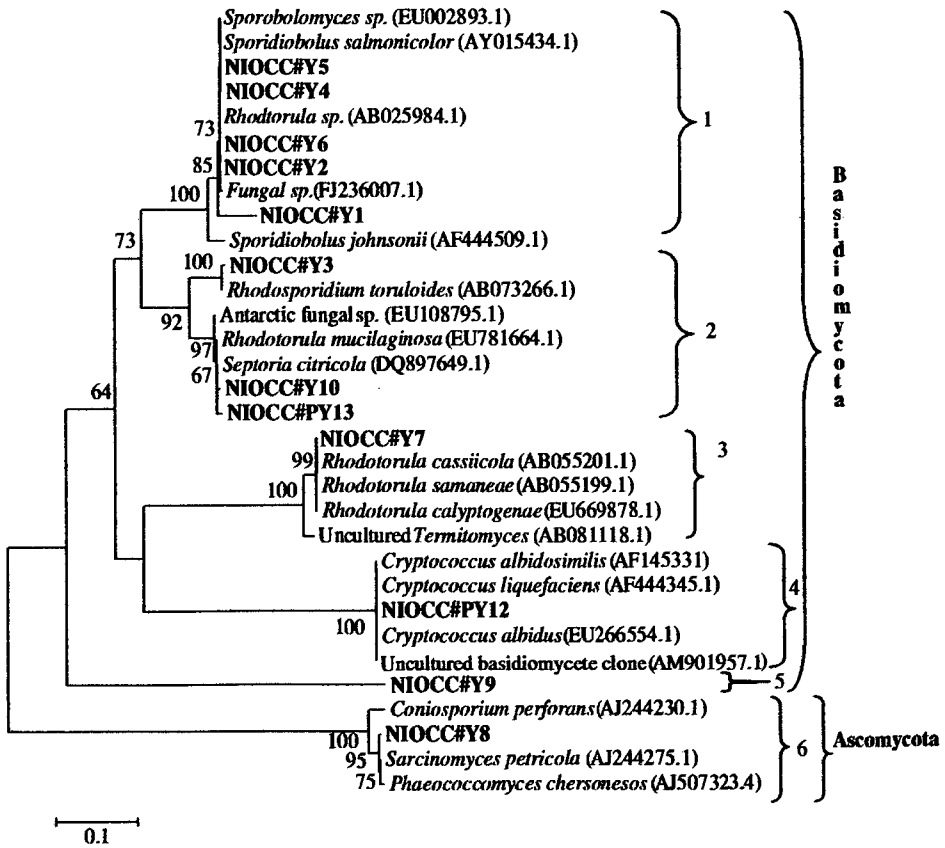
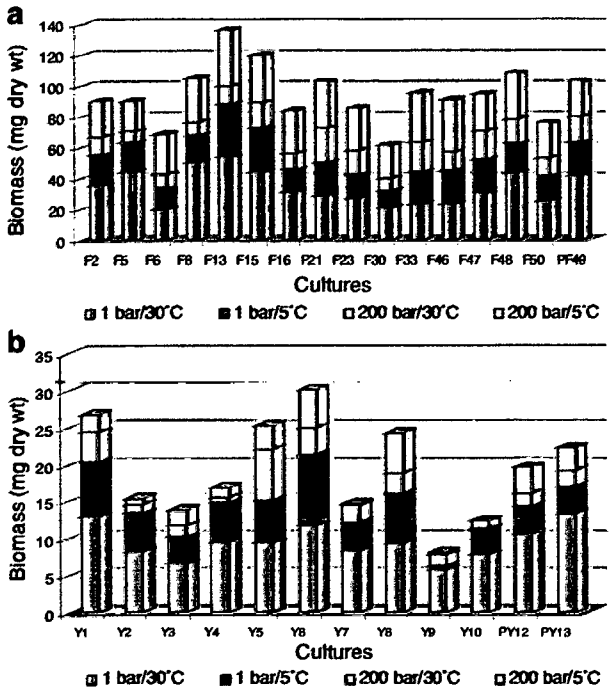


Fig. 4 Neighbor-joining phylogenetic tree for yeasts based on rDNA-ITS sequences (~600 bp). Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1,000 replicates (values below 50% not shown)



Results of 1-way ANOVA performed on biomass obtained under different growth conditions (Fig. 5a & 5b):

	Fungi	Yeast
F critical	2.8	2.8
F value	25.5	30.5
P value	9.06-E-11***	7.83E-11***
Degrees of freedom	63	47

*** significant at>0.01 %.

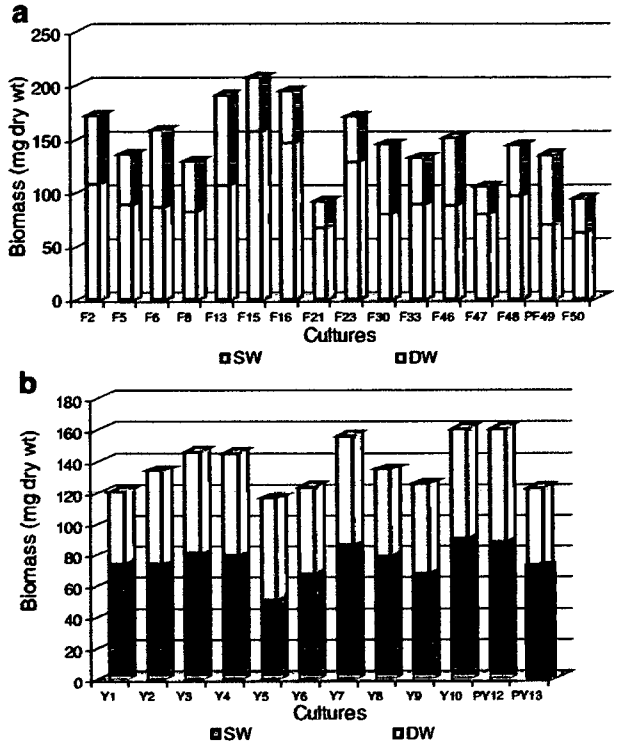
Fig. 5 a Biomass (mg dry wt/20 ml) of mycelial fungi after 20 days incubation in malt extract broth under different pressure and temperature conditions (SD values<10%). b Biomass (mg dry wt/20 ml) of yeasts after 20 days incubation in yeast extract, peptone, dextrose broth under different pressure and temperature conditions (SD values<10%)

with sea water than with distilled water (Fig. 6a and b). A one-way ANOVA clearly demonstrated a distinct difference in growth of fungi in seawater versus distilled water (see the legend for Fig. 6a and b). However, none of the cultures showed absolute requirement of seawater for growth. All of the sporulating fungi showed sporulation in seawater medium. Spores from four sporulating fungi showed germination of spores under 200–500 bar pressure at 30°C and no dependence on salinity (Fig. 7). A one-way ANOVA showed statistically significant effect of hydrostatic pressure on spore germination but salinity did not show such effect (see the legend for Fig. 7). With increasing hydrostatic pressure, a decrease in spore germination was observed. The four fungi appeared to be euryhaline because the spores germinated from 1.7 to 34 ppt salinity.

Discussion

Distribution

The fungi isolated from deep-sea sediments did not correspond to the fungi generally termed marine fungi (Jones et al. 2009). Ascomycetes dominated among the filamentous fungi whereas basidiomycetes were dominant among the yeast (unicellular fungi) in deep-sea sediments of the Central Indian Ocean. Bass et al. (2007) observed that yeast sequences dominated in deep-sea sediments of the Pacific Ocean at 1,500–4,000 m depth. On the other hand, in the present study as well as that of Damare et al. (2006) filamentous fungi were the dominant culturable



Results of 1-Way analysis of variance (ANOVA) performed for the data in Fig. 6a & 6b

	Fungi	Yeast
F critical	4.2	4.3
F value	30.8	13.1
P value	4.9E-06***	0.002***
Degrees of freedom	31	23

*** significant at >0.01 %

Fig. 6 a Biomass (mg dry wt/10 ml) of mycelial fungi after 6 days incubation in malt extract broth prepared in sea water (SW) and distilled water (DW) (SD values<10%). b Biomass (mg dry wt/10 ml) of yeasts after 6 days incubation in yeast extract peptone and dextrose broth prepared in sea water (SW) and distilled water (DW) (SD values<10%)

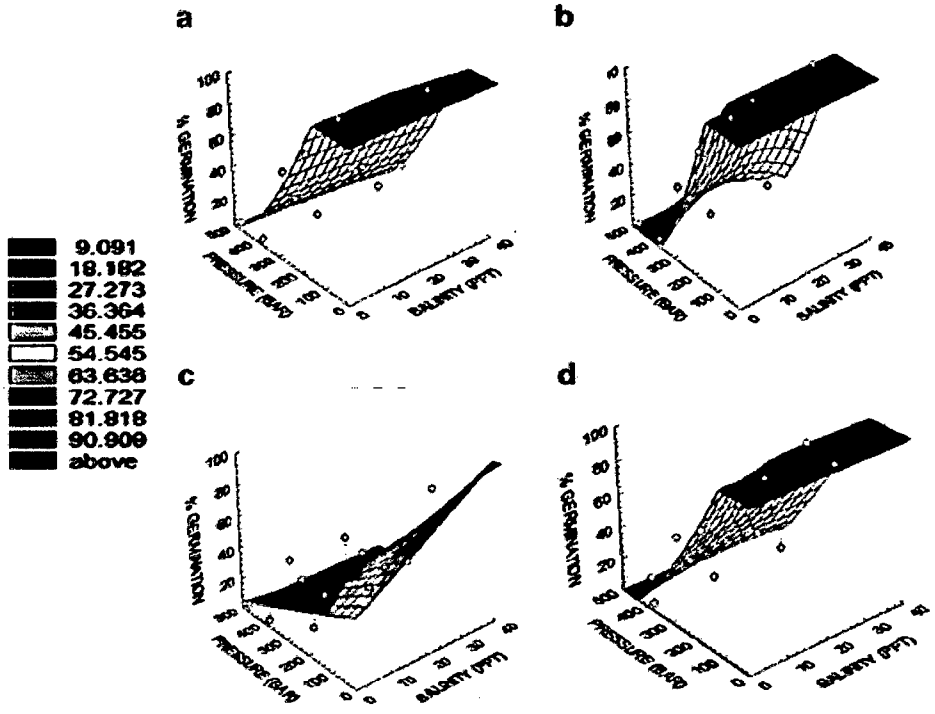


Fig. 7 3D Surface plot graph showing spore germination of four sporulating fungi, a NIOCC#F2, *Penicillium* sp. b NIOCC#F5, *Cladosporium* sp. c NIOCC#F6 *Aspergillus* sp. and (d) NIOCC#F13, *Cladosporium* sp. grown at 30°C under different hydrostatic pressure (200, 300, 400 and 500 bar) and salinity (1.7, 17 and 34 ppt) for 15 days (SD values<12%)

Results of 1-Way ANOVA performed for the culture NIOCC#F2

	Pressure/% germination	**Salinity/% germination**
F critical	4.3	4.3
F value	82.6	1.3
P value	6.7E-09***	0.3
Degrees of freedom	23	23

*** significant at >0.01 %

Results of 1-way ANOVA performed for the culture NIOCC#F5

	Pressure vs% germination	**Salinity vs% germination**
F critical	4.3	4.3
F value	78.3	1.9
P value	1.06E-08***	0.2
Degrees of freedom	23	23

*** significant at >0.01 %

Results of 1-way ANOVA performed for the culture NIOCC#F6

	Pressure vs% germination	**Salinity vs% germination**
F critical	4.3	4.3
F value	92.2	0.02
P value	2.5E-09***	0.9
Degrees of freedom	23	23

*** significant at >0.01 %

Results of 1-Way ANOVA performed for the culture NIOCC#F13

	Pressure vs% germination	**Salinity vs% germination**
F critical	4.3	4.3
F value	80.5	2.0
P value	8.4E-09***	0.2
Degrees of freedom	23	23

*** significant at >0.01 %

fungi. It is possible that deep-sea yeasts may require more specialized media for culturing.

The ITS and 18S sequences matched while identifying only some of the non-sporulating and sporulating mycelial cultures (Table 3). On the other hand, there was a 100% consensus between these two techniques while identifying most of the yeast cultures (Table 3). These results indicate that identification up to species level should be based on more than two techniques. Using a few other fungal specific primers as reported by Pang and Mitchell (2005), this problem of low diversity and sequence matches could be resolved.

The presence of fast growing sporulating forms such as *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Curvularia* sp., *Acremonium* sp., and *Fusarium* sp., have been reported as culturable forms (Roth et al. 1964; Raghukumar et al. 1992; Damare et al. 2006) and their molecular sequences have been detected in the oceanic environment (Bass et al. 2007). We are reporting for the first time the presence of *Sagenomella* sp., *Capronia coronata, Exophiala* sp., and *Tilletiopsis* sp., from deep-sea sediments. Several of the cultured fungi in our studies showed phylogenetic similarity to fungi reported in marine environment. The 18S sequences of the filamentous fungi NIOCC#F5 and F8 showed 99-100% homology to Pezizomycotina isolate EU594360, originally reported to have been isolated from a marine sponge *Haliclona simulans* (Table 4). The 18S sequences of the isolate NIOCC#F16 and #F30 showed homology to the isolates obtained from deep hydrothermal ecosystem of Mid-Atlantic Ridge and the isolate NIOCC#F13 to *Cladosporium* sp., reported from hypersaline environment (Table 4). The ITS sequences of the isolates NIOCC#F5, #F15, #F16, #Y5, and #Y7 showed homology to the cultures either from deep-sea or marine environment (Table 5).

Growth under simulated deep-sea conditions

Although most of the fungi isolated were terrestrial species, they seem to have adapted to growth in the deep-sea and evolved into distinct physiological species. This was inferred from the fact that all the cultures showed good growth at 200 bar pressure and 5°C. All the filamentous fungi and a few yeasts showed better biomass production at 200 bar/5°C than at 200 bar/30°C. Biomass production was much better in seawater medium than in medium prepared with distilled water in filamentous as well as unicellular fungi. However, none of these fungi showed absolute requirement for sea water or NaCl for growth as has been shown for a few deep-sea actinomycetes (Jensen et al. 2005). The fact that the culturable fungi from the deep-sea sediments showed homology to ITS and 18S sequences to those in GenBank does not necessarily imply that they are identical species. It is possible that they are distinct physiologically as was reported for *Aspergillus sydowii*, the fungal pathogen of seafan (Alker et al. 2001). Although a terrestrial fungus, it was metabolically distinct from non-pathogenic terrestrial strains.

Spores of four fungi (NIOCC#F2, F5, F6 and F13) germinated under 200–500 bar pressure at 30°C. Spores from none of these fungi germinated at 5°C under elevated hydrostatic pressure or even at 1 bar at this temperature. It has been demonstrated that low temperature and not the elevated hydrostatic pressure is the limiting factor for spore germination of deep-sea adapted fungi (Damare et al. 2006, 2008). Three terrestrial cultures (obtained from a culture collection centre) showed good mycelial growth under 200 bar pressure at 5°C as well as at 30°C (data not shown) but their spores failed to germinate under these conditions. Therefore, as reported by Damare et al. (2008) the effect of hydrostatic pressure and low temperature is different on mycelial fragments and fungal spores

Diversity

The low recovery of culturable fungi from deep-sea sediments was the most striking feature of the sampling carried out in 2006. A total of only 28 fungi, inclusive of filamentous and unicellular yeasts were recovered from 496 sediment samples (5.6% frequency) by methods such as particle plating, dilution plating, pressure incubation and low temperature incubation and four different culture media. At the same geographical positions (10–15°S and 73–76°E) of the Indian Ocean, Damare et al. (2006) isolated a total of 181 fungi from 672 sediment samples collected during three cruises from 2001–2003 (9% frequency). Almost similar methods and culture media for isolation of fungi were used by Damare et al. (2006). Such a difference in recovery of fungi may indicate large temporal variation or possibility of extremely patchy distribution of fungi in deep-sea sediments with pockets of high fungal population. Turner (1973) reported sinking waterlogged wood originating from offshore during monsoon runoffs creating "islands of wood" in the deep sea. Such persistent but shifting islands of wood might bring in saprophytic species, serve as dispersal centres and contribute to habitat diversity, niche specialization and enrichment. Similar route for several mussels in the deep-sea vents has been hypothesized (Distel et al. 2000). Patchy distribution of fungi seen in the Central Indian Basin appears to be similar to the scenario of high bacterial population associated with "whale fall" in deep sea (Smith and Baco 2003). The sinking particulate organic matter from the dynamic surface waters might also be contributing to this vast temporal difference in the almost static deep-sea sediments.

Species diversity was equally low with a total of 12 and 8 distinct species of filamentous and unicellular fungi

Table 4 Phylogenetic affiliations of fungal and yeast isolates on the basis of 18S sequences

Isolate ID	Phylogenetic nearest taxon, phylum (GenBank Accession No)	% identity	Source of phylogentic nearest taxon
Penicillium sp. (NIOCC#F2)	*Penicillium phialosporum*, Ascomycota (AF245266)	84	Not known
Cladosporium sp. (NIOCC#F5)	Pezizomycotina , Ascomycota (EU594360)	100	*Haliclona simulans* (a marine sponge)
Aspergillus sp. (NIOCC#F6)	Uncultured marine eukaryote clone UI12H09, Ascomycota (EU446322)	99	"anoxic deep hypersaline L'Atalante Basin, eastern Mediterranean Sea"
Cladosporium sp. (NIOCC#F8)	Pezizomycotina sp., Ascomycota (EU594360)	99	*Haliclona simulans* (a marine sponge)
Cladosporium sp. (NIOCC#F13)	*Cladosporium* sp. EXF228, Ascomycota (DQ780930)	99	hypersaline environments
Sagenomella sp. (NIOCC#F15)	*Sagenomella sclerotialis*, Ascomycota (AB024592)	99	Not known
Ascomycete sp. (NIOCC#F16)	Ascomycete sp. MV_26C, Ascomycota (EF638702)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
Tilletiopsis sp. (NIOCC#F21)	*Tilletiopsis albescens*, Basidiomycota (D83188)	99	Not known
Sagenomella sp. (NIOCC#F23)	*Sagenomella sclerotialis*, Ascomycota (AB024592)	99	Not known
Ascomycete sp. (NIOCC#F30)	Ascomycete sp. MV_25C, Ascomycota (EF638701)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
Tilletiopsis sp. (NIOCC#F33)	*Tilletiopsis albescens*, Basidiomycota (D83188)	100	Not known
Ascomycete sp. (NIOCC#F46)	Ascomycete sp. MV_26C, Ascomycota (EF638702)	100	Mid Atlantic Ridge in deep hydrothermal ecosystem
Aspergillus sp. (NIOCC#F47)	*Aspergillus restrictus*, Ascomycota (AB008407)	100	Culture collection
Sagenomella sp. (NIOCC#F48)	*Sagenomella sclerotialis*, Ascomycota (AB024592)	99	Culture collection
Capronia coronata (NIOCC#F50)	*Capronia coronata*, Ascomycota (AJ232939)	99	Not known
Acremonium sp. (NIOCC#PF49)	*Acremonium* sp., Ascomycota (AY392130)	99	Onychomyces
Rhodotorula sp. (NIOCC#Y1)	*Rhodotorula cassiicola*, Basidiomycota (AB055191)	99	Thailand
Sporidiobolus johnsonii (NIOCC#Y2)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Culture collection
Rhodosporidium toruloides (NIOCC#Y3)	*Rhodosporidium toruloides* , Basidiomycota (DQ832192)	100	Culture collection
Sporidiobolus johnsonii (NIOCC#Y4)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Culture collection
Sporidiobolus johnsonii (NIOCC#Y5)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Culture collection
Sporidiobolus johnsonii (NIOCC#Y6)	*Sporidiobolus johnsonii*, Basidiomycota (L22261)	100	Culture collection
Rhodotorula sp. (NIOCC#Y7)	*Rhodotorula cassiicola*, Basidiomycota (AB055191)	99	Thailand
Coniosporium perforans (NIOCC#Y8)	*Coniosporium perforans*, Ascomycota (EF137365)	100	Rocks
Graphiola cylindrica (NIOCC#Y9)	*Graphiola cylindrica*, Basidiomycota (D63929)	95	Not known
Rhodotorula sp. (NIOCC#Y10)	*Rhodotorula mucilaginosa*, Basidiomycota (EF218987)	100	Not known
Cryptococcus sp. (NIOCC#PY12)	*Cryptococcus vishniacii*, Basidiomycota (AB032657)	99	Culture collection
Cryptococcus sp. (NIOCC#PY13)	*Cryptococcus vishniacii*, Basidiomycota (AB032657)	99	Culture collection

respectively. Using classical morphology-based identification system Damare et al. (2006) reported only 8 identified filamentous fungi and several unidentified sporulating and non-sporulating fungi. We do not know how many of non-sporulating fungi reported by Damare et al. (2006) belonged to the same taxa. Using molecular-based identification system in the present study we could overcome some of these problems. Insufficient database for ITS sequences also is one of the reasons for reduced diversity assessment (Zachow et al. 2009; Anderson et al. 2003). Culture-based diversity assessments suffer from biases in isolate recovery, unknown culture conditions, competitive interference and over-growth by other fungi (Panebianco et al. 2002; Pang and Mitchell 2005). These factors might have played a role in underestimating fungal diversity in deep-sea sediments in the present study. Bass et al. (2007) also reported low fungal diversity of uncultured fungal sequences from deep-sea sediments. They reported only 18 fungal 18S-types in deep-sea samples. These results indicate that both, the cultured and uncultured fungal diversity appears to be low in deep sea. Our studies on diversity of yet-uncultured fungi in the same samples might throw some more light on

Table 5 Phylogenetic affiliations of fungal and yeast isolates on the basis of ITS sequences

Isolate ID	Phylogentic nearest taxon, Phylum (GenBank Accession No)	% identity	Source of phylogentic nearest taxon
Penicillium sp. (NIOCC#F2)	*Penicillium citreonigrum*, Ascomycota (AY373908)	99	Dust
Cladosporium sp. (NIOCC#F5)	*Cladosporium cladosporioides*, Ascomycota (EU497957)	100	Deep-sea
Aspergillus sp. (NIOCC#F6)	*Aspergillus* sp., Ascomycota (EF672304)	99	Coffea arabica
Cladosporium sp. (NIOCC#F8)	*Cladosporium cladosporioides*, Ascomycota (EF136373)	99	rice wine, wheat Qu
Cladosporium sp. (NIOCC#F13)	*Cladosporium* sp., Ascomycota (EF105367)	99	toxic mold *Stachybotrys chartarum*
Sagenomella sp. (NIOCC#F15)	Uncultured fungus clone, Eukaryota (DQ279836)	91	deep-sea marine sediments in South China Sea
Ascomycete sp. (NIOCC#F16)	*Exophiala spinifera*, Ascomycota (AM176734)	99	Sediment from deep sea of Pacific Ocean
Tilletiopsis sp. (NIOCC#F21)	*Tilletiopsis albescens*, Basidiomycota (AB025695)	98	Culture collection, Tokyo
Sagenomella sp. (NIOCC#F23)	Doubtful, Ascomycota	-	-
Ascomycete sp. (NIOCC#F30)	Hypocreales sp., Ascomycota (DQ682584)	100	coffee plants with the fungal entomopathogen *Beauveria bassiana*
Tilletiopsis sp. (NIOCC#F33)	*Tilletiopsis albescens*, Basidiomycota (AB025695)	98	Culture collection, Tokyo
Ascomycete sp. (NIOCC#F46)	*Exophiala xenobiotica*, Ascomycota (EF025407)	99	Fungus Testing Laboratory, Texas, US
Aspergillus sp. (NIOCC#F47)	*Aspergillus caesiellus*, Ascomycota (AY373865)	100	Not known
Sagenomella sp. (NIOCC#F48)	*Phialemonium dimorphosporum*, Ascomycota (DQ403199)	99	Lesions excised from *Mugil gyrans*
Capronia coronata (NIOCC#F50)	*Exophiala dermatitidis*, Ascomycota (EF568099)	100	Clinical sample
Acremonium sp. (NIOCC#PF49)	*Trichothecium roseum*, Ascomycota (EU622273)	100	Culture collection, china
Rhodotorula sp. (NIOCC#Y1)	*Sporobolomyces* sp., Basidiomycota (EU002893)	95	peduncle endophyte
Sporidiobolus johnsonii (NIOCC#Y2)	*Sporidiobolus salmonicolor*, Basidiomycota (AY015434)	100	Not known
Rhodosporidium toruloides (NIOCC#Y3)	*Rhodosporidium toruloides*, Basidiomycota (AB073266)	99	Not known
Sporidiobolus johnsonii (NIOCC#Y4)	*Sporobolomyces* sp., Basidiomycota (EU002893)	100	coffee plants
Sporidiobolus johnsonii (NIOCC#Y5)	*Rhodotorula* sp., Basidiomycota (AB025984)	99	deep-sea environments around the northwest Pacific Ocean
Sporidiobolus johnsonii (NIOCC#Y6)	*Sporidiobolus* sp., Basidiomycota (DQ317366)	100	soils and historic wood from the Ross Sea Region of Antarctica
Rhodotorula sp. (NIOCC#Y7)	*Rhodotorula calyptogenae*, Basidiomycota (EU669878)	99	seawater
Coniosporium perforans (NIOCC#Y8)	*Sarcinomyces petricola*, Ascomycota (AJ244275)	98	Meristematic tissues
Graphiola cylindrica (NIOCC#Y9)	*Tilletiopsis oryzicola*, Basidiomycota (AB045708)	95	leaves
Rhodotorula sp. (NIOCC#Y10)	*Rhodotorula mucilaginosa*, Basidiomycota (EU781664)	98	stem from 5 year old tree growing in a subtropical monsoon climate at an elevation of 600-800 m
Cryptococcus sp. (NIOCC#PY12)	*Cryptococcus albidosimilis*, Basidiomycota (AF145331)	100	Antarctica
Cryptococcus sp. (NIOCC#PY13)	*Rhodotorula mucilaginosa*, Basidiomycota (EU781664)	99	stem from 5 year old tree growing in a subtropical monsoon climate at an elevation of 600–800 m

this issue. New culture media and methodologies need to be used as is done for bacterial diversity studies.

Using 18S and ITS sequences, all the fungi isolated in the present study were identified, sequences and cultures deposited in respective repositories, thus increasing the data base for fungi from extreme environment. This study is the first report of molecular phylogenetics to assess the diversity of culturable fungi in deep-sea sediments. Molecular identification facilitated in identifying species which are new to the deep-sea habitat. Low diversity of culturable fungi might also be due to the failure to culture the yet-uncultured fungi, a problem similar to the bacterial diversity in deep-sea environment and many other environments. Direct sequencing of environmental samples would improve our understanding of the fungi present in deep seas as unculturable fungi would be revealed and many methods are becoming available (e.g. DGGE, Duong et al. 2006; Seena et al. 2008). We are now working on 18S and ITS amplification of DNA directly from these deep-sea sediments to estimate diversity of such yet-uncultured fungi.

Marine fungi play a role in macroaggregate formation in deep-sea sediments, possibly carbon sequestration (Damare and Raghukumar 2008), and decomposition of particulate organic matter (Kimura et al. 2001). Marine-derived fungi are also a treasure trove of bioactive molecules (Bugni and Ireland 2004; Raghukumar 2008). Therefore continued efforts to improve culturing techniques will help in discovering the "missing fungi" as a source for new molecules.

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Review

A review on deep-sea fungi: occurrence, diversity and adaptations

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Abstract

The few studies on deep-sea fungi in recent years (using either culturing or molecular signatures) have provided evidence on their occurrence. A majority of culture-dependent and culture-independent fungi recovered have homology to terrestrial species, indicating possible arrival in deep-sea either via wind or terrestrial runoffs. However, the presence of novel fungal sequences with <97% similarity to previously identified fungal sequences in public databases has also been reported. Several filamentous fungi and yeasts recovered from deep-sea sediments of the Central Indian Basin from ~5000 m grew under hydrostatic pressures of 20–40 MPa and 5°C. Some of these fungi had unusual morphology during initial culturing and also when grown at 20 MPa/5°C. Direct detection of fungal hyphae in deep-sea sediments is a daunting task as they are present in low abundance. We demonstrated the occurrence of fungal hyphae in deep-sea sediments by direct staining of the sediments with Calcofluor White, a fluorescent optical brightener. This review presents data on the occurrence, diversity and adaptations of fungi to various deep-sea habitats, with special emphasis on their possible ecological role under these extreme environmental conditions.

Keywords: adaptations; culturing; deep-sea; fungi; hydrostatic pressure.

Introduction

Conditions like low temperature, elevated hydrostatic pressure, and low nutrient availability combine to make the deep-sea an extreme environment. Most of the deep-sea bottom is stable, cold and dark, and it was first assumed that most of the life forms may be present in a state of suspended animation in this largest refrigerator in the world. ZoBell and Johnson (1949) first coined the term barophile and ZoBell and Morita (1957) obtained the first evidence of piezophilic growth in mixed microbial cultures recovered from the deep-sea. Yayanos (1979) reported the first isolate of pressure-adapted bacteria. Subsequently, many psychrophilic piezophiles with various optimal growth pressures have been isolated and characterized physiologically and genetically (see Kato 1999). Marine sediments overlay two-thirds of the earth's surface and harbor diverse and abundant communities (Snelgrove et al. 1997). Early estimates calculated the presence of 3.8×10^{30} prokaryotes in unconsolidated subsurface sediments, with 97% or 3.7×10^{30} occurring at depths shallower than 600 m. The estimated number of prokaryotes in deeper sediments is only 0.13×10^{30} cells (Whitman et al. 1998). This value was uncertain because it was based on extrapolation, but it still represents considerable microbial biomass. What are the functions, metabolism and biogeochemical roles of microbes at these depths?

Barghoorn and Linder (1944) demonstrated the presence of an indigenous marine mycota, with growth and reproduction on submerged wood after defined periods of time. Subsequently, filamentous fungi have been recovered from a variety of materials in the sea. They appear to be associated with decomposing algal and plant tissues including intertidal and benthic algae, seagrasses and mangroves as well as a wide variety of cellulosic materials from land, such as driftwood, and leaves (see Raghukumar 2008). Calcium carbonate deposits are often and apparently actively reworked by filaments of boring fungi (Golubic et al. 2005), which are more widespread and abundant than endolithic algae. The presence of fungi in oceanic waters and deep-sea sediments have been sporadically reported in the past (see Raghukumar and Damare 2008). This review aims to present current knowledge of fungi in the deep-sea with an emphasis on techniques of isolation, direct detection, diversity, adaptations and ecological roles.

Techniques for collection of sediment and water samples

The prerequisite for studying fungi in deep oceans is collection of samples. Many sampling devices have been designed and used for collection of water and sediments. Several bacteriological water samplers have been described that introduce new principles or modifications, but they all have limitations, which restrict their usefulness. The majority of samplers that have been described are suitable only for collecting surface samples or samples from shallow depths. Niskin bottles are used universally for collecting water samples in a sterile container at any oceanic depth (Figure 1), while

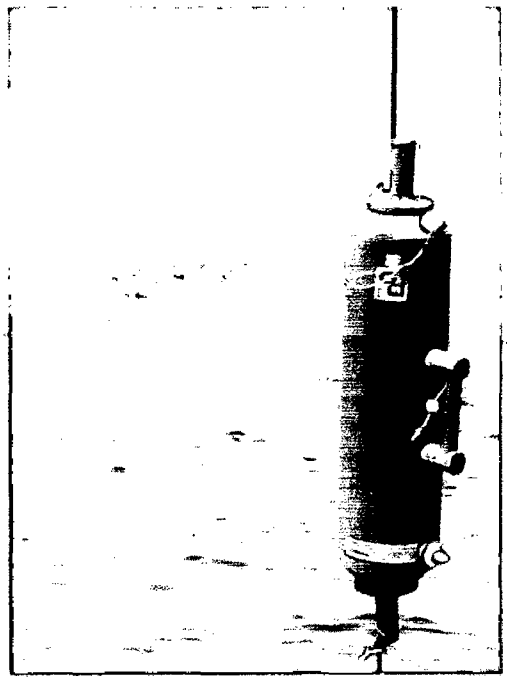

Figure 1 Niskin sample (5 l volume) being lowered into water column to collect water samples.
Several such samplers can be attached on the winch wire for collecting water samples from different depths.

sediments are collected by a variety of corers. The first pressure-retaining sampler for collecting water sample from the deep-sea was successfully tested and used by Jannasch et al. (1973). A deep-sea workstation was developed under the Deep-Star program at JAMSTEC, Japan for retrieving and culturing deep-sea organisms under simulated deep-sea conditions. This system also contains a pressure-retaining sediment sampler, which is able to obtain a sediment sample while maintaining ambient pressure and low temperature after collection from the deep-sea bottom (Yanagibayashi et al. 1999). The most common samplers for collecting deep-sea sediments are box corers, multiple corer or long gravity corers. Sampling with box corers is efficient where the topography of the ocean floor is more or less flat. Raghukumar et al. (2001) used USNEL box corer (50×50×50 cm) during cruises to the Central Indian Basin (CIB) to collect sediment samples (Figure 2). Sub-cores were collected from the center of the box corer with alcohol-sterilized PVC cylinders 5 cm in diameter. Subsections of 2 cm down to 10 cm depth and, thereafter, every 5 cm were extruded from these sediment cores of approximately 30–40 cm length directly into sterile plastic bags to avoid any aerial contaminants. The bags were closed with rubber bands and carried to the working area in the microbiology laboratory on board. Long gravity cores up to 5 m in length were also used for collecting sediment samples (Raghukumar et al. 2004). Precautions to maintain aseptic conditions during sampling are of importance. Samplers need to be sterilized by washing thoroughly with hot water and surface sterilization with steam and alcohol. More recently, samples have been collected with titanium syringes or sediment samplers attached to robotic arms on deep-sea submersibles in hydrothermal vent sites (Burgaud et al. 2009, 2010, Le Calvez et al. 2009).

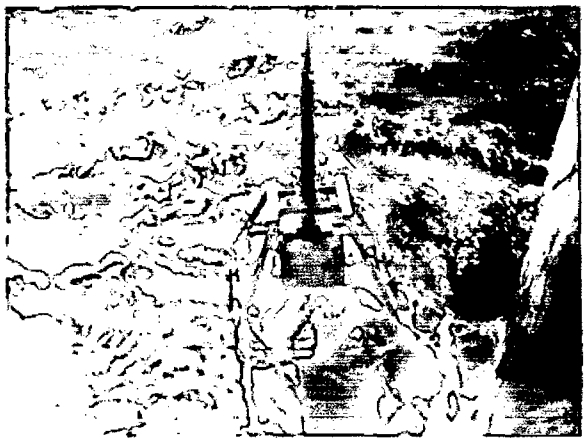

Figure 2 USNEL Box corer (50 cm^3) being lowered to collect sediment samples.

Techniques for culturing deep-sea fungi

It is best to isolate deep-sea fungi on board ship, immediately after the sample is retrieved. This maximizes the chances of getting more culturable fungi, which sometimes may not remain viable during transport to land-based facilities for isolation. Strict procedures to monitor aerial contamination are required during culturing on board. The working area should be alcohol-sterilized each time before handling the sediment cores. The culturing is always done in a closed low-temperature room. In order to check for aerial contaminations during culturing on board the research vessel, nutrient plates should be exposed in the sampling space and in the culturing facilities (e.g., laminar hood and laboratory) during each sampling. The culturing or sediment enrichment should always be carried out in a laminar flow hood in front of gas or spirit burners. Damare (2007) and Singh et al. (2010) isolated filamentous fungi and yeasts from deep-sea sediments and water column, respectively, using such stringent procedures on board the research vessels. For isolating yeasts from the water column, a membrane filtration technique was used (S. Damare, V. Damare, P. Verma, C. Raghukumar, unpublished results). One hundred milliliter water samples were filtered through sterile cellulose ester membranes with a pore size of 0.45 μm (Millipore, Billerica, MA, USA). These membranes were then placed on solid mycological media plates and supplemented with antibiotics to suppress bacterial growth. The agar plates were incubated at a desired temperature (10°C) until visible colonies appeared on the membrane. Le Calvez et al. (2009) and Burgaud et al. (2009) isolated fungi associated with hydrothermal vent animals by crushing them in sterile seawater and plating on to several mycological media.

Apart from the conventional dilution plating method, a modified particle plating method along with pressure incubation methods have also been used to obtain culturable fungi (Damare et al. 2006a, Damare 2007, Singh et al. 2010). In the dilution plating method, ~0.1 g of sediment is suspended in sterile seawater, vortexed for 1 min and 100 µl aliquots are spread-plated. In a particle plating technique (Bills and Polishook 1994), approximately 1 g of sediment slurry is sieved successively through screens with mesh sizes of 200 and 100 µm. Particles that pass through the 200 µm mesh but retained on the 100 µm mesh are spread-plated. For both the above techniques, the plates are incubated at 5°C at 0.1 MPa pressure (1 bar pressure) for 15–20 days. In the pressure incubation method, approximately 0.5 g of sediments are placed in sterile plastic pouches (4×4 cm) containing 2 ml of sterile seawater malt extract broth (MEB); the open ends of the pouches are sealed with an electrical sealing machine (Quickseal, Sevana, India). The bags are placed in a deep-sea culture vessel (Tsurumi and Seiki Co., Yokohama, Japan) filled with sterile water and pressurized. The pressure vessels is immediately placed at 5°C and incubated for 30 days. At the end of this incubation period, 100 µl of the sediment is spread-plated on nutrient media prepared in seawater, and the plates are incubated at 0.1 MPa and 30°C until fungal colonies appear (within 8–10 days). The percentages of culturable fungi obtained by incubation of deep-sea sediments under elevated hydrostatic pressure are higher than those obtained by dilution plating in three cruises to the Central Indian Basin (Table 1).

Bacteriologists have used several techniques to culture previously uncultured bacteria. One such method, called dilution-to-extinction, has yielded difficult-to-culture bacteria like SAR11 (Giovannoni and Stingl 2007). In this technique, the sediment is diluted to give a final concentration of 1–5 bacterial cells ml^{-1} and plated in a modified very low nutrient medium prepared in ambient seawater. Zengler et al. (2002) reported a microencapsulation technique for culturing uncultured bacteria from environmental samples. It involved encapsulation of cells in environmental samples in gel microdroplets (GMD). The gel microdroplets were incubated in amended seawater medium for several weeks, resulting in encapsulated microcolonies. These gel microdroplets were sorted out by flow cytometry, based on their distinct light-scattering signatures. This property enabled discrimination between unencapsulated single cells, empty or singly occupied GMDs and GMDs containing a microcolony. The GMDs were further used for massive cultivation under various low nutrient conditions. This technique helped in culturing previously uncultured phylotypes. Such culturing techniques for culturing deep-sea fungi needs to be developed to tap this immense reservoir of fungal diversity.

Detection and diversity of fungi in the deep-sea

Fungi in shells collected from deep-sea waters of 4610 m depth (Höhnk 1969) were the first identified from the deep-sea. This was followed by isolation of fungi from water samples (collected from surface to 4500 m depth in subtropical Atlantic Ocean waters) using sterile van Dorn bags or Niskin samplers (Roth et al. 1964). Deep-sea fungi have been obtained by directly submerging wooden panels at 1615–5315 m depth (Kohlmeyer 1977). These fungi were, however, not cultured. Four of the fungi were found growing on wooden panels and one on chitin of a hydrozoan. Mycelial fungi growing inside shells of mollusks at 4830 m depth in the Atlantic were documented by Poulicek et al. (1986). Several filamentous fungi were isolated from surface sterilized calcareous fragments collected from 300–860 m depth in the Bay of Bengal (Raghukumar et al. 1992). These fungi were isolated using 1/5 diluted malt extract medium prepared with seawater. Kohlmeyer and Kohlmeyer (1979) observed that "tests for the tolerance of high pressures and low temperatures can indicate whether the isolated fungal species are indigenous deep-sea forms or aliens from other habitats". In concordance with this view, conidia of *Aspergillus ustus* (Bainier) Thom *et* Church and *Graphium* sp. isolated from the calcareous sediments germinated at 10 and 20 MPa pressure in Czapek-Dox medium and on shells suspended in seawater (Raghukumar and Raghukumar 1998).

Recovering culturable fungi from deep-sea sediments and other such extreme environments always carries the risk of contamination. Therefore, only detection by direct examination of sediments provides proof of existence. Direct microscopic detection of fungi in deep-sea sediments has seldom been attempted. Detection of fungal filaments in formalin-preserved calcareous fragments obtained from 965 m depth in the Arabian Sea confirmed that they were actively growing in these shells. These calcareous fragments were treated with EDTA (ethylenediaminetetraacetic acid) to dissolve calcium carbonate, and subsequently stained with the fluorescent brightener Calcofluor White to visualize fungal filaments under an epifluorescence microscope (Raghukumar and Raghukumar 1998). Isolation and direct detection of fungi was reported in deep-sea sediments collected from the Chagos Trench in the Indian Ocean at a depth of 5900 m

Table 1 Number of fungi isolated from deep-sea sediments by different methods during 3 cruises in the Central Indian Basin (CIB).

	Particle plating	Dilution plating	Pressure incubation*	Direct incubation in MEB** at 5°C
Total no. of sediment samples used	532	260	263	153
Number of fungi recovered***	101	50	94	3
Percentage recovery	19	19	36	2

*Incubated at 30 MPa pressure; **malt extract seawater broth. Fungi were isolated as described in detail by Damare et al. (2006a) and Singh et al. (2010); ***number of distinct morphological types obtained during the three cruises in the CIB.

(Raghukumar et al. 2004). Fungal hyphae could not be detected in sediments by bright field microscopy but were visible by epifluorescence microscopy after staining sediments with Calcofluor White (Raghukumar et al. 2004). Damare et al. (2006a) detected fungal mycelia in deep-sea sediments by using polyclonal antibodies. Polyclonal antibodies were produced against *Aspergillus terreus*, the most commonly isolated fungus from these sediments. Presence of *A. terreus* in the sediment was confirmed by immunofluorescence probes. These studies indicate that fungi in deep-sea sediments might have been undetected by conventional microscopy.

Very recently, Burgaud et al. (2010) used a fluorescent *in situ* hybridization (FISH) technique to detect the presence of yeasts in animals collected from deep-sea hydrothermal vents. Although yeasts were obtained from some of the vent fauna, no FISH signal was observed in them. FISH, which uses singly labeled rRNA-targeted oligonucleotide probes, suffers from low signal intensity in the targeted microbes. Therefore, the absence of a FISH signal does not rule out the presence of isolated yeasts in these vent fauna. These authors, however, detected FISH signals in concentrated seawater, indicating that the yeast population is relatively small in deep-sea vents. To detect fungi in deep-sea animals and sediments, it should be feasible to use improved techniques such as CARD-FISH (Amann and Fuchs 2008) or DOPE-FISH (Stoecker et al. 2010). In catalyzed reporter deposition (CARD)-FISH, a horseradish peroxidase-labeled oligonucleotide probe is used, resulting in several-fold higher sensitivity than standard FISH. A further improvement of this technique labeled oligonucleotide probes with multiple fluorescent dyes to increase the FISH signal intensity. Double-labeling-of-oligonucleotide-probes (DOPE)-FISH increases the signal intensity of standard FISH probes by at least a factor of 2, without causing specificity problems. We are planning to use such advanced techniques to detect fungi in CIB sediments and anoxic sediments of oxygen minimum zone (OMZ) in the Arabian Sea, off Goa, India.

The culturable fungi *Penicillium lagena* (Delitsch) Stolk *et* Samson and *Rhodotorula mucilaginosa* (A. Jörgensen) F. C. Harrison were retrieved from a depth of 10,500 m sediment samples from the Mariana Trench in the Pacific Ocean (Takami 1999). Using classical morphology-based taxonomy, Damare et al. (2006a) reported *Aspergillus* sp. are the most dominant form, followed by unidentified non-sporulating cultures from the Central Indian Basin (CIB). Using ITS and 18S sequences of SSU rDNA to identify culturable fungi, Singh et al. (2010) reported 16 filamentous fungi and 12 yeast species from the CIB. They reported for the first time the occurrence of *Sagenomella* sp., *Exophiala* sp., *Capronia coronata* Samuels and *Tilletiopsis* sp. from deep-sea sediments (Table 2). These authors observed that most culturable filamentous fungi belonged to ascomycetes, whereas most of the yeast isolates belonged to basidiomycetes. Burgaud et al. (2009) isolated 62 filamentous fungi, mostly ascomycetes associated with animals, from various deep-sea hydrothermal vent sites. Isolation of fungi associated with deep-sea dwelling macrofauna and zooplankton should be targeted in future to describe their diversity and ecological roles. A new genus and species of deep-sea ascomycete, *Alisea longicola* J. Dupont *et* E.B.G. Jones, was described by analyses of 18S and 28S rDNA sequences and morphological characters (Dupont et al. 2009). The species was isolated from sunken wood obtained from Pacific Ocean off Vanuatu Islands. Connell et al. (2009) isolated eight yeasts and yeast-like fungal species from cold hydrothermal environment and basalt rock surfaces from an active deep-sea volcano, Vailulu'u Seamount, Samoa. Recently, Burgaud et al. (2010) obtained 32 isolates of yeasts belonging to the ascomycota (n=11) and basidiomycota (n=21) phyla associated with deep-sea fauna at hydrothermal vents. Ascomycetous yeasts belonged to seven different taxonomic clusters, whereas basidiomycetous yeasts belonged to five.

Table 2 Diversity of culturable fungi isolated from the deep-sea sediments during three cruises AAS 46, AAS 61 and ABP 26 in the CIB (Damare et al. 2006a, Singh et al. 2010).

Identification of isolates	No. of isolates
Aspergillus sp.	31
Non-sporulating mycelial fungi	26
Aspergillus terreus Thom.	25
Aspergillus restrictus G. Sm.	22
Cladosporium sp.	14
Penicillium sp.	12
Aspergillus sydowii (Bainier *et* Sartory) Thom *et* Church	5
Unidentified ascomycetes	4
Sagenomella sp.	3
Tilletiopsis sp.	2
Acremonium sp.	2
Exophiala sp.	2
Capronia coronata	1
Fusarium sp.	1
Sporidiobolus johnsonii Nyland	4
Rhodotorula sp.	3
Cryptococcus sp.	2
Rhodosporidium toruloides Banno	1
Graphiola cylindrica Kobayasi	1
Coniosporium perforans Sterf.	1
Aureobasidium sp.	1

The occurrence and diversity of microbial eukaryotes from the deep-sea in the aphotic zone at 250–3000 m depth in the Antarctic polar front (Lopez-Garcia et al. 2001), the Guaymas Basin hydrothermal vent (Edgcomb et al. 2002), hydrothermal sediment at the Mid-Atlantic Ridge and Lost City hydrothermal field (Lopez-Garcia et al. 2003, 2007) and the hypersaline anoxic deep-sea basin of L'Atalante (Alexander et al. 2009) have been reported using PCR amplification of SSU rRNA genes and sequence analyses. As these reports demonstrated fungi as one of the eukaryotic groups in these sediments, methods employing amplification of sediment DNA with fungal specific primers to study culture-independent diversity started gaining popularity. Le Calvez et al. (2009) investigated culture-independent fungal diversity by analyzing the small-subunit rRNA gene sequences amplified by PCR using DNA extracts from hydrothermal vent sam-

ples. They reported unsuspected diversity, with new species in three fungal phyla. Many of the species identified are unknown, even at higher taxonomic levels in the chytridiomycota, ascomycota and basidiomycota. On the contrary, Bass et al. (2007) reported very low diversity of filamentous fungi, with only 18 fungal 18S-types from 11 deep-sea sediment samples. These reports were based on direct amplification of small-subunit ribosomal RNA genes from water. Thus, it can be concluded that combining culturing with phylogenetic analysis will give us a better picture of the diversity of fungi in the deep-sea environment. Further, diversity of fungi in deep-sea sediments reported by culture-independent methods (Lai et al. 2007, Takishita et al. 2007, Biddle et al. 2008, Le Calvez et al. 2009, Edgcomb et al. 2010, Nagano et al. 2010) emphasizes the challenge that culture-dependent methods must overcome.

A majority of the fungi reported to date from the deep-sea are terrestrial-like fungi. However, Alker et al. (2001) and Zuccaro et al. (2004) have isolated from marine habitats "so called terrestrial fungi" that may have evolved into marine forms. We need to determine whether they are physiologically different from their terrestrial counterparts in nutritional requirements and enzyme activities. No true piezophilic fungi have been reported so far. They are most likely to be found associated with deep-sea dwelling marine fauna. Detection will be possible only when we have instruments to retrieve deep-sea samples without depressurization. Some of the novel clones or operational taxonomic units (OTUs) described by several research groups (Lopez-Garcia et al. 2001, Gadanho and Sampaio 2005, Lai et al. 2007, Le Calvez et al. 2009, Nagano et al. 2010, P. Singh, C. Raghukumar, P. Verma, Y. Shouche, unpublished data) from various deep-sea locations may indeed be true piezophiles. We also need to devise newer culturing methods and media for obtaining yet-uncultured forms in culture. The association of many eukaryotic phylotypes with parasitic organisms may have significant impacts on host population and diversity in the deep-sea environment (Brown et al. 2009).

Sampling of deep marine seafloor sediments by the ocean drilling program (ODP) has enabled examination of microbial abundance and diversity in this extreme environment (Parkes et al. 2005). Biddle et al. (2005) reported recovery of ascomycetous fungi belonging to the genera *Cladosporium, Penicillium* and *Acremonium* spp. by direct plating and by enrichment culturing technique from sediment core collected at 200 m below sea floor (mbsf) from 252 m water depth on the outer shelf edge of the Peru Margin. These cultured fungi were identified by ITS sequencing. From the deep-sea sediment cores down to 37 m below the seafloor of the Peru Margin and Peru Trench, Edgcomb et al. (2010) have recovered fungal sequences from both DNA- and RNA-based clone libraries. Basidiomycetous fungi were the most consistent phylotypes recovered from these sites. Working with RNA-based clone libraries, these authors were also able to identify the active members of the community. Further, the presence of fungi has been reported from some more extreme environments. The basidiomycetous yeast *Cryptococcus curvatus* (Diddens *et* Lodder) Golubev was found to be the dominant fungal phylotype in oxygen-depleted sediments from deep-sea methane seeps (Takishita et al. 2006, 2007). The seeping fluids in such areas contain methane and hydrogen sulfide. Fungal sequences have been recovered from gas-hydrate bearing sediments (Cao 2010). Gas hydrates are ice-like minerals that have crystallized under low temperature, elevated hydrostatic pressure and methane concentrations. In gas-hydrates, methane is the dominant hydrocarbon in the gas mixture held in water molecules. Lai et al. (2007) reported several fungal sequences in methane hydrate-bearing deep-sea sediments that were not associated to any known fungi or fungal sequences in the public databases. Moreover, clones phylogenetically similar to *Phoma, Cylindrocarpon, Hortaea, Cladosporium, Emericella, Aspergillus, Malassezia, Cryptococcus, Lodderomyces, Candida* and *Pichia* were also recovered.

Physiological characterization of fungi isolated from deep-sea hydrothermal vents has shown the presence of true halophiles and those that are halotolerant (Burgaud et al. 2009, 2010, Le Calvez et al. 2009). Most of the recovered fungi from the CIB sediments were mesophiles or psychrotolerant, but no true halophiles or psychrophiles were reported (Damare et al. 2006a, Singh et al. 2010). Several of the fungi isolated had similarities with fungi that are animal parasites (e.g., Burgaud et al. 2009, Le Calvez et al. 2009, Nagano et al. 2010) and thus these may play an important role as facultative parasites of deep-sea animals and impact host population and diversity in the deep-sea environment (Moreira and Lopez-Garcia 2003, Brown et al. 2009). It is also likely that some of these fungi are in symbiotic association with deep-sea fauna or have stimulating effects on host defense responses. A *Cryptococcus* sp. associated with the healthy coral *Pocillopora damicornis* Linnaeus elicited a cryoprotective response from the soft tissue of the coral. The fungus was demonstrated to extend the survival of coral cells by two days, selectively maintaining skeletogenic cell types (Domart-Coulon et al. 2004). Nagano et al. (2010) reported a special group designated DSF-Group 1, containing at least 14 OTUs, some of which have been reported from oxygen-depleted deep-sea environments like methane cold seeps, anoxic bacterial mats and below the sea floor, but not from shallow seas or surface waters (Bass et al. 2007, Takishita et al. 2007). It is likely that they may be anaerobic or facultatively anaerobic fungi. Several of the yeasts isolated by Connell et al. (2009) produced siderophores, a class of molecules used to assimilate and utilize Fe(III) from the environment, and one isolate was capable of oxidizing Mn(II). These results indicate that fungi may play an active role in biomineralization processes in the deep-sea. Methylotrophic yeasts present in methane seeps (Lai et al. 2007) may play a significant role in global methane and carbon cycles and might find application in diagnostic processes.

Growth of deep-sea isolated fungi under simulated deep-sea conditions

The basic requirement for these studies is an instrument able to simulate the conditions in the deep-sea. Different types of

instruments have been used, but the majority of the work has focused on bacteria (Jannasch and Wirsen 1977, Helmke 1979, Bernhardt et al. 1987). Lorenz and Molitoris (1992) produced a system, modified from equipment developed for bacteria, to cultivate fungi, especially yeasts, under simulated deep-sea condition. ZoBell and Johnson (1949) and Yamasato et al. (1974) showed that yeasts isolated from terrestrial and marine environments grew at elevated hydrostatic pressure, with an upper limit of approximately 50 MPa. However, they grew the cultures in closed glass tubes, and only the oxygen dissolved in the medium was available for growth of the cells. This was also true for other methods used for bacteria, such as cultivation in thick-walled silicone tubing (Groß et al. 1994) and in plastic syringes (Alongi 1990). Helmke (1979) demonstrated that such cultivation techniques did not produce sufficient biomass for growth assessment of actinomycetes. To overcome this limitation, Helmke cultured actinomycete cells in polypropylene bags in a fluorocarbon-filled pressure vessel, as fluorocarbon has high oxygen solubility. Berger and Tam (1970) cultured aerobic bacteria in plastic bags, but used water, which had low oxygen solubility; due to lack of oxygen exchange, the system became anaerobic very rapidly. In all our growth-related studies, we used oxygen-permeable polypropylene bags resulting in a good biomass yields as dry weight of filamentous fungi or yeasts (Damare et al. 2006a, Singh et al. 2010).

Under simulated laboratory conditions, we compared the growth and production of biomass by deep-sea filamentous fungi and yeasts (measured as mg dry weight in 20 ml culture medium). Growth measurements of yeasts were carried out by biomass estimation and not by measuring optical density because yeast cells tended to stick to the surface of culture pouches and scraping them off with medium resulted in dilution of the turbidity. Most of the fungi were piezotolerant, with growth under elevated hydrostatic pressure. A majority of filamentous fungi and yeasts had higher biomass production at 0.1 MPa and 30°C than under elevated hydrostatic pressure (20 MPa) at 5 and 30°C (Table 3). Six filamentous fungi produced biomass in the range of 10–15 mg dry weight in 20 ml culture medium at 20 MPa/30°C, and six fungi produced biomass in the range of 25–30 mg in 20 ml culture medium at 20 MPa/5°C (Table 3). Five yeasts produced biomass in the range of 2–4 mg dry weight in 20 ml culture medium at 20 MPa/30°C and at 20 MPa/5°C (Table 3). These cultures had better growth in media prepared in seawater than in distilled water. However, none of the cultures showed an absolute requirement for seawater for growth (Damare et al. 2006a). In one experiment, of the 32 filamentous fungi compared for biomass production in media prepared in seawater and distilled water, equal numbers had biomass maxima in seawater and distilled water (Singh et al. 2010). However, biomass production was higher in seawater than in distilled water. Similar results were obtained with deep-sea yeasts (Singh et al. 2010). We require development of bioreactors [similar to those used for bacteria (Miller et al. 1988)] to measure accurately the biomass of yeasts and filamentous fungi continuously in order to calculate specific growth rates under simulated deep-sea conditions. Terrestrial fungi also grew and produced biomass under hydrostatic pressures of 20 MPa at 5° and at 30°C (Damare et al. 2006a).

Table 3 Biomass of filamentous fungi and yeasts produced under varying hydrostatic pressures and temperatures.

Biomass range (mg dry weight in 20 ml culture medium)	No. of filamentous fungi*			
	0.1 MPa/30°C	0.1 MPa/5°C	20 MPa/30°C	20 MPa/5°C
5–10	0	0	4	0
10–15	0	3	6	0
15–20	1	5	5	1
20–25	5	5	1	4
25–30	2	2	0	6
30–35	1	1	0	4
35–40	1	0	0	1
40–45	4	0	0	0
45–50	1	0	0	0
50–55	1	0	0	0
	No. of yeast cultures*			
0–2	0	1	6	5
2–4	0	5	5	5
4–6	1	3	0	2
6–8	2	2	1	0
8–10	5	1	0	0
10–12	2	0	0	0
12–14	2	0	0	0

*Number of filamentous fungi and yeast showing the biomass production in a particular range under specific growth condition. Culturable fungi and yeasts were isolated during cruise #ABP26 (Singh et al. 2010). Biomass as dry weight for mycelial fungi was determined after 20 days incubation in malt extract broth under different pressure and temperature conditions. Biomass as dry weight for yeasts was determined after 20 days incubation in yeast extract, peptone, dextrose broth under various pressure and temperature conditions as described in detail by Singh et al. (2010).

Daniel et al. (2006) opined that most, if not all, organisms can live under a large range of hydrostatic pressures. Surface organisms can survive and grow up to a pressure of 20 MPa (equivalent to 2 km depth in oceans). These authors believe that as life originated in the deep-sea environment, tolerance of elevated hydrostatic pressure is a physical/chemical attribute found in all organisms. We need to verify whether all, or at least a majority of terrestrial fungi are able to survive under deep-sea conditions.

Response of spores and mycelia to low temperature and elevated hydrostatic pressure

Germination of fungal spores in the deep-sea may be hindered by several obstacles, such as the mycostatic effect of seawater (Kirk 1980), low temperature, elevated hydrostatic pressure and low nutrient conditions. A defining characteristic of spores is their ability to develop into a new individual without fusion with another reproductive cell. The first step in this is spore germination, which is the sequence of events that converts the resting/dormant spore into a rapidly growing germ tube from which the mycelium is produced by elongation, septum formation and branching. A spore is defined as having germinated if the germ tube is at least as long as the width of the spore. The effect of extreme conditions on the germination of conidia and viability of deep-sea *Aspergillus* isolates has been examined by Damare et al. (2008). Spores of three deep-sea and terrestrial *Aspergillus* isolates and a deep-sea *Cladosporium* isolate germinated much better in sediment extracts of different dilutions at 20 MPa pressure at 30°C, but not at 5°C (Table 4). However, percentage germination decreased gradually with increasing pressure. Spores failed to germinate at 5°C, even at 0.1 MPa pressure, through an incubation period of 20 days. Although all of these fungi produced biomass at elevated hydrostatic pressure and low temperature when initiated with mycelial inoculum, spores did not germinate under these conditions (Damare 2007). Thus, it appears that low temperature and not elevated hydrostatic pressure is a limiting factor for spore germination and further biomass production in the deep-sea. A temperature shock of 15 min at 50°C may break the dormancy of spores in three *Aspergillus* species, resulting in their germination at 0.1 MPa at 5°C but not at 20 MPa/5°C (Table 5), indicating that this shock was not sufficient in the face of combined negative effects of low temperature and elevated hydrostatic pressure. Thus, fungal mycelial fragments that appear to be metabolically more active are more tolerant to elevated hydrostatic pressure and low temperature than the dormant fungal spores. In conclusion, mycelial fragments have better chances of propagation under deep-sea conditions than spores. As observed by Ivanova and Marfenina (2001), we suspect that the critical size of mycelial fragments may also influence the survival of fungal species in stressful environments.

Pressure effects on yeasts

The term piezophysiology is used to describe the effects of high pressure on growth, viability and cellular responses in living cells (Abe 2004). *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen, a piezotolerant yeast is used as a model organism to study the effects of hydrostatic pressures. Growth and

Table 4 Effect of sediment extract dilutions and elevated hydrostatic pressures on spore germination of deep-sea isolated and terrestrial fungi.

Concentrations of sediment extract**	% Germination of spores							
	Aspergillus terreus (deep-sea isolate)		*Aspergillus ustus* (deep-sea isolate)		*Cladosporium* sp. (deep-sea isolate)		*Aspergillus terreus* (terrestrial isolate)	
	0.1 MPa/ 30°C	20 MPa/ 30°C	0.1 MPa/ 30°C	20 MPa/ 30°C	0.1 MPa/ 30°C	20 MPa/ 30°C	0.1 MPa/ 30°C	20 MPa/ 30°C
1 (undiluted)	100	36	100	23	100	19	100	28
1:10	100	31	100	26	114	20	90	25
1:20	93	30	100	19	78	21	82	21
1:40	77	35	100	24	77	17	77	20
1:60	73	27	96	20	73	10	79	13
1:80	65	22	88	10	70	10	67	7
1:100	51	12	63	10	60	9	52	9

Hydrostatic pressure (MPa)***	% Germination of spores at varying hydrostatic pressures at 30°C*			
0.1	94	100	92	90
10	10	29	22	21
20	9	25	20	19
30	5	16	12	5
50	3	8	5	6

*No germination observed at 5°C for all the pressures; **data taken from Damare and Raghukumar (2008); ***data taken from Damare et al. (2006a).

Table 5 Effect of varying hydrostatic pressures and temperatures on spore germination.

Incubation conditions	% Spores germinated								
	Aspergillus terreus (deep-sea isolate)			*Aspergillus ustus* (deep-sea isolate)			*Aspergillus terreus* (MTCC479, terrestrial isolate)		
	A	B	C	A	B	C	A	B	C
20 MPa/30°C	14±4	12±3	16±4	6±2	9±2	8±3	13±3	16±4	18±4
20 MPa/5°C	0	0	0	0	0	0	0	0	0
0.1 MPa/30°C	100	100	100	100	100	100	100	100	100
0.1 MPa/5°C	0	0	100	0	D	100	0	0	100

A, malt extract broth (MEB); B, MEB with 20% sucrose; C, spores given a heat shock of 50°C for 15 min and then incubated in MEB with 20% sucrose; D, doubtful germination. Data taken from Damare and Raghukumar (2008). ± refers to SD.

cellular activity in *S. cerevisiae* is unaffected at pressures lower than 20–30 MPa. Higher pressures induce various stress response through pressure-inducible genes and proteins. Piezotolerance depends on the duration of high-pressure application. Yeast cells in stationary phase are more resistant to pressure than actively growing cells (Abe 2004, Fernandes 2005). Hydrostatic pressure of 200 MPa for 30 min induce several morphological changes in wild-type and trehalose-6-phosphate synthase (*tsp1*) mutant cells of *S. cerevisiae* (Fernandes et al. 2001). Such mutant cells are unable to accumulate trehalose, a disaccharide, a well-known cell membrane protectant against adverse environmental conditions. When the cells were subjected to preheat treatment at 40°C for 60 min before application of the pressure treatment, both the types of cells (wild and mutant) acquired resistance to the pressure. Such induced barotolerance in *S. cerevisiae* by heat shock was earlier reported by Iwahashi et al. (1991).

Effect of lethal pressures on yeast cells have been studied by several groups (Iwahashi et al. 1991, 2003, Fernandes et al. 2004). Iwahashi et al. (2003) studied DNA microarrays of *Saccharomyces cerevisiae* and analyzed expression levels of ~6000 genes. The genome-wide expression profiles suggested that high pressure (180 MPa at 4°C for 2 min) causes damage to cellular organelles similar to the damage caused by detergents, oils, freezing and thawing. Fernandes et al. (2004) studied gene expression patterns in response to hydrostatic pressure of 200 MPa for 30 min in *S. cerevisiae* by whole genome microarray hybridization. Among the 6200 genes analyzed by these authors, ~5% were affected by hydrostatic pressure treatment, 131 were >2-fold induced, while 143 were <2-fold down-regulated. The majority of the up-regulated genes were involved in stress defense and carbohydrate metabolism, while a large number of down-regulated genes were in the categories of cell cycle progression and protein synthesis. The effects of pressures that cause growth inhibition in *S. cerevisiae* are different from those caused by lethal pressures (Abe 2004). Accordingly, Iwahashi et al. (2005) studied genome-wide mRNA expression profiles of *S. cerevisiae* grown under 30 MPa, which was shown to cause growth inhibition. They reported up-regulation of genes involved in membrane metabolism, and the responses were essentially different from the response to pressures that cause cellular death.

Pressure effects on filamentous fungi

Microbial activity is inhibited under elevated hydrostatic pressure in comparison with activity at 0.1 MPa. All components of activity, including growth, respiration, and specific biochemical processes, appear to be affected by elevated hydrostatic pressures (Abe and Horikoshi 1995, Fernandes et al. 2004, Daniel et al. 2006, Abe 2007). Pressure-inducible genes, which aid in pressure acclimatization, have been proposed for marine bacteria that experience large vertical transport in the water column (Bartlett 1991). In bacteria, pressure effects on gene expression, membranes, membrane proteins, DNA structure and function, cell division, protein and enzyme functions have been studied in detail, while in fungi the studies have been restricted to detection in deep-sea sediments and the ability to grow under elevated hydrostatic pressure and produce extracellular enzymes active under elevated hydrostatic pressure (see Raghukumar and Damare 2008).

Exposure of both prokaryotic and eukaryotic cells and/or tissues to a variety of physiological stresses results in the rapid synthesis of a specific class of proteins. This phenomenon is known as the "stress response", and the newly formed transient proteins are termed "stress proteins". These proteins play a significant role in cells and tissues in the manifestation of adaptation and may serve as a defense mechanism against a variety of stress conditions (Lindquist and Craig 1988). As the adaptation of other kinds of stress or shock condition is often mediated by the production of such specific stress proteins (e.g., heat shock, cold shock or antifreeze proteins), a suitable approach to high-pressure response would be to look for alterations in the protein patterns of microorganisms grown at different hydrostatic pressures, ranging from atmospheric pressure to the limit of viability.

Some fungi have abnormal morphology immediately after isolation from deep-sea sediments of the CIB (Damare et al. 2006a). They had extremely long conidiophores and vesicles covered by long hyphae, instead of phialides of metulae or conidia, as is typical of the genus *Aspergillus*. Most of these abnormal features disappeared after repeated (~4–6 times) subculturing. Several fungi isolated from deep-sea sediments were initially non-sporulating, but formed spores after repeated (~6–8 times) subculturing at 0.1 MPa pressure. Several of these fungi had distinct swellings when grown

under elevated hydrostatic pressure. The type of nutrients used for culturing also seemed to affect the morphology. When deep-sea fungi were grown in malt extract seawater broth, the hyphae had several swellings, while they grew quite normally in sediment extract medium (Damare and Raghukumar 2008). Two deep-sea fungi have microcyclic conidiation when grown at 10 MPa (Raghukumar and Raghukumar 1998). In this case, immediate conidiation followed conidial germination, without the growth of a vegetative mycelium. Microcyclic conidiation is reported to occur under nutrient-limiting conditions and may help fungi complete their life cycles quickly. This may result from the arrest of apical growth followed by lateral differentiation of conidium-producing cells.

Possible ecological roles in the deep-sea environment

Aggregate formation and carbon contribution

Bacteria and fungi are major players in transformation of organic matter in terrestrial soil. In contrast to land, however, most studies on deep-sea sediments have focused exclusively on bacteria and have demonstrated their intense metabolic activities (Turley and Dixon 2002). The fungi and their role in the deep-sea sediments have remained neglected, mainly due to the fact that they are not easily observable. This is because fungi mostly remain embedded in aggregates and hence go unnoticed (Damare and Raghukumar 2008). Treatment with EDTA increases detection of fungi. EDTA is commonly used to dissolve calcareous substrates for extraction of living cells. It is sufficiently acidic for dissolution of carbonates, but does not kill algal or fungal cells. EDTA is a chelating agent that is effective in solubilizing the polysaccharides as well (Liu et al. 2002). It was further suggested that fungi in deep-sea sediments may be involved in humic aggregate formation by processes very similar to those in terrestrial sediments. Reducing sugars and amino acids formed as by-products of microbial metabolism in terrestrial sediments are known to undergo non-enzymatic polymerization and form brown-colored products, constituting humus (Tisdall and Oades 1982). Humic material combines with soil particles to form microaggregates. Fungal hyphae further act as binding agents to form macroaggregates by trapping fine particles into the microaggregates (Kandeler et al. 1999). Fungi or bacteria can thus remain protected in certain particle size classes (Suberkropp and Meyers 1996). Cations such as Si^{4+}, Fe^{3+}, Al^{3+} and Ca^{2+} form bridges between particles in terrestrial microaggregates (Bronick and Lal 2005).

Although fungi are generally believed to be more effective at aggregate stabilization than other soil microflora, several studies have concluded that their primary contribution to aggregation is through hyphal entanglement of soil particles (Molope et al. 1987). Damare and Raghukumar (2008) demonstrated formation of microaggregates by fungi grown in sediment extract medium under 20 MPa pressure. The microaggregates stained positively for humic substances indicating that fungal activities are responsible for the formation of humus, perhaps in deep-sea sediments as well (Stevenson 1994). Fungi present, concealed inside these microaggregates contribute to the nutrient cycling in the deep-sea habitat, by acting upon them from within the aggregates. Aggregation protects soil organic matter in a biotically regulated mechanism for accumulation and maintenance of soil organic matter (Beare et al. 1994). This is a particularly helpful feature in natural environments as it prevents extracellular enzymes from diffusing away from secreting cells. Thus, these humic-enzyme complexes have an important role in overall nutrient dynamics of the sediments (Burns 1978). Fungi produce a large amount of exopolysaccharides (Selbmann et al. 2003), which in turn may enrich the aggregates. Hyphal sheaths often have an adhesive role in the attachment of fungal mycelium to surfaces and particle entrapment (Hyde et al. 1986).

Fungal biomass constitutes a large portion of the potentially mineralizable organic matter associated with forest and grassland soils (Chiu et al. 2006). Damare and Raghukumar (2008) estimated fungal biomass in deep-sea sediments based on fungal biovolume. By microscopically measuring hyphal lengths and diameters of fungi in Calcofluor-stained sediments and assuming a cylindrical shape, fungal biovolume was calculated using the formula: $\pi r^2 \times$hyphal length. Biovolume was converted to carbon assuming an 1.0 pg C μm^{-3} (Van Veen and Paul 1979). Based on these values, the fungal organic carbon contribution to deep-sea sediments of Central Indian Basin was estimated to be 2.3–6.3 $\mu g\ g^{-1}$ dry sediment. The bacterial carbon contribution for the same site was reported in the range of 1.0–4.0 mg g^{-1} dry sediment (Raghukumar et al. 2001). The fungal carbon contribution in the coastal waters off Goa ranged from 10–150 pg g^{-1} sediment (Jebraj and Raghukumar 2009). In contrast, the fungal carbon contribution in soil from grassland and forests is in the range 453–3375 $\mu g\ g^{-1}$ dry sediment (Chiu et al. 2006). The results we obtained may be large underestimates as fungal biomass estimated by hyphal length may often fall below values obtained by biochemical indicators like phospholipids (Balser et al. 2005), hexosamine (Gessner and Newell 2001) or ergosterol (Mille-Lindblom et al. 2004). Melanin and chitin, the polymers of fungal cell walls are not easily degradable, whereas phospholipids of bacterial cell walls are easily degraded. Thus, fungus-mediated C storage is expected to be more persistent than that sequestered by bacteria (Bailey et al. 2002). A comparative quantitative study using ^{14}C-labeled sugars and amino acids with pure cultures of deep-sea bacteria and fungi to measure growth, uptake kinetics and carbon sequestration under simulated deep-sea conditions would improve quantitative estimation of deep-sea fungal roles.

Extracellular enzymes

Several enzymes are involved in the cycling of nutrients in the deep-sea and can be used as potential indicators of nutrient cycling processes. In aquatic ecosystems such as the deep-sea, alkaline phosphatase activity (APA) plays an important role in the regeneration of inorganic phosphate through its catalysis of organic esters to inorganic P (Chróst 1991). Organic P content in the CIB measured during three

cruises varied from 0.007–0.011%, and sediment alkaline phosphatase activity was in the range 0.06–7.80 U, which is equivalent to the release of 1.0 μmol phosphate phosphorus h^{-1} g^{-1} dry sediment (Raghukumar et al. 2006). Damare (2007) tried to differentiate the contributions of fungi and bacteria to APA in deep-sea sediments of the CIB in simulated deep-sea conditions in laboratory microcosm experiments (Table 6). The plain sediment without any additional nutrients had very low APA. On addition of detritus, APA increased 20-fold, indicating sharp increases in microbial activity, typical of a "feast and famine" deep-sea habitat, where the nutrient supply is mostly in the form of pulses; nutrients are sometimes available in plenty and sometimes not available for days in a row. APA was higher in the presence of antibacterial antibiotic, indicating a major share of fungi towards APA (Table 6). This indicates that fungi might play an important role in degradation of detritic material, as reported in the terrestrial and mangrove environments (Newell 1996). In contrast, addition of autoclaved *Artemia* larvae and cysts had no effect on APA. A protease enzyme might play a direct role in degradation of such animal detritus. The deep-sea fungal isolate *Aspergillus terreus* (#A4634) in the presence of antibacterial antibiotics had very high APA at both incubation pressures (in comparison with deep-sea bacterial APA in the presence of a fungicide in the same microcosm experiment) (Table 6). Hence, fungi may be playing an important role in the P cycle in the deep-sea.

Deep-sea microorganisms growing under extreme conditions are likely to be a good source of industrially useful enzymes with novel properties (Synnes 2007, Dang et al. 2009). Protease is one of several extracellular enzymes produced by the fungi that have major roles in their nutritional requirements. Damare et al. (2006b) demonstrated that 11% of the total fungi (n=221) isolated from CIB deep-sea sediments have low temperature-active protease production. These fungi when grown under elevated hydrostatic pressure synthesized extracellular protease, but in very low quantity compared with that produced under 0.1 MPa. As enzyme production is governed by growth, reduced growth at 5°C and elevated hydrostatic pressure appeared to be responsible for low-levels of enzyme production. One of the fungi isolated from the deep-sea produced 1639 Azocasein units ml^{-1} of alkaline protease with optimum activity at pH 9.5, with 45% and 25% of activity at 20°C and 15°C, respectively. It was active in the presence of several commercial detergents and 0.5 M NaCl, equivalent to a seawater salinity of 29. These features are desirable for commercial application as a detergent additive in cold laundry washes (Raghukumar et al. 2009). The proteases from deep-sea fungi might prove to be a valuable source of detergent enzymes.

Fungal polygalacturonases (PGase) are useful enzymes for clarification of fruit juices in the food industry. Two novel endopolygalacturonases active at 0–10°C were purified from the culture supernatant of a deep-sea yeast (strain N6) isolated from the Japan Trench at a depth of 4500–6500 m (Miura et al. 2001, Abe et al. 2006). The hydrolytic activity of N6-PGases remained almost unchanged up to a hydrostatic pressure of 100 MPa at 24°C (Abe et al. 2006). Interestingly this strain of *Cryptococcus* was tolerant to $CuSO_4$ up to a concentration of 50 mM and had high activity of superoxide dismutase, an enzyme responsible for scavenging superoxide radicals (Abe et al. 2001). High pressure can shift the temperature requirement for a given chemical reaction towards lower temperature (Daniel et al. 2006). Therefore, it might be possible for a reaction requiring a thermophilic enzyme to perform effectively with a mesophilic enzyme at elevated hydrostatic pressure.

Table 6 A comparison of bacterial and fungal contribution to alkaline phosphatase activity (APA) under simulated deep-sea conditions in the presence and absence of additional nutrients.

Experimental condition	Release of MUF (μg l^{-1} h^{-1})**	
	20 MPa/5°C	50 MPa/5°C
1 Plain sediment	5.6±0.3	4.6±0.9
2 Sediment+antibacterial antibiotic	4.5±1.2	4.9±0.1
3 Sediment+fungicide	1.5±0.9	0.2±0.1
4 Sediment+detritus	101.2±25.4	98.6±21.1
5 Sediment+detritus+antibacterial antibiotic	90.0±8.4	97.0±15.9
6 Sediment+detritus+fungicide	73.5±34.9	70.1±34.9
7 Sediment+artemia larvae	5.0±1.6	2.6±0.4
8 Sediment+artemia larvae+antibacterial antibiotic	3.9±0.6	2.0±0.7
9 Sediment+artemia larvae+fungicide	3.4±1.5	2.6±1.2
10 Sediment+detritus+deep-sea fungus*+antibacterial antibiotic	108.0±0	93.9±9.3
11 Sediment+detritus+deep-sea bacteria+fungicide	84.9±16.6	67.4±0.6

**Aspergillus terreus*; **APA activity was estimated by measuring the released fluorescent compound 4-methylumbelliferone (MUF) from MUF-phosphate at 340 nm excitation and 450 nm emission. The pH of the reaction mixture was 8.3 (Hoppe 1983). Ampicillin (0.1 g in 100 ml of the medium) and penicillin (4000 U in 100 ml of the medium) were used as the antibacterial agents. Bavistin™ at 0.5% final concentration was used as an antifungal compound. The concentrations of the antibiotic and the fungicide used in the study were standardized by growing bacteria and fungi in the presence of varying concentrations of both the inhibitors. The second lowest concentration not showing cross inhibition (fungicide not inhibiting bacteria and vice versa) were used in the study. To simulate sporadic enrichment of the deep-sea sediments, phytodetritus and dead *Artemia* larvae were used for spiking the sediments. Detritus was collected from shallow waters of Dona Paula Bay (Goa, India) with a 200 μm plankton net. It was a mixture of phyto- and zooplankton (Damare 2007). ± refers to SD.

Conclusion

Deep-sea sediments appear to be habitats of hidden fungal diversity; accordingly a systematic survey of world oceans for culture-dependent and culture-independent fungi is recommended. Newer methods and media to isolate and culture require development. Their role in macroaggregate formation is important in biogeochemical cycling of nutrients and carbon sequestration in the deep-sea. They may be a source of novel secondary metabolites and enzymes with industrially desirable properties (Pettit 2010).

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ENVIRONMENTAL MICROBIOLOGY

Fungal Community Analysis in the Deep-Sea Sediments of the Central Indian Basin by Culture-Independent Approach

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Abstract Few studies have addressed the occurrence of fungi in deep-sea sediments, characterized by elevated hydrostatic pressure, low temperature, and fluctuating nutrient conditions. We evaluated the diversity of fungi at three locations of the Central Indian Basin (CIB) at a depth of ~5,000 m using culture-independent approach. Community DNA isolated from these sediments was amplified using universal and fungal-specific internal transcribed spacers and universal 18S rDNA primer pairs. A total of 39 fungal operational taxonomic units, with 32 distinct fungal taxa were recovered from 768 clones generated from 16 environmental clone libraries. The application of multiple primers enabled the recovery of eight sequences that appeared to be new. The majority of the recovered sequences belonged to diverse phylotypes of Ascomycota and Basidiomycota. Our results suggested the existence of cosmopolitan marine fungi in the sediments of CIB. This study further demonstrated that diversity of fungi varied spatially in the CIB. Individual primer set appeared to amplify different fungal taxa occasionally. This is the first report on culture-independent diversity of fungi from the Indian Ocean.

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Introduction

Over 70% of the earth is covered by oceans, and ocean bottom which is covered with sediments of varying texture, form a major habitat for benthic organisms. Most marine sediments are located in cold, dark, high-hydrostatic-pressure habitats where food comes from distant surface waters [33]. Several studies have described the presence of diverse forms of microbes from this habitat [24, 34, 36]. Besides bacteria fungi also play a major role in the global biogeochemical cycle of nutrients and have been reported from various terrestrial [11] and marine habitats [16], including deep-sea environments [8]. There is a growing interest in understanding the microbial diversity in extreme environments. However, diversity of fungi, their ecological role and abundance in the deep-sea which is one such environment, is poorly understood. Sporadic reports of fungi cultured from deep-sea sediments from various locations exist. Species of *Phoma, Lodderomyces, Malassezia, Cryptococcus, Cylindrocarpon, Hortaea, Pichia, Aspergillus*, and *Candida* have been cultured from methane hydrate bearing deep-sea sediments collected from water depths in South China down to ~3,000 m [19]. While culturable fungi obtained from deep-sea sediments of the Central Indian Basin from ~5,000 m depth were identified by classical morphology [8], those isolated by Singh et al. [32], were identified by molecular taxonomy. In both the cases, the fungi obtained showed growth under elevated hydrostatic pressure and low temperature. These studies may not depict the actual diversity of fungi in deep-sea sediments because of limitations in culturing methods and viability of fungal propagules. A few studies applying targeted environmental sequencing have reported existence of fungal species in deep-sea Antarctic plankton [23]. Using fungal-specific 18S rRNA

gene libraries, Bass et al. [2] showed yeasts to be the dominant forms at several locations in the deep oceans. 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 [22] whereas culturable fungi, identified with universal ITS primers from several hydrothermal vent sites were reported recently [5]. While studying microeukaryotic diversity in an acidic iron river, Gadanho and Sampaio [10] reported new fungal phylotypes with fungal-specific primers that were not detected when universal eukaryotic primers were used. These studies suggested that fungal diversity could have been underestimated in such extreme environments. In view of the above, we constructed gene libraries with universal and fungal-specific primer sets to assess the fungal diversity in the deep-sea sediments of the Central Indian Basin (CIB) through a culture-independent approach in the present study. Such primers have been known to amplify a variety of fungal sequences from mixed environmental samples [26]. Furthermore, fungal community composition analysis was carried out for distantly located stations as well as two depths at each of these stations.

Methods

Sampling

Sediment samples were collected on the cruise ABP-26 on board the Russian research vessel Akademik Boris Petrov in December 2006 from three stations namely, SVBC-04 (station A), SVBC-31 (station B), and SVBC-37 (station C) of the Central Indian Basin (Fig. 1) at depths of ~5,000 m (Table 1). The seafloor of the CIB is characterized by the presence of homogenous, soft to slightly compact, dark brown sediments with yellowish sediments at deeper depths. The station SVBC-37 was in a seamount area and the sediment texture was calcareous. The other two stations were characterized by the presence of siliceous sediments. The sampling procedure described by Raghukumar et al. [29] and Damare et al. [8] was followed. The samples were collected with an USNEL-type box corer of 50 cm^3 size. Thus, the collected sediments were mostly undisturbed and compact. The average length of sediment cores obtained from these locations was ~30 cm. Sub-cores of sediments were collected using alcohol-sterilized PVC cylinder of 5-cm inner diameter. Subsections of 2 cm down to a depth of 10 cm and thereafter every 5 cm length down to 30 cm depth were cut from the sediment cores and directly introduced into sterile plastic bags to avoid any aerial contamination. The sediments were stored at −20°C immediately after sampling. DNA was isolated from each frozen sub-section of the sediment cores under sterile conditions to avoid cross-contamination. The DNA samples

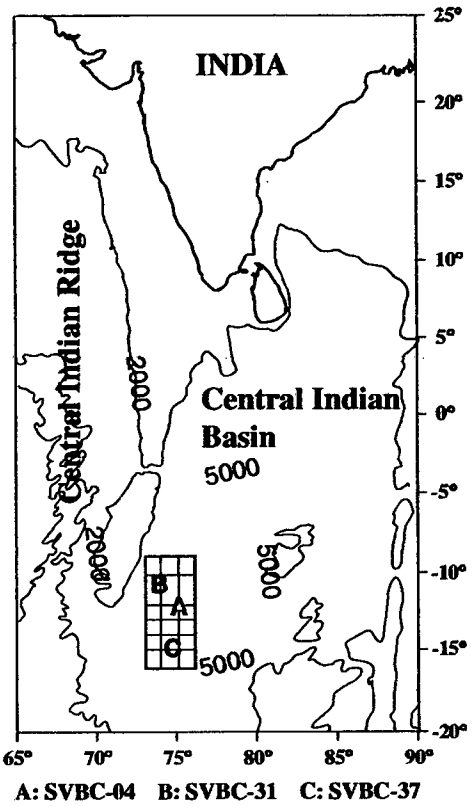

Figure 1 Geographic location of the sampling sites in the Central Indian Basin

from the subsections were pooled into two parts of 0–15 and 15–30 cm for each of the three stations in order to minimize the number of clone libraries. Thus, finally a total of six DNA samples were processed. In order to monitor the contamination by air-borne fungi, control media plates were exposed for 10 min on the deck of the research vessel where the cores were received and in the microbiology laboratory on board the research vessel during every sampling.

DNA Extraction, Environmental PCR, and Clone Library Construction

DNA was isolated from 0.5 g of the sediment sample using the Q-Bio gene Soil DNA extraction kit (MP Biomedicals, OH, US) according to the manufacturer's instructions. DNA samples from the three stations were amplified using fungal-specific ITS1F/ITS4 [13], primer pair a as well as universal ITS1/ITS4, primer pair b, and universal 18S rDNA NS1/NS2 primer set [41], primer pair c. The conditions for PCR included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 15 min. The PCR reaction mixture (50 μl) consisted of

Table 1 Sampling locations in the Central Indian Basin

Sample number	Touch positions		Water depth (m)	Sediment texture
	Lat (°S)	Long (°E)		
SVBC-04 (station A)	13,00.000	76,29.950	5,377	Siliceous
SVBC-31 (station B)	12,00.000	74,59.900	5,153	Siliceous
SVBC-37 (station C)	12,59.980	74,59.960	3,992	Calcareous, seamount area

50 μg bovine serum albumin (New England Biolab), 0.6 U *Taq* DNA polymerase (Bangalore Genei, India), 1.5 mM $MgCl_2$, dNTPs (0.2 mM each), primers (0.5 μM each), and 1× PCR buffer (Roche, Switzerland). Reaction mixture without template DNA was used as a negative control, and sediments spiked with fungal DNA was used as a positive control. Amplified products were gel-purified and ligated with pGEM-T easy vector (Promega, USA) and transformed into *E. coli* cells (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Transformants were grown ovemight at 37°C in Luria-Bertani broth containing 100 $\mu g ml^{-1}$ of ampicillin. The presence of insert was confirmed by PCR with M13 forward and reverse primers. One μl of the broth containing the clone was added to 25 μl of PCR reaction mixture. PCR protocol included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. Clones containing positive insert were further processed for plasmid isolation and purification using Millipore plasmid preparation kit (Millipore, USA). Sequencing of the plasmids was done at the National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3,730 DNA Analyzer, Applied Biosystems, USA). A total of 18 environmental gene libraries were constructed from the DNA samples from three stations, each with two depths and three primer pairs (3×2×3). A total of 48 clones were screened from each library.

Phylogenetic Analyses

Forward and reverse sequences were assembled using Chromas Pro version 1.34 (Technelysium Pty Ltd, Tewantia, Queensland, Australia). Sequences obtained with ITS and 18S rDNA primers were analyzed separately. All the sequences were checked with Ribosomal Database Project [7] for the presence of chimeras. These chimeric sequences were eliminated from subsequent analyses. Pairwise alignment of the sequences was carried out using Clustal W2 software [37]. Conserved motifs were identified, and sequences were trimmed manually. Clones were grouped into operational taxonomic units (OTUs) by using sequence similarity cut-off value of 98% [26] by using MOTHUR software version 1.4.1 [30]. The results are presented as OTU_01 to OTU_09 (blue fonts) obtained with fungal-specific ITS primer pair, OTU_10 to OTU_22 (green fonts) retrieved with universal ITS primer pair and OTU_23- to OTU_39 (red fonts) with universal 18S rDNA primer pair (Table 2).

A representative sequence from each OTU was queried against NCBI-GenBank BLASTN search [1]. Out of the 18 environmental gene libraries, two showing biased amplification of a single sequence type due to PCR artifact were eliminated from the further analyses. Multiple alignments were done for all the sequences along with their closest match in ClustalW [37]. Gaps and ambiguously aligned sequences were removed from the further analyses. A phylogenetic analysis was conducted using distance setting (Maximum Parsimony) in MEGA 4 [17] with 1,000 bootstrap replicates. Phylogenetic trees were constructed with sequences obtained with ITS (primer pairs a and b) and 18S rDNA primer set (primer pair c) individually.

Diversity Analyses

All diversity analyses were carried out using MOTHUR software. Shannon–Wiener diversity indices were calculated for OTUs obtained from each library constructed with three individual primer sets. Species effort curves were constructed to determine the sensitivity of the observed richness to the number of clones sampled. Community composition was plotted as relative abundance of taxonomic subclasses at different stations and depths.

Nucleotide Sequence Accession Numbers

Representative sequences of each OTU were deposited in NCBI-GenBank under the accession numbers GU370716 to GU370774.

Results

Physico-chemical Properties of the Sediments

The area of sampling was characterized by the presence of polymetallic nodules. The nodule abundance ranged

Table 2 Phylogenetic affiliation of the fungal OTUs obtained with different primer sets (sequences showing <97% similarity values are represented in bold)

Serial no.	Phylum	Closest relative (GenBank accession no.)	Percent similarity	Originally reported habitat	OTUs with fungal-specific ITS primers	OTUs with universal ITS primers	OTUs with universal 18S rDNA primers
1	Ascomycota	*Sagenomella* sp. (FJ357782)	99	Deep-sea sediment of CIB	*OTU_02*	**OTU_11**	–
2	Ascomycota	*Dothideomycete* sp. (EU680535)	99	Moth exoskeleton	–	**OTU_21**	–
3	Ascomycota	*Aspergillus penicillioides* (DQ985959)	97	Lacaziosis patient	–	**OTU_15**	*OTU_23*
4	Ascomycota	*Aspergillus restrictus* (AB008407)	99	Soil	–	–	*OTU_24*
5	Ascomycota	*Hortaea* sp. F 47 (FJ755827)	95	Marine sponge	*OTU_01*	**OTU_10**	–
6	Ascomycota	*Stenella musicola* (EU514294)	99	*Musa acuminata*	*OTU_04*	**OTU_13**	–
7	Ascomycota	*Candida* sp. F15 (FJ755821)	100	Coastal waters of China	*OTU_05*	**OTU_14**	–
8	Ascomycota	*Aspergillus restrictus* (AB002079)	94	Not known	–	**OTU_16**	*OTU_27*
9	Ascomycota	*Debaryomyces yamadae* (AB054266)	98	Not known	–	**OTU_19**[a]	–
10	Ascomycota	*Pichia jadinii* (EU568927)	99	Pilot-scale municipal compost	*OTU_06*[a]	–	–
11	Ascomycota	*Nodulisporium* sp. (EF600033)	98	Stroma of *Epichloe typhina*	*OTU_07*[a]	–	–
12	Ascomycota	*Aspergillus niger* (GQ228449)	96	Soil from Kalabagh iron ore deposit	–	–	*OTU_25*
13	Ascomycota	*Candida glucosophila* (AB013519)	97	Not known	–	–	*OTU_28*
14	Ascomycota	*Aspergillus fumigatus* (GQ169424)	99	Fossa cheese	–	–	*OTU_29*[a]
15	Ascomycota	*Aspergillus* sp. (FJ864683)	99	Root of *Angelica sinensis*	–	–	*OTU_30*[a]
16	Ascomycota	*Phoma herbarum* (AY293777)	99	Not known	–	–	*OTU_32*
17	Ascomycota	*Aspergillus unguis* (EF067336)	99	Culture collection	–	–	*OTU_35*
18	Ascomycota	*Ulospora bilgramii* (DQ384071)	98	Dung	–	–	*OTU_37*[a]
19	Ascomycota	*Capnodium coffeae* (DQ247808)	93	Maize rhizosphere soil	–	–	*OTU_39*[a]
20	Ascomycota	*Bionectriaceae* sp. (FJ821507)	97	Basidioma of *Phellinus gilvus*	–	**OTU_22**[a]	–
21	Ascomycota	Fungal sp. ARIZ L365 (FJ612791)	99	Tree seed	*OTU_08*[a]	–	–
22	Ascomycota	Uncultured (GQ120138)	98	Arabian Sea sediment	–	–	*OTU_26*
23	Ascomycota	Uncultured (AB074653)	98	Arabian Sea sediment	–	–	*OTU_31*[a]
24	Ascomycota	Uncultured (AJ515945)	97	Maize rhizosphere soil	–	–	*OTU_33*
25	Ascomycota	*Candida parapsilosis* (GQ395610)	99	Feeding production sample	–	**OTU_20**[a]	–
26	Basidiomycota	*Malassezia pachydermatis* (DQ457640)	96	Not known	–	**OTU_17**	–
27	Basidiomycota	*Trichosporon asahii* (AB369919)	99	Skin of patient	*OTU_03*	**OTU_12**	–
28	Basidiomycota	*Wallemia sebi* (AY741380)	97	Hypersaline water of salterns	–	–	*OTU_34*
29	Basidiomycota	*Rhodosporidium sphaerocarpum* (EF643587)	100	Deep-sea of Pacific Ocean	*OTU_09*[a]	–	–
30	Basidiomycota	Fungal sp. FCAS11 (GQ120154)	97	Arabian Sea sediment	–	–	*OTU_38*
31	Basidiomycota	Uncultured (AJ515927)	99	Contaminated aquifer sediment	–	–	*OTU_36*
32	Basidiomycota	Uncultured (FJ889114)	94	Hawaiian coastal waters	–	**OTU_18**	–

Primer pairs: ITS1F/ITS4 (fungal-specific) are in italics, ITS1/ITS4 (Universal ITS) in bold, and NS1/NS2 (Universal 18S rDNA) in bold italics

[a] Singletons

0–10.7 kg m^{-2}. The approximate salinity, temperature, and pH were found to be 35 PSU (practical salinity units), 3°C, and ~7.0 respectively. Among other parameters, the total proteins, carbohydrates, and lipids ranged from 0–1.5, 0–1, and 0–1 mg g^{-1} dry sediment, respectively. Total bacterial counts in this area ranged from 10^7–10^9 cells g^{-1} dry sediment. Organic carbon ranged from 0.3–0.4 mg g^{-1} dry sediment (unpublished data).

Environmental Libraries

A total of 768 clones were sequenced from the 16 environmental libraries. Of the resultant sequences, 257 sequences were found to be fungal, resulting in a total of 39 OTUs, after clustering on a basis of 98% sequence identity criterion. The other 511 clones (~66.5%) were bacterial, eukaryotic, or chimeric in nature and thus were excluded from the study. The 39 fungal OTUs comprised 32 distinct fungal taxa (Table 2).

Analyses of Fungal Diversity Obtained with Different Primer Sets

Out of the 39 fungal OTUs, four were exclusively amplified by primer set a, six with primer set b, and 15 by the primer set c (Table 2). Ten OTUs were simulta-

neously recovered with primer pairs a and b (Table 2) and four with b and c. The number of singletons, i.e., detected only once in libraries constructed with primer pairs a, b, and c were four, three, and five, respectively (marked with an asterisk in Table 2). Apart from these singletons, the remaining OTUs were represented ≥2 times in different libraries.

The fungal-specific primer set ITS1F/ITS4 (primer pair a) and universal fungal primers ITS1/ITS4 (primer pair b) amplified sequences exclusively from a diverse group of fungi belonging to Ascomycota and Basidiomycota (Figs. 2 and 3). Primer pair a and primer pair b recovered seven and ten OTUs respectively belonging to Ascomycota. Both the ITS primer sets detected Sordariomycetes, Dothideomycetes, and Saccharomycetes (Figs. 2 and 3). In addition, the primer set b amplified members of Eurotiomycetes as well. Among Ascomycota, the primer pair a and the primer pair b amplified one (OTU_01) and two (OTU_10, OTU_16) new sequence types respectively having similarities less than 97% to the known fungal taxa (Table 2).

Primer pair a and primer pair b recovered two and three OTUs belonging to Basidiomycota, respectively. This group was found to include members from Tremellomycetes, Microbotryomycetes, and Ustilaginomycetes. Out of these, only Tremellomycetes was the common clade shared by both the primer sets (Fig. 3). Primer pair b amplified two new sequence type of Basidiomycota (OTU_17, OTU_18).

Out of the total 22 OTUs recovered with these two primers sets, eight OTUs affiliated with sequences reported from marine environment (Table 2). Out of these eight OTUs, three were new sequence types. Five fungal taxa were simultaneously recovered with universal and fungal-specific ITS primer sets (Table 2).

A major part of the sequences recovered with universal 18S rDNA primer set NS1/NS2 (primer pair c) belonged to Ascomycota (Fig. 4). The number of OTUs belonging to Ascomycota and Basidiomycota were 14 and three,

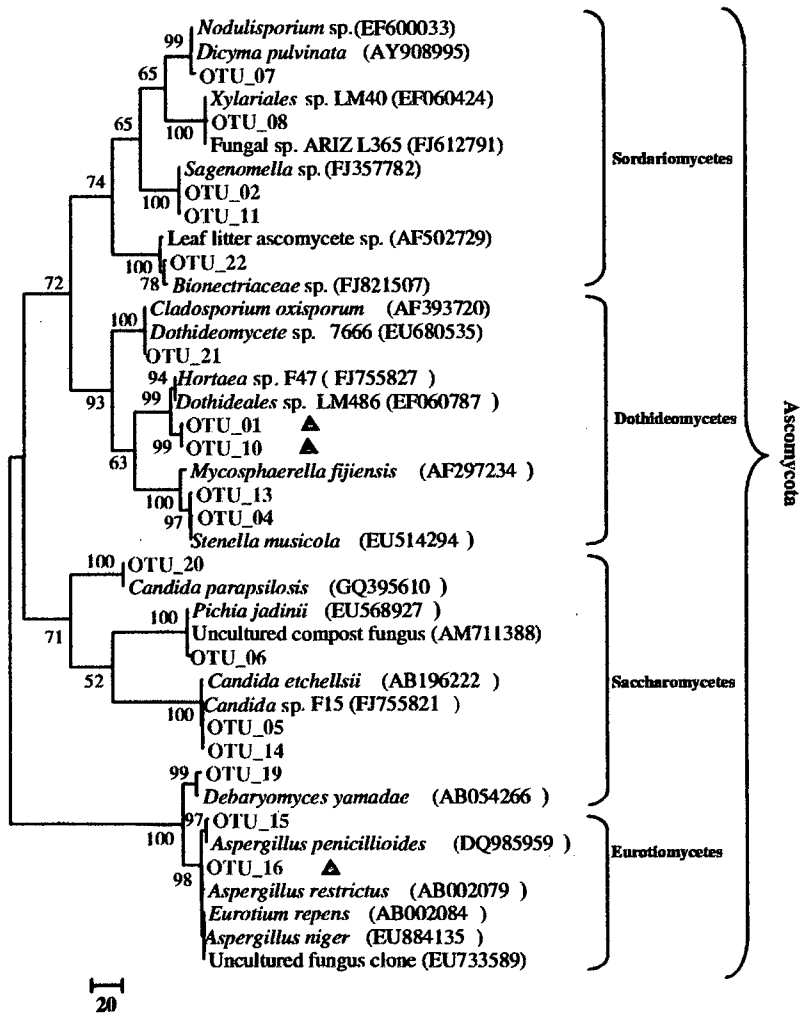

Figure 2 Maximum parsimony phylogenetic tree based on fungal ITS gene sequences of phylum Ascomycota after amplification with primer set, ITS1F/ITS4 (fungal-specific, marked with *blue color*) and ITS1/ITS4 (universal ITS, marked with *green color*). Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. *Numbers above* or *below* branches indicate bootstrap values (>50%) from 1,000 replicates. New sequence types are marked with *triangle*

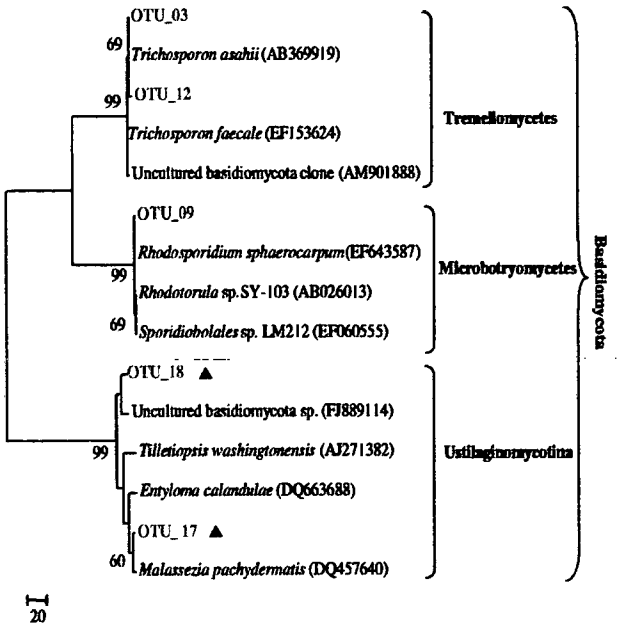

Figure 3 Maximum parsimony phylogenetic tree based on fungal ITS gene sequences of phylum Basidiomycota after amplification with primer set, ITS1F/ITS4 (fungal-specific, marked with *blue color*) and ITS1/ITS4 (universal ITS, marked with *green color*). Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. *Numbers above* or *below* branches indicate bootstrap values (>50%) from 1,000 replicates. New sequence types are marked with *triangle*

respectively. Out of these, three new sequence types showing less than 97% similarity with the existing data base, OTU_25 and OTU_27 (with less than 50% bootstrap value) and OTU_39 (with bootstrap value of 91%) branched deeply within Eurotiomycetes (Fig. 4). OTU_34 belonging to the subclass Wallemiomycetes clustered with *Wallemia sebi* and OTU_38 clustered with an unclassified species of fungus with a 99% bootstrap value (Fig. 4). Only two fungal taxa namely *Aspergillus penicillioides* and *Aspergillus restrictus* were simultaneously amplified with universal ITS and 18S rDNA primers (Table 2).

Fungal Diversity at Different Stations

Fungal taxa were more or less unevenly distributed at different stations (Table 3). Some of these OTUs were found exclusively in station A, B, or C. Distribution of fungal taxa in stations A, B, and C was 10, 21, and 16, respectively, indicating richness of diversity in station B. Species of *Sagenomella*, *A. penicillioides*, *Stenella musicola*, and *Candida* sp. occurred in high frequency (Table 3).

Distribution of Different Subclasses and Species Richness

Seven and five major subclasses of fungi were recovered with ITS and 18S rDNA primers, respectively (Fig. 5). Compositional difference was observed between stations and occasionally with different depths of the same station. In a few instances, individual primer sets amplified different subclasses from the environmental clone libraries. For example, Sordariomycetes, Tremellomycetes, and Ustilaginomycetes were amplified with ITS primer sets, whereas Wallemiomycetes was amplified only with 18S rDNA primer set (Figs. 2, 3, and 4). Members of Eurotiomycetes were present in all the stations. Members of Sordariomycetes and Dothideomycetes were found to be the major forms in stations A and C, whereas yeasts were dominant in station B. Sequences belonging to Wallemiomycetes were found only in station C. Shannon–Wiener diversity values (H) were 2.5, 3.0, and 4.5 for libraries constructed with universal ITS, fungal-specific ITS, and universal 18S rDNA primers, respectively. The species effort curves for libraries with all the primers were found to show positive slope, and thus no evidence of saturation was found (data not shown).

Discussion

Diversity with Multiple Primers

Primer sets for the amplification of two different segments of rRNA gene were chosen because 18S rDNA is supposed to be conserved in nature and thus discrimination between closely related species is difficult to be resolved. On the other hand, ITS region of rRNA gene is known to give better resolution of taxonomic species, but phylogenetic analyses of unknown sequences cannot be resolved due to its less conserved nature [26]. Therefore, to overcome this problem and get a better diversity estimate, we resorted to amplify both of these segments from environmental DNA samples. The use of different primers enabled the recovery of larger and varied number of fungal OTUs from deep-sea sediments than would have been possible otherwise [15, 26]. Thus, a total of 22 and 17 OTUs were recovered exclusively with ITS and 18S rDNA primer sets, respectively. Primer sets for ITS and 18S rDNA region exclusively identified 15 fungal taxa each. Two fungal taxa were commonly recovered with ITS and 18S rDNA primer sets (Table 2). Jebaraj et al. [15] have also recovered a large number of fungal OTUs from anoxic sediments using multiple primer approach. In contrast, yeasts were found to be dominating forms in deep oceans including distantly related forms in a study conducted by the amplification of SSU region with fungal-specific 18S rDNA primers [2]. In the previous study employing culture-dependent approach, only 12 filamentous fungi and eight species of yeasts from a total of 188 sediment samples from 20 stations of CIB were isolated [32]. Therefore, diversity observed through culture-independent method (32 distinct

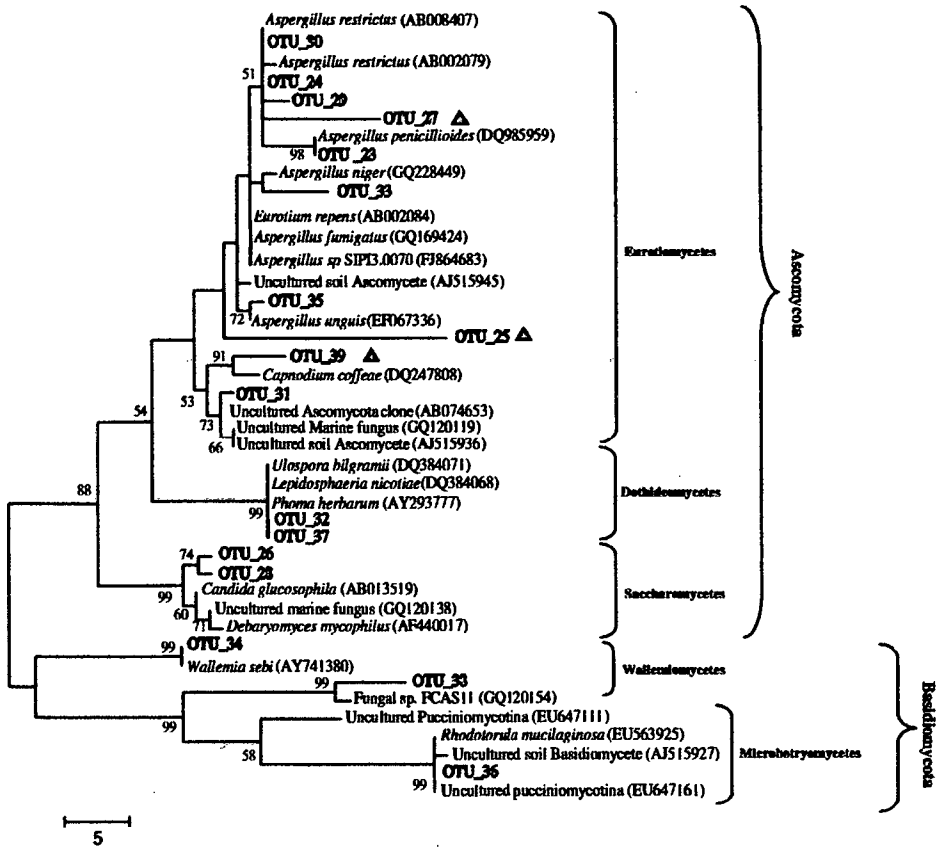

Figure 4 Maximum parsimony phylogenetic tree based on fungal 18S rDNA gene sequences of phylum Ascomycota and Basidiomycota after amplification with universal 18S rDNA primer set, NS1/NS2 (marked with *red color*). Topology was built using Mega v.4.1 from a ClustalW 1.83 alignment. *Numbers above* or *below* branches indicate bootstrap values (>50%) from 1,000 replicates. New sequence types are marked with *triangle*

species) with multiple primer sets was ~38% higher than that obtained through culture-dependent (20 fungal species) approach from the same geographical location. The need to use different primer sets is also highlighted by the fact that the phylogenetic affinities of OTUs recovered with different primer sets seemed to be different (Table 2). In the present study, Asco- and Basidiomycota were the most common phylogenetic sequences identified with ITS and 18S rDNA primers as observed by O'Brien et al. [26] using the same primer sets. In contrast to our results, O'Brien et al. [26] recovered sequences belonging to Chytridiomycota and Zygomycota with these primer pairs. Lai et al. [19] also reported the presence of Asco- and Basidiomycota from methane hydrate seeps using only the fungal-specific ITS primer pair.

Multiple sequence analysis approach resulted in identifying eight OTUs showing less than 97% similarity to the existing fungal taxa and thus appeared to be novel. Out of these, six were affiliated to Ascomycota and two to Basidiomycota. Thus, these new sequences add to the existing database for probable novel fungal signatures.

Spatial Distribution and Heterogeneity

This study suggests that fungal diversity may be heterogeneous in deep-sea sediments as some of the subclasses were restricted to a few stations (Fig. 5). Biological communities in deep sea have shown dependence on variables such as substrate availability and type, biogeochemistry, nutrient input, productivity, and hydrological conditions on regional scale [20]. Substrate heterogeneity was shown to play an important role in structuring nematode diversity in deep-sea sediments [40]. These authors reported nematode assemblages that varied in hard nodule substratum from soft sediments beneath the nodules. Such small-scale habitat heterogeneity may also impact fungal diversity in deep-sea sediments. Sequences belonging to Chytridiomycota and Zygomycota were recovered by using fungal-specific 18S rDNA primers from marine sources [2]. Therefore, their absence in the present study suggests that their distribution may be site-specific. Microbial diversity has been shown to be impacted by temporal and spatial dimensions of deep-sea [34].

Phylogenetic Affiliation with Marine Fungal Taxa

Out of 39 OTUs, 12 OTUs showed affiliation with sequences already reported from marine sources (Table 2), thus adding support to the view that these were possibly typically marine. Out of these 12, two OTUs representing *Sagenomella* sp. and the yeast *Rhodosporidium* sp. were recovered in culture from the CIB in the earlier study [32]. These two cultures showed

Table 3 Distribution of closest identified fungal taxa in different stations

OTU no.	Closest relative(GenBank accession no.)	Number of clones recovered			Total number of clones recovered
		Station A	Station B	Station C	
OTU_02, **OTU_11**	*Sagenomella* sp.(FJ357782)	33	11	27	71
OTU_15, ***OTU_23***	*Aspergillus penicillioides* (DQ985959)	21	20	1	42
OTU_05, **OTU_14**	*Candida* sp. *F15* (FJ755821)	8	26	0	34
OTU_04, **OTU_13**	*Stenella musicola* (EU514294)	6	10	7	23
OTU_16, ***OTU_27***	*Aspergillus restrictus* (AB002079)	4	6	4	14
OTU_25	*Aspergillus niger* (GQ228449)	3	6	4	13
OTU_01, **OTU_10**	*Hortaea* sp. F 47 (FJ755827)	2	0	4	6
OTU_28	*Candida glucosophila* (AB013519)	0	3	2	5
OTU_24	*Aspergillus restrictus* (AB008407)	3	2	0	5
OTU_03, **OTU_12**	*Trichosporon asahii* (AB369919)	3	1	0	4
OTU_26	Uncultured Ascomycota (GQ120138)	4	0	0	4
OTU_21	*Dothideomycete* sp. (EU680535)	0	3	0	3
OTU_18	Uncultured Basidiomycota sp. (FJ889114)	0	3	0	3
OTU_35	*Aspergillus unguis* (EF067336)	0	0	3	3
OTU_36	Uncultured soil Basidiomycete (AJ515927)	0	0	3	3
OTU_38	Fungal sp. FCAS11 (GQ120154)	0	0	3	3
OTU_17	*Malassezia pachydermatis* (DQ457640)	0	3	0	3
OTU_32	*Phoma herbarum* (AY293777)	0	0	2	2
OTU_33	Uncultured soil Ascomycete (AJ515945)	0	0	2	2
OTU_34	*Wallemia sebi* (AY741380)	0	0	2	2
OTU_19	*Debaryomyces yamadae* (AB054266)	0	1	0	1
OTU_06	*Pichia jadinii* (EU568927)	0	1	0	1
OTU_07	*Nodulisporium* sp. (EF600033)	0	1	0	1
OTU_29	*Aspergillus fumigatus* (GQ169424)	0	1	0	1
OTU_30	*Aspergillus* sp. (FJ864683)	0	1	0	1
OTU_37	*Ulospora bilgramii* (DQ384071)	0	0	1	1
OTU_39	*Capnodium coffeae* (DQ247808)	0	0	1	1
OTU_22	*Bionectriaceae* sp. (FJ821507)	0	1	0	1
OTU_08	Fungal sp. ARIZ L365 (FJ612791)	0	1	0	1
OTU_31	Uncultured fungal clone (AB074653)	0	0	1	1
OTU_20	*Candida parapsilosis* (GQ395610)	0	1	0	1
OTU_09	*Rhodosporidium sphaerocarpum* (EF643587)	0	1	0	1

Primer pairs: ITS1F/ITS4 (fungal-specific) in italics, ITS1/ITS4 (Universal ITS) in bold, NS1/NS2 (Universal 18S rDNA) in bold italics

growth under simulated deep-sea conditions of 200 bar pressure, temperature of 5°C, and salinity of 35 PSU suggesting either their adaptation to the deep-sea conditions or their autochthonous existence. This is the first report of occurrence of *W. sebi* from deep-sea sediments. It is a halophilic and osmophilic fungus isolated from hypersaline water of salterns and is described to have successfully adapted to life in extremely saline environments [18]. Its sequence was detected in a marine sponge from Hawaii [12] and was also isolated in culturable form from different species of marine sponges of the South China Sea [21], suggesting its marine nature. Cantrell et al. [6] identified a series of halotolerant fungi, including *Aspergillus*, *Penicillium*, *Cladosporium*, and *Hortaea* sp. from solar salterns using ITS1/ITS4 primer set. Using the same primer set, some of these were recovered in the present study also. We are reporting here for the first time on the recovery of sequences showing affiliation with Microbotryomycetes and Tremellomycetes from deep-sea sediments. The percentage abundance of these subclasses was very low in comparison with other groups, suggesting their low diversity under deep-sea conditions. The class Microbotryomycetes (represented by *Rhodotorula, Rhodosporidium*) includes mycoparasites, phytopathogens, and putative saprotrophs with a diversity of micromorphological characters. Many species contain organelles, termed colacosomes, which are associated with mycoparasitism [3]. The recovery

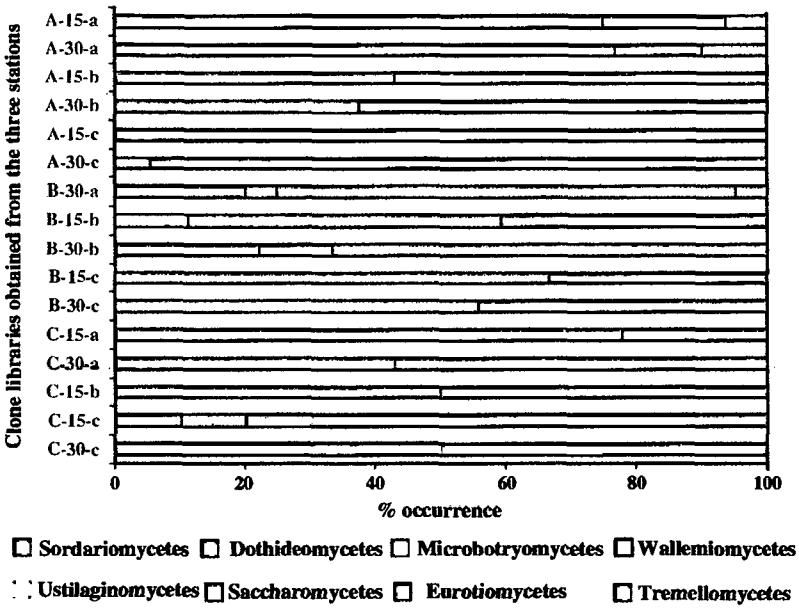

Figure 5 Proportional distribution of different taxonomic groups in various clone libraries. Percentage occurrence of sequences of each taxonomic group is indicated on *X*-axis. Clone libraries are represented on *Y*-axis. The clone libraries are represented in *bold*, designated with station number *A* (SVBC-04), *B* (SVBC-31), and *C* (SVBC-37) followed by depth of sediment sample (15 or 30 cm), primer pair used were *a* (ITS1F/ITS4), *b* (ITS1/ITS4), and *c* (NS1/NS2). The clone libraries *B-15-a* and *C-30-b* showed biased amplification of a single sequence type due to PCR artifact and were eliminated from the analyses

of other pathogenic yeasts like *Candida* and *Debaromyces*, suggests that deep-sea sediments may act as reservoir of such forms. Pathogenic black yeast in mussel and other animal parasitic forms have been reported from hydrothermal vents [25, 39]. These results are in accordance with earlier observation that oceans are incubators and conveyor belt for pathogenic microorganisms [14].

Terrestrial Fungi in the Deep-Sea Sediments

A large proportion (~ 69%) of fungal sequences recovered in high frequency (Table 3) matched with terrestrial taxa, supporting the earlier hypothesis [8, 9, 29] that they are transported into the deep sea and subsequently adapt to the extreme conditions. One of the most common terrestrial fungus *Aspergillus* has been recovered both by culture-dependent [4, 8] and culture-independent methods from various marine habitats [2, 15, 19]. Therefore, the ecological role and adaptations of these ubiquitous terrestrial forms in such extreme conditions need to be further investigated. Takishita et al. [36] and Shao and Sun et al. [31] have emphasized the role of sedimentation in accumulating facultative marine fungi in deep-sea sediments. Irrespective of their mode of transport, these facultative marine fungi offer an excellent tool to study physiology of deep-sea-adapted fungi. Fungi-like *Sagenomella* which is reported for the first time from deep-sea by culture-dependent [32] as well as culture-independent approach from the same site can be one such model organism. Several of these terrestrial taxa were isolated from marine sponges too, and they exhibited in vitro anti-fungal properties [28] and anti-tumor and anti-bacterial activities [21], suggesting that terrestrial taxa found in the marine environment could be a rich source of new biologically active natural products [21].

In conclusion, the multiple primer approach used in the present study provided an insight to the diversity of fungi in the deep-sea sediments of the CIB. The lack of saturation level with all the primer sets (data not shown) indicates that the actual diversity of fungi must be greater than detected here. Therefore, exhaustive sampling to obtain near-accurate estimation of fungal diversity in deep-sea is recommended. In addition, designing more fungal-specific primers to identify novel species obtained by culture-independent approach is required. It is not appropriate to speculate that all the fungal diversity obtained by using DNA amplification represent viable fungi because low temperature conditions of deep-sea environment will favor preservation of DNA. Thus, it is possible that only a fraction of the uncultured fungi will be viable. Culture-independent approach provides valuable indication of the presence of the organism/community, and in turn information gained could be utilized for their recovery in culturable form using targeted culturing methods. The methods to isolate the yet-to-be-cultured fungi from deep-sea sediments such as improvement of isolation media, incubation techniques should be evolved so that rare and slow-growing forms could be obtained.

Biological diversity in this extreme ecosystem may have enormous potential in the development of new products such as pharmaceuticals, molecular probes, enzymes, cosmetics, nutritional supplements, and agrichemicals [35]. A recently described antibiotic-resistance enzyme from the deep-sea bacterium *Oceanobacillus iheyensis* isolated from 1,050 m

depth in the Pacific Ocean [38] and a novel λ-carrageenase with no similarity to any reported protein, obtained from a deep-sea bacterium isolated from Suruga Bay, Japan, at a depth of 2,409 m [27] are some of the examples. Deep-sea sediments may harbor new fungal taxa that are tolerant to low temperature and elevated hydrostatic pressure which may lead to discovery of new metabolites or enzymes that are useful for novel biotransformation processes.

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ORIGINAL PAPER

Assessment of fungal diversity in deep-sea sediments by multiple primer approach

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Abstract Increasing evidence of the fungal diversity in deep-sea sediments has come from amplification of environmental DNA with fungal specific or eukaryote primer sets. In order to assess the fungal diversity in deep-sea sediments of the Central Indian Basin (CIB) at ~5,000 m depth, we amplified sediment DNA with four different primer sets. These were fungal-specific primer pair ITS1F/ITS4 (internal transcribed spacers), universal 18S rDNA primers NS1/NS2, Euk18S-42F/Euk18S-1492R and Euk18S-555F/Euk18S-1269R. One environmental library was constructed with each of the primer pairs, and 48 clones were sequenced per library. These sequences resulted in 8 fungal Operational Taxonomic Units (OTUs) with ITS and 19 OTUs with 18S rDNA primer sets respectively by taking into account the 2% sequence divergence cut-off for species delineation. These OTUs belonged to 20 distinct fungal genera of the phyla Ascomycota and Basidiomycota. Seven sequences were found to be divergent by 79–97% from the known sequences of the existing database and may be novel. A majority of the sequences clustered with known sequences of the existing taxa. The phylogenetic affiliation of a few fungal sequences with known environmental sequences from marine and hypersaline habitat suggests their autochthonous nature or adaptation to marine habitat. The amplification of sequences belonging to Exobasidiomycetes and Cystobasidiomycetes from deep-sea is being reported for the first time in this study. Amplification of fungal sequences with eukaryotic as well as fungal specific primers indicates that among eukaryotes, fungi appear to be a dominant group in the sampling site of the CIB.

Keywords Uncultured fungi · 18S rRNA gene · ITS primers · Deep-sea sediments · Central Indian Basin

Introduction

Microbial communities existing in deep-sea oceanic environments account for a total cellular carbon content of $\sim 3 \times 10^{17}$ g (Whitman et al. 1998). These deep-sea microbial communities, mainly of bacteria, archaea, protists and fungi play an important role in the recycling of the nutrients (Snelgrove et al. 1997). Among these, bacteria and archaea have been studied in detail (Stackebrandt et al. 1993; Urakawa et al. 1999; Li et al. 1999; Takai and Horikoshi 1999; DeLong and Pace. 2001; Sogin et al. 2006; Hongxiang et al. 2008; Luna et al. 2009). A few recent studies have reported eukaryotic diversity from extreme environments such as hydrothermal vents (López-García et al. 2003, 2007), anoxic environments (Stoeck and Epstein 2003, Stoeck et al. 2003, 2006; Jebaraj et al. 2010) and deep-sea sediments (Bass et al. 2007; Edgcomb et al. 2011).

The eukaryotic diversity studies have been executed by molecular survey of ribosomal genes using culture-independent approach in most of the recent reports. An unexpected high diversity of eukaryotic rDNA sequences was reported from the deep-sea (López-García et al. 2001). Using eukaryotic specific primers, several studies have reported only a small fraction of total rDNA sequences affiliating with fungi in comparison with other eukaryotic

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lineages (López-García et al. 2001; Stoeck et al. 2003, 2006). However, Edgcomb et al. (2011), while using eukaryotic specific primers, reported fungi to be dominant in marine deep-sea subsurface. The use of multiple primer approach for studying diversity has revealed recovery of diverse fungal forms from oxygen depleted marine environments (Jebaraj et al. 2010).

Using culture-dependent approach, fungi were isolated and identified from the deep-sea sediments of the CIB (Raghukumar et al. 2004; Damare et al. 2006; Singh et al. 2010). Further Singh et al. (2011) analyzed the fungal diversity in deep-sea sediments using culture-independent approach by targeting universal 18S as well as fungal specific and universal ITS (internal transcribed spacers) regions of rRNA genes from three locations in the CIB. It is known that some of the primer pairs designed for amplification of fungal sequences may co-amplify non-fungal templates from environmental samples leading to inaccurate estimation of fungal diversity (Borneman and Hartin 2000). In addition, the specific primers designed for amplification of fungal 18S rDNA and ITS regions may be biased towards certain fungal taxonomic groups. In order to overcome these problems, we used a multiple-primer approach to study fungal diversity in the present work. The efficiency of primers for amplification of fungal as well as other eukaryotic sequences from mixed environmental samples was evaluated by targeting ITS and 18S rDNA regions. The primer set used for ITS region was fungal specific, whereas all the three 18S rDNA primer sets used were universal. Fungal and eukaryotic 18S rDNA region share some common sequences resulting in cross amplification by the primers designed for this region (Anderson et al. 2003). Such primers may vary in their specificity in amplifying different fungal taxonomic groups. Therefore, we hypothesized that using several 18S rDNA primer sets may help in estimating fungal diversity with greater accuracy.

Materials and methods

Sampling

Sediment sample was collected on the cruise ABP-26 on board the Russian research vessel Academic Boris Petrov in December 2006 from the station SVBC-33 at a depth of ~5,000 m. The seafloor at this site is characterized by the presence of homogenous, soft to slightly compact, dark brown coloured sediments with yellowish bands at shallower and mottling of dusky brown bands at deeper depths. The sediment was of siliceous nature. The sampling procedure described by Raghukumar et al. (2004) and Damare et al. (2006) was followed. Sediment was collected with an USNEL-type box corer of 50 cm^3 size. The sample thus collected was undisturbed and compact. Sub-cores of sediment were collected using a sterile PVC cylinder of 5 cm inner diameter. The length of the sediment core obtained was 30 cm. Subsections of 2 cm down to a depth of 10 cm and thereafter every 5 cm length down to 30 cm depth were cut from the sediment core and directly introduced into sterile plastic bags to avoid exposure to aerial contamination. The sediment was stored at −20°C immediately after sampling. In order to monitor the contamination by air-borne fungi, fungal media plates were exposed to the air for 10 min on the deck of the research vessel where the cores were received and in the microbiology laboratory on board the research vessel.

DNA extraction, environmental PCR and clone library analyses

Environmental DNA was isolated from each frozen subsection of the sediment cores under sterile conditions to avoid cross contamination. DNA was isolated from 0.5 g of the sediment sample from each subsection of the core using the Q-Bio gene Soil DNA extraction kit (MP Biomedicals, OH, USA) according to the manufacturer's instructions. The DNA samples from all the subsections were pooled together and was amplified using fungal-specific primer pair, ITS1F/ITS4 as well as universal 18S rDNA primers, NS1/NS2, Euk18S-42F/Euk18S-1492RE and Euk18S-555F/Euk18S-1269R (Table 1). The conditions for PCR included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 15 min. The PCR reaction mixture (50 µl) consisted of 50 µg bovine serum albumin (New England Biolab), 0.6 U *Taq* DNA polymerase (Bangalore Genei, India), 1.5 mM $MgCl_2$, dNTPs (0.2 mM each), primers (0.5 µM each), and 1× PCR buffer (Roche, Switzerland). Reaction mixture without template DNA was used as a negative control, and sediments spiked with fungal DNA was used as a positive control. Amplified products were gel-purified and ligated with pGEM-T easy vector (Promega, USA) and transformed into *E. coli* cells (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Transformants were grown overnight at 37°C on Luria–Bertani agar containing 100 µg ml^{-1} of ampicillin. White colonies were screened for the presence of insert by single colony lysis PCR with M13 forward and reverse primers. PCR protocol included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. A total of 4 environmental gene libraries, one each from the four primer sets was obtained. Forty eight clones were screened from each library. Clones containing positive

Table 1 Details of the primers used

Primer name	Sequences	References
ITS1F	5′-CTTGGTCATTTAGAGGAAGTAA-3′	Lai et al. (2007)
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	Lai et al. (2007)
NS1	5′-GTAGTCATATGCTTGTCTC-3′	O' Brien et al. (2005)
NS2	5′-GGCTGCTGGCACCAGACTTGC-3′	O' Brien et al. (2005)
Euk18S-42F	5′-CTCAARGAYTAAGCCATGCA-3′	López-García et al. (2003)
Euk18S-1492R	5′-ACCTTGTTACGRCTT-3′	Stoeck et al. (2006)
Euk18S-555F	5′-AGTCTGGTGCCAGCAGCCGC-3′	López-García et al. (2003)
Euk18S-1269R	5′-AAGAACGGCCATGCACCAC-3′	López-García et al. (2003)

insert were further processed for plasmid isolation and purification using Millipore plasmid preparation kit (Millipore, USA). Sequencing of the plasmids was done at the National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3730 DNA Analyzer, Applied Biosystems, USA).

Phylogenetic analyses

Forward and reverse sequences were assembled using Chromas Pro version 1.34. Sequences obtained with ITS and 18S rDNA primers were analyzed separately. All the sequences were checked with Ribosomal Database Project for the presence of chimeras (Cole et al. 2004). These chimeric sequences were eliminated from subsequent analyses. Pairwise alignment of the sequences was carried out using Clustal W2 software (Thompson et al. 1994). Conserved motifs were identified, and sequences were trimmed manually. Clone sequences were grouped into operational taxonomic units (OTUs) by using sequence similarity cut-off value of 98% (O'Brien et al. 2005) by using MOTHUR software (Schloss et al. 2009). A representative sequence from each OTU was queried against NCBI-GenBank BLASTn search. Details are shown for the phylogenetic affiliation of the representative sequence from each OTU (Table 2). Multiple alignments were done for all the sequences along with their closest match in Clustal W. Gaps and ambiguously aligned sequences were removed from the further analyses. A phylogenetic analysis was conducted using distance setting maximum likelihood (ML) in MEGA 5.03 (Kumar et al. 2008) with 1000 bootstrap replicates. Individual phylogenetic trees were constructed with sequences obtained with ITS and each of the 18S rDNA primer set.

Nucleotide sequence accession numbers

The GenBank accession numbers of the nucleotide sequences determined in this study are presented in Table 2.

Results

Physical parameters of the sampling site

The sediment sample collected for the present study was characterized by the presence of polymetallic nodules. The nodules abundance was found to be 0.4 kg m^{-2}. The approximate salinity, temperature and pH were found to be 35 psu (practical salinity units), 3°C and 7.0 respectively. Among other parameters, the total proteins, carbohydrates and lipids were approximately 1.5, 1.0 and 1.0 mg g^{-1} sediment respectively. Total bacterial counts (Acridine Orange Direct Counts) in this area ranged from 10^7 to 10^9 cells g^{-1} dry sediment. Organic carbon ranged from 0.3 to 0.4 mg g^{-1} dry sediment (Sharma 2008).

Analysis of libraries

No growth of aerial mycoflora was observed on media plates that were exposed on the deck and in the microbiology laboratory on board the research vessel. A total of 48 and 144 clones (3 × 48) from ITS and 18S primer pairs respectively, were sequenced from the environmental libraries. This resulted in 27 fungal OTUs, eight with ITS (17% relative frequency) and nineteen with 18S rDNA primer pairs (13% relative frequency) after clustering on a basis of 98% sequence identity criterion. A total of 20 distinct fungal species were obtained from these 27 OTUs. The results are presented as **OTU_01** to **OTU_08** obtained with fungal-specific ITS primer pair, **OTU_9** and **OTU_10** with universal 18S rDNA primer pairs, NS1/NS2, **OTU_11** to **OTU_20** with 18S rDNA primer pair Euk18S-42F/Euk18S-1492RE and **OTU_21** to **OTU_27** with 18S rDNA primer pairs Euk18S-555F/Euk18S-1269R (Table 2).

None of the 18S rDNA primers amplified non-fungal eukaryotic sequences. Out of the 48 and 144 total clones for ITS and 18S rDNA primer sets, 23 (47.9%) and 39 (27.1%) respectively were bacterial or chimeric in nature and thus were excluded from the analysis. The fungal OTUs mostly belonged to the phyla Ascomycota and Basidiomycota (Table 2). The number of singletons i.e. detected only once were four and ten respectively for the

Table 2 Phylogenetic affiliations of the OTUs obtained with different primer sets for station SVBC-33

OTU no.	Primer set used	Accession no.	Best BLAST hit				Isolation source	% Similarity
			Taxon	Phylum	Class	Accession no.		
OTU_01	ITS1F/ITS4	HM572248	Unidentified isolate f4Fc56	Ascomycota	Dothideomycetes	EU680535	Filter dust	99
OTU_02		GU370751	Unidentified fungal clone S3	Basidiomycota	Wallemiomycetes	FJ820491	Air sample	**79**
OTU_03		GU370752	Uncultured basidiomycete clone	Basidiomycota	Exobasidiomycetes	AM901800	House dust	99
OTU_04*		GU370753	Uncultured *Wallemia* isolate	Basidiomycota	Wallemiomycetes	GU931736	House dust	99
OTU_05*		GU370754	*Aphyllophorales* sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_06*		GU370755	*Nectria mauritiicola*	Ascomycota	Sordariomycetes	AJ557830	Not known	99
OTU_07*		GU370756	*Rhodotorula calyptogenae*	Basidiomycota	Cystobasidiomycetes	EU669878	Sea water	99
OTU_08		HM572249	*Trichosporon asahii*	Basidiomycota	Tremellomycetes	AB369919	Not known	99
OTU_09	NS1/NS2	HM572252	Uncultured *Malassezia* clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	99
OTU_10		HM572251	Uncultured *Aspergillus* clone	Ascomycota	Eurotiomycetes	FJ393417	Gut of HBV infected patients	99
OTU_11	18S-42F/Univ1492RE	GU370757	*Fungal* sp. NIOCC#F15	Ascomycota	Sordariomycetes	FJ357782	Deep-sea sediments	99
OTU_12		GU370758	Unidentified isolate f3Fc79	Basidiomycota	Wallemiomycetes	GU721564	Filter dust	99
OTU_13*		GU370759	Unidentified isolate f4Fc56	Ascomycota	Dothideomycetes	EU680535	Filter dust	99
OTU_14*		GU370760	*Aphyllophorales* sp. LM82	Basidiomycota	Agaricomycetes	EF060457	Sea water	98
OTU_15*		GU370761	Unidentified fungal clone S3	Basidiomycota	Wallemiomycetes	FJ820491	Air sample	**79**
OTU_16*		GU370762	*Nectria mauritiicola*	Ascomycota	Sordariomycetes	AJ557830	Not known	99
OTU_17		GU370763	*Wallemia* sp. F53	Basidiomycota	Wallemiomycetes	FJ755832	Marine sponge	**80**
OTU_18*		GU370765	Unidentified basidiomycete clone	Basidiomycota	Exobasidiomycetes	AM901883	House dust	99
OTU_19*		GU370766	*Rhodotorula* sp. SY-74	Basidiomycota	Microbotryomycetes	AB025984	Deep-sea	99
OTU_20*		GU370767	Dothideomycete sp.	Ascomycota	Dothideomycetes	EU680530	Leaf	**94**
OTU_21	EK555F/EK1269R	GU370768	Uncultured *Malassezia* clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	**96**
OTU_22		GU370769	Uncultured *Aspergillus* clone	Ascomycota	Eurotiomycetes	FJ393417	Gut of HBV infected patients	**97**
OTU_23		HM587247	*Aspergillus restrictus*	Ascomycota	Eurotiomycetes	AB008407	Not known	99
OTU_24*		GU370771	*Wallemia sebi*	Basidiomycota	Wallemiomycetes	AY741380	Hypersaline water of saltern	98
OTU_25*		GU370772	*Candida orthopsilosis*	Ascomycota	Saccharomycetes	FN812686	Not known	99
OTU_26*		GU370773	*Aspergillus penicillioides*	Ascomycota	Eurotiomycetes	AB002077	Not known	**91**
OTU_27		GU370774	Uncultured *Malassezia* clone	Basidiomycota	Exobasidiomycetes	FJ393445	Gut of HBV infected patients	99

Similarity of <98% is indicated in bold

Singletons are represented with *

ITS and 18S rDNA primer sets (marked with an asterisk in Table 2). The remaining OTUs were present ≥2 times in different libraries.

Diversity observed with different primer sets

The fungal specific primer set ITS1F/ITS4 amplified eight OTUs belonging to seven different classes of Ascomycota and Basidiomycota. Of these, the majority belonged to Basidiomycota (Fig. 1a). A larger proportion of the sequences affiliated with the unidentified and uncultured fungal clones (Fig. 1a; Table 2). Among Basidiomycota, OTU_02 clustered with an uncultured clone belonging to the class Wallemiomycetes with 79% similarity (Table 2). Two basidiomycetes, OTU_05 and OTU_07 affiliated with the sequences (Table 2) which have been isolated from sea water in the previous studies (NCBI data base).

Universal fungal 18S rDNA primer pair NS1/NS2 amplified sequences showing 99% similarity to two phylotypes i.e. uncultured *Malassezia* and *Aspergillus* clone, belonging to the class Exobasidiomycetes and Eurotiomycetes respectively (Table 2; Fig. 2a).

The universal eukaryotic primer set 18S-42F/Univ1492RE, recovered fungal sequences belonging to ten different phylotypes and these belonged to six different classes of the phyla Ascomycota and Basidiomycota (Table 2). Three OTUs (OTU_15, OTU_17 and OTU_20) showed very low similarity (79, 80 and 94%) with their closest relative in NCBI database and thus may be novel phylotypes. Most of the sequences amplified by this primer set clustered with uncultured fungal clones. OTU_15 and OTU_17 branched out forming a separate cluster within Basidiomycota (Fig. 3a).

The other universal eukaryotic primer set EK555F/EK1269R, amplified seven fungal phylotypes clustering with four different classes of Ascomycota and Basidiomycota (Fig. 4a). Three phylotypes OTU_21, OTU_22 and OTU_26 showed <97% similarity with existing sequences in the database and thus may be new (Table 2). Out of these three, OTU_21 and OTU_22 affiliated with the sequences which were also amplified by NS1/NS2 primer set but with a similarity of 99% with sequences in the public database (Table 2). These three OTUs clustered separately from the sequences of their closest relative, with OTU_26 showing a maximum divergence (Fig. 4a).

Proportional distribution of different taxonomic groups in various clone libraries

The proportional distribution (shown in percentage) of fungal taxonomic groups varied in different clone libraries (Figs. 1b, 2b, 3b, 4b). *Trichosporon asahii* (32%) formed a major portion of sequences amplified by the ITS primer set (Fig. 1b). Two sequences, namely uncultured clones of *Aspergillus* and *Malassezia* sp. amplified with 18S rDNA primers set NS1/NS2, showed distribution of 75 and 25% respectively (Fig. 2b). Maximum number of distinct fungal sequences was obtained with eukaryotic primer set, 18S-42F/Univ1492RE, out of which *Wallemia* sp. (28%) was the dominant phylotypes (Fig. 3b). The four phylotypes, *Aphyllophorales* sp., *Nectria mauritiicola*, unidentified isolate f4Fc56 and unidentified fungal clone S3 amplified by this primer set were amplified by ITS1F/ITS4 primer set as well (Figs. 1b, 3b). A majority of the sequences amplified by these two primer sets belonged to Basidiomycota. The major phylotypes amplified by the primer set EK555F/EK1269R belonged to the class Eurotiomycetes of the phylum Ascomycota (Fig. 4b). The two sequences, *Aspergillus* and *Malassezia* sp amplified by this primer pair were amplified by NS1/NS2 as well (Figs. 2b, 4b). There were no common OTUs shared by the primer sets 18S-42F/Univ1492RE and EK555F/EK1269R (Figs. 3b, 4b).

Discussion

The aim of the present study was to obtain maximum diversity of fungi from the deep-sea sediments. Therefore, four different sets of primers were used to amplify fungal sequences from a single core of deep-sea sediment from the Central Indian Basin (CIB). Previous studies on fungal diversity from the same area in three distantly located stations did not show a rich fungal diversity (Singh et al. 2011) and was perhaps due to primer bias towards specific fungal taxonomic groups and limited number of primers used. Therefore, an attempt was made to study fungal diversity from a single core using four primer sets which included three 18S rDNA primers besides one fungal-specific ITS primer set. The percentage frequency of fungal phylotypes obtained in the present study with ITS and 18S rDNA primer pairs was 17 and 13% respectively. This was higher than in the earlier study conducted in the same area (Singh et al. 2011), where the frequency obtained was 4 and 6% respectively with ITS and 18S rDNA primer pairs. Therefore, a multiple-primer approach appears to be better for assessment of fungal diversity. Anderson et al. (2003) obtained high diversity of fungi from grassland soil samples by using four ITS and 18S rDNA primer sets.

The primers used in the present study have been reported to amplify diverse forms of eukaryotic lineages from mixed environmental samples from various habitats (López-García et al. 2003; Takishita et al. 2005; Stoeck et al. 2006; Edgcomb et al. 2011). However, the absence of non-fungal eukaryotic sequences in the present study was intriguing. All the recovered sequences showed affiliation either with Ascomycota or Basidiomycota phyla whereas,

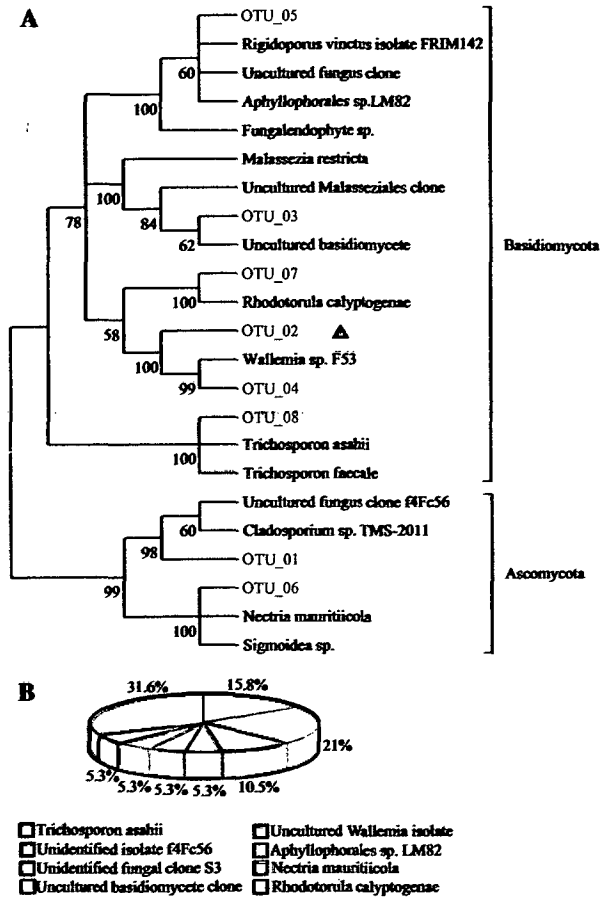

Fig. 1 a Maximum likelihood (ML) phylogenetic tree for the fungal OTUs constructed based on fungal ITS gene sequences obtained using fungal specific primer set ITS1F/ITS4. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. *Numbers* below branches indicate bootstrap values (>50%) from 1,000 replicates. New sequence types are marked with *triangle*. b Proportional distribution of different fungal taxa in the clone library constructed with fungal specific primer set ITS1F/ITS4

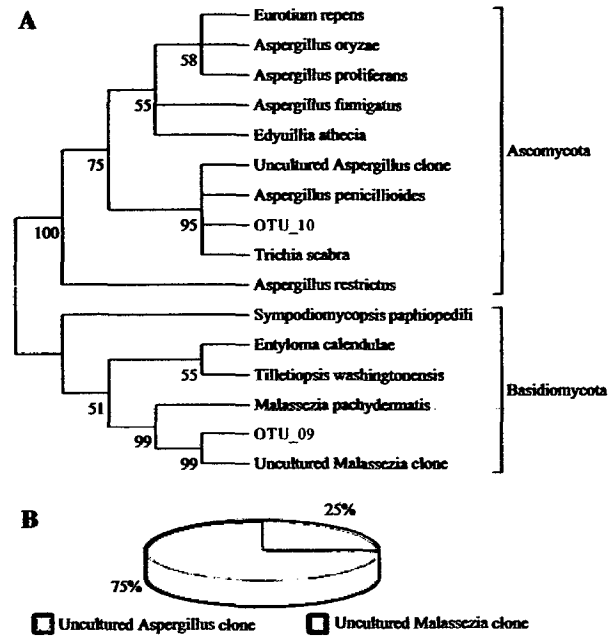

Fig. 2 a ML phylogenetic tree for the fungal OTUs constructed based on fungal 18S rDNA gene sequences obtained using universal fungal 18S rDNA primer set, NS1/NS2. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. *Numbers* below branches indicate bootstrap values (>50%) from 1,000 replicates. New sequence types are marked with *triangle*. b Proportional distribution of different fungal taxa in the clone library constructed with 18S rDNA primer set NS1/NS2

the same fungal specific primers have been shown to amplify sequences from Zygomycota and Chytridiomycota also (O'Brien et al. 2005; Lai et al. 2007; Nagano et al. 2010). Non-detection of these two groups either by culture-dependent (Singh et al. 2010) or by culture-independent approach (Singh et al. 2011) suggests their absence or low abundance in sediments of the sampling sites. Additionally, as suggested by Gao et al. (2010) the fungal primers used may have low specificity towards amplification of 18S rDNA sequences of Zygomycota and Chytridiomycota from mixed environmental samples. Out of the eight fungal OTUs obtained with ITS primer set, 4 were singletons and out of the nineteen OTUs obtained with 18S rDNA primer sets, 10 were singletons. Thus, ~50% of the sequences were singletons indicating low abundance of these phylotypes. It also points out that diversity analysis is far from saturation in the present study and a greater number of sampling and clone libraries is required.

The sequences recovered with ITS1F/ITS4 primer set showed high number of phylotypes belonging to Basidiomycota which is concordant with the previous studies where basidiomycetous yeasts were found to be dominating forms in various deep-sea environments (Takishita et al. 2006; Bass et al. 2007).

One of the noticeable points in the present study was amplification of only two major phylotypes of unidentified *Malassezia* and *Aspergillus* species by the 18S rDNA primer set NS1/NS2. However, PCR artefact or primer bias cannot be attributed to this because these two phylotypes contributed to a large share in the phylotypes amplified by EK555F/EK1269R primer pair too (Fig. 4b). These species have been reported as potential pathogenic forms of animals (Lai et al. 2007). In addition, *Aspergillus* species was reported to play an important role in denitrification process in anaerobic marine sediments off Goa (Jebaraj et al. 2010). This suggests a possible versatile role of fungi in major ecological processes in the deep-sea.

The eukaryotic primer sets, 18S-42F/Univ1492RE and EK555F/EK1269R amplified a total of 17 fungal OTUs. Among these, 5 phylotypes (OTU_11, OTU_14, OTU_17, OTU_19 and OTU_24) affiliated with the sequences

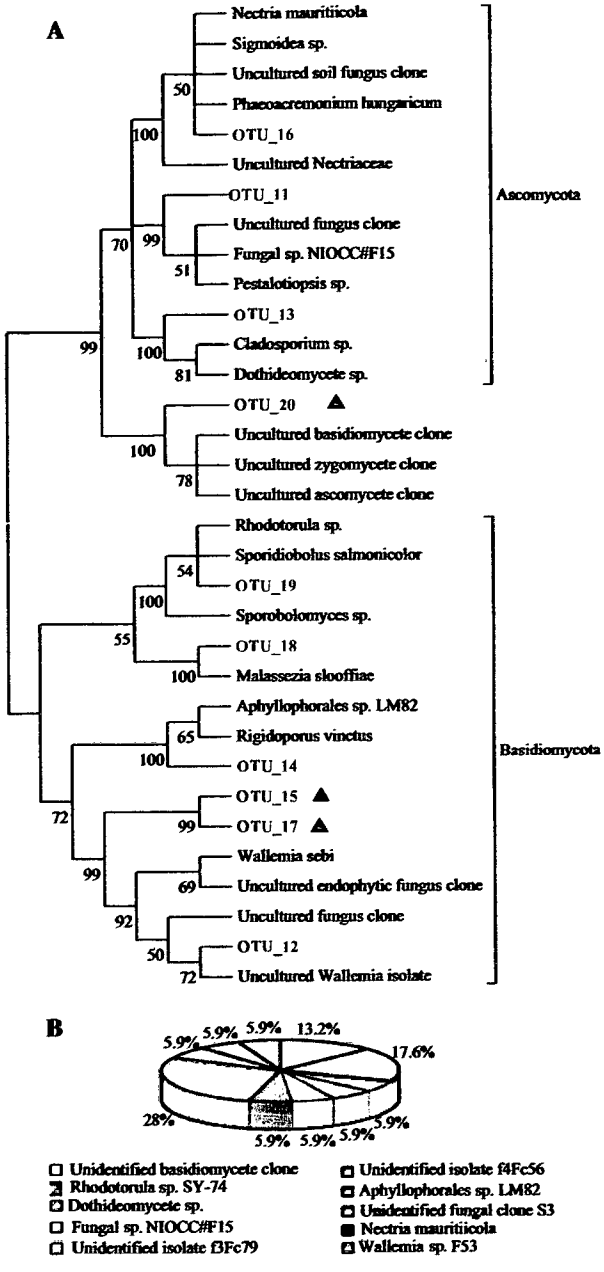

Fig. 3 **a** ML phylogenetic tree for the fungal OTUs constructed based on 18S rDNA gene sequences obtained using universal eukaryotic 18S rDNA primer set, Euk18S-42F/Euk18S-1492RE. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. *Numbers* below branches indicate bootstrap values (>50%) from 1,000 replicates. New sequence types are marked with *triangle*. **b** Proportional distribution of different fungal taxa in the clone library constructed with 18S rDNA primer set Euk18S-42F/ Euk18S-1492RE

reported from marine habitat. Out of these, OTU_11 affiliated with the fungal sp. NIOCC#F15, which was obtained in culture from the CIB sediments and was identified as *Sagenomella* species by amplification of its

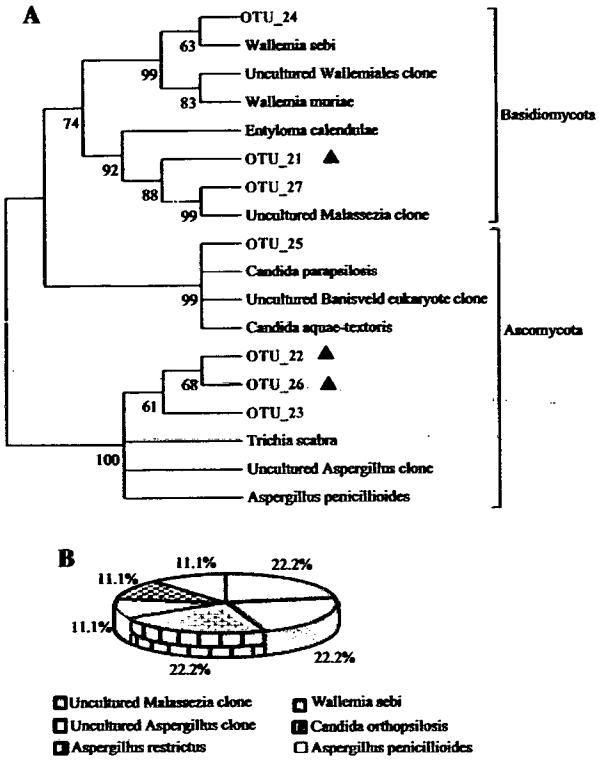

Fig. 4 **a** ML phylogenetic tree for the fungal OTUs constructed based on 18S rDNA gene sequences obtained using universal eukaryotic 18S rDNA primer set, Euk18S-555F/Euk18S-1269R. Topology was built using Mega v.5.03 from a ClustalW 1.83 alignment. *Numbers* below branches indicate bootstrap values (>50%) from 1,000 replicates. New sequence types are marked with *triangle*. **b** Proportional distribution of different fungal taxa in the clone library constructed with 18S rDNA primer set Euk18S-555F/ Euk18S-1269R. Uncultured *Malassezia* clone (22.2%) includes OTU_21 (11.1%) and OTU_27 (11.1%)

18S rDNA gene (Singh et al. 2010). The other phylotypes which were obtained in culturable form were OTU_07 and OTU_19 (*Rhodotorula* spp.) and OTU_23 and OTU_26 (*Aspergillus* spp.) in the previous studies (Singh et al. 2010). Growth and spore germination of these species under simulated conditions of deep sea proved their adaptation to such extreme conditions (Singh et al. 2010). One of the sequences, (OTU_24), amplified with EK555F/ EK1269R showed high similarity with *Wallemia sebi* which was isolated from hypersaline waters of saltern and was demonstrated to be very efficient halophilic and osmophilic species (Kunčič et al. 2010). This species was also isolated from marine sponges (Gao et al. 2008; Liu et al. 2010). These findings suggest presence of salt tolerant species under such extreme conditions. There were no overlapping sequences amplified by these two universal eukaryotic primer sets suggesting that each primer pair amplified specific 18S rRNA gene sequences.

None of the fungal phylotypes except *Trichosporon asahii*, obtained during the present study matched with the phylotypes obtained from the cores at different locations in the CIB, during the same sampling period (Singh et al. 2011). This suggests a spatial variation in distribution of fungal phylotypes, even in not so dynamic habitat such as deep-sea at ~5,000 m depth. The sequences belonging to Exobasidiomycetes amplified by all the four primer sets is being reported here for the first time from the deep-sea environment. Its presence in anoxic sediments off Goa (Jebaraj et al. 2010) and in coastal Hawaiian waters (Gao et al. 2010) was reported and is known to consist of species which are plant pathogens (Begerow et al. 2006). *Rhodotorula* sp. belonging to the subphylum Cystobasidiomycetes, reported in the present study is known to be a mycoparasite (Bauer and Oberwinkler 1991).

Proportional distribution of fungal sequences recovered with different primer sets varied (Figs. 1b, 2b, 3b, 4b). Among the four primer sets, the universal eukaryotic primer set 18S-42F/Univ1492RE amplified maximum number of diverse fungal phylotypes. These results are in contrast to the previous studies (López-García et al. 2003; Stoeck et al. 2006) where only a few fungal OTUs were obtained. Only a few sequences overlapped between different primer sets resulting in detection of high diversity of fungal phylotypes by multiple-primer approach (Figs. 1b, 2b, 3b, 4b).

A total of seven OTUs affiliating with a percentage similarity of <97% with the existing sequences reported in the database were obtained in the present study. This indicates the efficient nature of the primers used to amplify such novel forms present in deep-sea sediments. These novel forms comprised mostly of the sequences matching with *Aspergillus*, *Malassezia* and unidentified fungal clones suggesting their possibility of being novel marine variants of the existing species. Jebaraj et al. (2010) also reported recovery of novel fungal sequences by using multiple primer approach.

The polymetallic nodules reported in the CIB are rich in Ba, Co, Cu, Fe, Mg and Mn (Nath et al. 1989). The recovery of fungi from this area suggests their tolerance to metals. In fact, Connell et al. (2009) have reported metal-tolerant yeasts producing siderophores from deep-sea basalt rocks in Vailulu'u Seamount, Samoa. These findings indicate active role of fungi in biogeochemical cycles in the deep sea.

In conclusion, the use of multiple primer approach enabled the recovery of diverse fungal phylotypes from mixed environmental sediment sample of the CIB. The high diversity obtained in spite of less number of clones screened suggested the abundance of fungi under such extreme conditions. Some of the fungal sequences obtained in the present work have been earlier reported from marine environment. This supports their presence and ecological role in various biological processes in this environment. The presence of halophilic forms like *Wallemia* sp. from culture-independent study should be proved by culture-dependent approach using various culturing techniques which may enhance our understanding of salt tolerance in deep-sea fungi. Fungi such as *Sagenomella* species obtained by culture-dependent as well as culture-independent approach could be used as a model organism to understand pressure tolerance in fungi.

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