

Chapter 3

Fungi

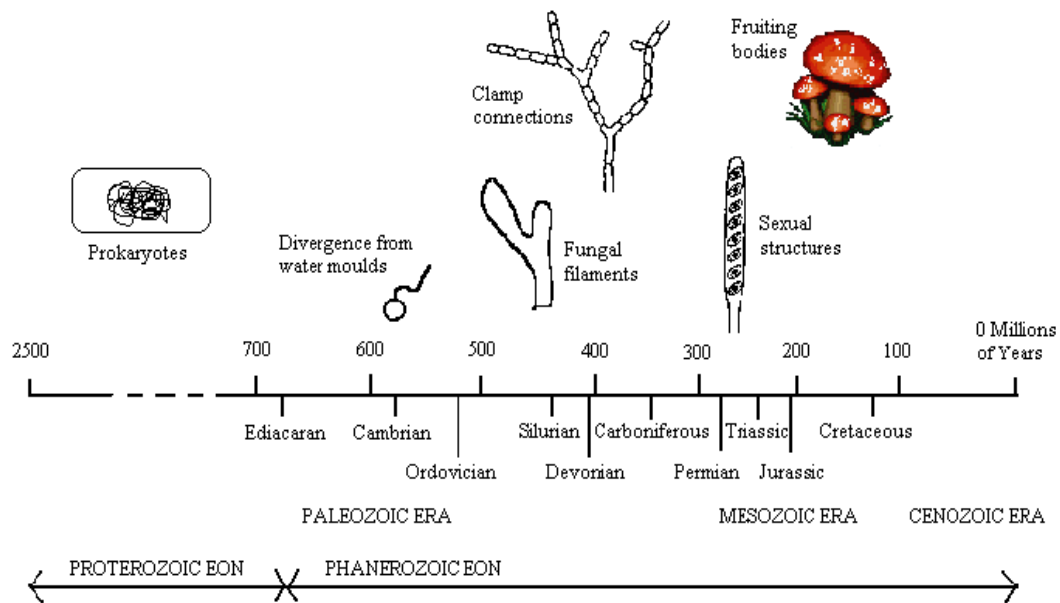
in

Marine

Environments

From evidence inferred from molecular sequence data, it appears that eukaryotes and bacteria shared their last common ancestor around 2000 million years ago. Plants, animals and fungi then began to diverge from one another in the region of 1000 million years ago. The divergence of animals from fungi has been estimated at 965 million years ago. The oldest known fossilized fungal spores have been found in amber dating back to 225 millions of years ago. Fossilized fungal spores in sediments of around 50 - 60 million years old can be found with relative ease.

Fig. 3.1 Geological time-line illustrating key events during fungal evolution, including structural and period data



Source: http://www.world-of-fungi.org/Mostly_Mycology/Jon_Dixon/fungi_timeline.htm

Fossil evidence for eukaryotic organisms (presumably protists) dates back to about 2000 million years ago, and in spite of the existence of records of presence of only marine life at that time, the existence of true saprobic marine fungi was often

questioned, for instance, by [Bauch \[1936\]](#), who wrote: “Saprobic Ascomycetes which play an important role in forest and soil in deterioration of organic material, especially of wood, appear to be completely absent in seawater”. The first facultative marine fungus, *Phaeosphaeria typharum*, was described by [Desmazières \[1849\]](#) as *Sphaeria scirpicola* var. *typharum* from typha in freshwater. [Durieu & Montagne \[1869\]](#) discovered the first obligate marine fungus on the rhizomes of the sea grass, *Posidonia oceanica*, and marveled at the most remarkable life - style of *Sphaeria posidoniae* (*Halotthia posidoniae*), which spends all stages of its life-style at the bottom of the sea. One single publication influenced the development of marine mycology more than any other paper, namely, “Marine Fungi: Their Taxonomy and Biology” by [Barghoorn & Linder \[1944\]](#). These authors demonstrated that there was an indigenous marine mycota, showing growth and reproduction on submerged wood after defined periods of time. This publication stimulated worldwide research in marine mycology. [Johnson & Sparrow](#) published the first monograph on marine mycology in 1961.

There have been various definitions of marine fungi in the literature. While some authors defined marine fungi based on their ability to grow at certain seawater concentrations [[Johnson & Sparrow, 1961](#); [Tubaki, 1969](#)], other workers have determined the physiological requirements for the growth of marine fungi in sea water, or in particular concentrations of sodium chloride [[Meyers, 1968](#); [Jones & Jennings, 1964](#)]. The most widely accepted definition for marine fungi is that of [Kohlmeyer & Kohlmeyer \[1979\]](#), according to which *obligate marine fungi* are those that grow and sporulate exclusively in a marine or estuarine habitat; *while facultative marine fungi* are those from freshwater or terrestrial habitats able to grow and possibly also sporulate in the marine environment. They further suggest that a valid criterion for the

definition of a marine fungus might be its ability to germinate and to form mycelium under natural marine conditions although such conditions may vary, depending on the species. Thus, marine fungi are not a taxonomically, but an ecologically and physiologically defined group.

The number of fungi described worldwide is estimated at around 70,000, but their total number may be as high as 1.5 million species. However, the share of marine fungi is a measly 1000 to 1500 only. Marine fungi comprise saprobic forms present in the open ocean waters (pelagic) and in bottom (benthic) zones. However, majority of the studies on marine fungi are related to forms occurring in various types of submerged materials in waters and sediments nearest to land, the neritic and littoral zones. Little knowledge exists of fungi present in oceanic deep waters and associated sediments.

Most fungi found in marine habitats are microscopic. The largest ascocarps occur in *Amylocarpus encephaloides*, which do not exceed 3 mm. The basidiomycetes *Digitatispora marina* and *Nia vibrissa* have fruiting bodies 4 mm in length and 3 mm in diameter, respectively. This is because the marine environment does not permit the development of large, fleshy fruiting bodies, because abrasion of waves and grains of sand does not allow formation of such structures.

Higher marine fungi occur as parasites on plants and animals, as symbionts in lichenoid associations with algae and as saprobes on dead organic material of plant or animal origin [Kohlmeyer & Kohlmeyer, 1979]. Fungi from coastal and marine ecosystems are neglected but contribute a significant part of marine biodiversity. Fungi in general are able to degrade a wide range of recalcitrant biological molecules and particularly in coastal ecosystems, fungal activity may be critical in the early

stages of biodegradative pathways. Fungi in the marine environment have only been fully recognized since about 1960, and within the group, marine fungi have shown highest decadal indices (% increases in species number over a 10 year period). Between 1981 and 1991, [Hawksworth \[1991\]](#) calculated that marine fungi had the highest decadal index (49%) for any fungal group. Furthermore, Hawksworth put forward the opinion that less than 10% of fungal biodiversity has been discovered.

Higher marine fungi constitute Ascomycotina, Basidiomycotina and Deuteromycotina. Majority of them being ascomycetes, their spores show adaptation to the marine ecosystem through the production of appendages, which facilitate buoyancy in water, entrapment and adherence to substrates. Marine filamentous fungi have been reported on a variety of detritus: decaying wood, leaves, seaweeds, seagrasses, calcareous and chitinous substrates. Studies on marine fungi were initiated in the temperate parts of the world. Subsequently, tropical locations were the centres of interest to understand their abundance and diversity [[Jones, 1993](#); [Kohlmeyer & Kohlmeyer, 1979](#)]. Tropical regions of Atlantic and Pacific Oceans were investigated more intensively than the Indian Ocean [[Kohlmeyer & Kohlmeyer, 1979](#)].

Fungi were not the object of serious biogeographical studies until 1980s [[Pirozynski & Walker, 1983](#)]. Early workers concentrated primarily on fungal distributions, producing long lists of fungi collected from particular localities or regions around the globe. Although abundant, accurate distribution data were a prerequisite to biogeographical analyses, such listings of fungi from particular geographic localities were not in themselves biogeographical studies. The earliest study to consider fungal distribution in any real ecological sense was that of [Fries \[1857\]](#). Fries considered “heat and humidity” as the determiners of global distribution

patterns and suggested that differences in fungal distribution were caused by variations in the amount of atmospheric moisture and rain. He divided the whole environment in two environments for fungaceous growth as temperate and tropical zone. The largest group of filamentous fungi is the Ascomycetes, which produce sexual spores within an ascus. The filamentous Basidiomycetes have sexual spores borne externally on a basidium, with only three species described from the marine environment [Kohlmeyer & Kohlmeyer, 1971]. The filamentous Deuteromycetes are imperfect fungi, and most of them are presumably asexual forms of the Ascomycetes.

The 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 the intertidal and benthic algae, seagrasses and mangroves as well as a wide variety of cellulosic materials from land, such as driftwood, pinecones, and leaves. Certain aquatic fungi from freshwater streams have processes on their spores that enable them to be preferentially concentrated in foam [Ingold, 1973]. Similar ecological observations on the spores of marine species from sandy habitats (arenicolous fungi) have been made by Kohlmeyer [1966]. Calcium carbonate deposits are often and apparently actively reworked by filaments of the boring fungi [Kohlmeyer, 1969], which are more widespread and abundant than the endolithic algae.

Filamentous fungi, especially those growing on plant materials, attract animal predators and are important in the food chain. The fungal mycelium of Ascomycetes and Deuteromycetes growing on cellulosic debris is able to support the requirements for growth and reproduction of one species of nematode [Meyers *et al.*, 1964], and to attract the gravid females of another species of nematode [Meyers & Hopper, 1966].

The commonest habitat for marine fungi is submerged wood, as suggested by all the studies, during the early years of marine fungi, i.e. in 1960s. Ascomycetes, often forming uniquely appendaged spores [Kohlmeyer, 1961], and dematiaceous Fungi Imperfecti predominate. Doguet [1962] found a hemibasidiomycetes on submerged wood, the first record of a saprobic representative of this class in a strictly marine environment. Wood either placed in sea water as panels [Johnson & Sparrow, 1961] or as structural material such as pilings, also becomes inhabited by fungi that are not normally considered part of the marine biota.

Calcareous shells comprise one of the major habitats for colonization by fungi in the sea [Kohlmeyer & Kohlmeyer, 1979; Raghukumar *et al.*, 1992]. Endolithic fungi, growing within such calcareous shells have been reported from intertidal beaches [Porter & Zebrowski, 1937; Cavaliere & Alberte, 1970; Kohlmeyer, 1969], coral rocks [Kohlmeyer & Volkmann-Kohlmeyer, 1987; 1989] and also live corals [Kendrick *et al.*, 1981; Ravindran *et al.*, 2001].

Extensive fungal borings of calcareous sediments of biological origin has been reported from all over the world [Perkins & Halsey, 1971; Rooney & Perkins, 1972; Perkins & Tsentas, 1976]. But very few experiments were carried out in order to understand the role of endolithic fungi in mineralization of calcium carbonate. Raghukumar *et al.* [1992] first reported isolation and quantification of fungi from the calcareous animal shells collected from various depths in the Bay of Bengal, the deepest being 860 m.

In this study, the fungal diversity and its role in the deep-sea sediments of the Central Indian Basin, the tropical zone has been reported.

Chapter 4

Fungal Diversity in Deep-sea Sediments

4.1 Introduction

Presence of fungi in oceanic waters and deep-sea have been sporadically reported in the past. Presence of fungi in shells collected from deep-sea waters of 4610m depth [Höhnk, 1961 & 1969] was the first report on deep-sea fungi. This was followed by isolation of fungi from water samples collected from subtropical Atlantic Ocean, from surface to 4500 m depth using sterile van Dorn bags or Niskin samplers [Roth *et al*, 1964]. Deep-sea fungi were obtained by directly submerging wooden panels at 1615-5315 m depth [Kohlmeyer, 1977]. These fungi were not cultured and only preserved specimens are available [Kohlmeyer & Kohlmeyer, 1979]. Four of these fungi were found growing on wooden panels and one on chitin of hydrozoa (Table 4.1). Mycelial fungi growing inside shells of mollusks at 4830 m depth in the Atlantic were documented [Poulicek *et al*, 1986]. A study spanning over 10 years showed a distinct succession pattern of fungi as endolithic community in molluskan shells [Poulicek *et al*, 1988]. 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. It was observed, “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” [Kohlmeyer & Kohlmeyer, 1979]. In accordance, it was demonstrated that conidia of *Aspergillus restrictus* isolated from the calcareous sediments germinated at 30 MPa pressure in Czapek-Dox medium and on shells suspended in seawater [Raghukumar & Raghukumar, 1998]. Detection of fungal filaments in formalin-preserved calcareous fragments obtained

from 965 m depth in the Arabian Sea further confirmed that they were actively growing in these shells. These calcareous fragments were treated with EDTA to dissolve the calcium carbonate and subsequently stained with the fluorescent brightener Calcofluor to visualize fungal filaments under the epifluorescence microscope [Raghukumar & Raghukumar, 1998]. Calcofluor was originally used to detect fungal infections of phytoplankton in natural waters [Müller & Sengbusch, 1983]. The marine yeasts, *Debaryomyces hansenii*, *Rhodotorula rubra* and *Rhodospiridium sphaerocarpum* were cultured at temperatures from 7° to 34°C and pressures from 0.1

Table 4.1 Fungi reported or isolated from deep-sea sources

Fungi reported	Source	Depth (m) & Location	Reference	Remarks
<i>Abyssomyces hydrozoicus</i>	Hydrozoa chitin	631-641, Atlantic	Kohlmeyer & Kohlmeyer, 1974	Only preserved specimens
<i>Bathyascus vermisporus</i>	Wood	1615 & 1720, Pacific		
<i>Oceanitis scuticella</i>		3975, Atlantic		
<i>Allescheriella bathygena</i>		1720		
<i>Periconia abyssa</i>		3975 & 5315, Atlantic		
<i>Aureobasidium pullulans</i>	Water	1000-4500, subtropical Atlantic	Roth <i>et al.</i> , 1964	Colony Forming Units (CFU)
<i>Cladosporium spp.</i>				
<i>Alternaria spp.</i>				
<i>Aspergillus sydowii</i>				
<i>Nigrospora spp.</i>				
<i>Penicillium solitum</i>				

<i>Aspergillus ustus</i>	Calcareous shells	965, Bay of Bengal	Raghukumar et al., 1992	CFU
<i>Penicillium citrinum</i>				
<i>Cladosporium</i> sp.				
<i>Scopulariopsis</i> sp.				
Non-sporulating fungus				
<i>Aspergillus fumigatus</i>				
<i>Cladosporium herbarum</i>				
<i>Rhodotorula mucilaginosa</i>	Sediments	10500, Mariana Trench	Takami et al., 1999	In culture
<i>Penicillium lagena</i>				
<i>Gymnascella marismortui</i>	Water	Depth not mentioned, Dead Sea	Buchalo et al., 1997	In culture
<i>Phoma pomorum</i>	Water	Depth not mentioned, Dead Sea		In culture
<i>Penicillium westlingii</i>				
<i>Aspergillus sydowii</i>	Sediments	5100, Central Indian Ocean	Raghukumar et al., 2004	In culture
Non-sporulating unidentified sp.				
<i>Aspergillus</i> sp.	Sediments	~5000, Central Indian Basin	Damare et al., 2006	CFU
<i>Aspergillus terreus</i>				
<i>A. restrictus</i>				
<i>A. sydowii</i>				
<i>Penicillium</i> sp.				
<i>Cladosporium</i> sp.				
<i>Curvularia</i> sp.				
<i>Fusarium</i> sp.				
Non-Sporulating fungi				
Unidentified fungi				

to 80 MPa [Lorenz & Molitoris, 1997]. Germination of fungal spores under simulated deep-sea conditions of low temperature and elevated hydrostatic pressures was also demonstrated [Zaunstöck & Molitoris, 1995]. Fungi have been retrieved from a depth of 10,500 m sediment samples from the Mariana Trench in the Pacific Ocean [Takami *et al*, 1997]. Sequences belonging to fungi have been reported from the aphotic zone at 250-3000 m depth in the Antarctic polar front [Lopez-Garcia *et al*, 2001]. These reports were based on direct amplification of small-subunit ribosomal RNA genes from water.

Recently, isolation and direct detection of fungi was reported from deep-sea sediments in the Chagos Trench in the Indian Ocean at a depth of 5900 m [Raghukumar *et al*, 2004]. The age of the sediments from which fungi were isolated and detected was estimated to range from >0.18 to 0.43 million years, being the oldest recorded age for recovery of culturable fungi. Fungal hyphae could not be detected by bright field microscopy but were visible by epifluorescence microscopy. The relation between sediment particles and fungal hyphae was clear when viewed under the combination of bright field and epifluorescence light. These studies indicated that presence of fungi in deep-sea sediments might have gone undetected by conventional microscopy. This chronology of the events in the research methodologies and strategies clearly indicates the increasing attention being paid to the presence of fungi in deep-sea.

4.1.1 Differentiation of fungal isolates based on molecular methods

Presently, along with the classical morphological and/or biochemical methods, DNA based molecular methods are increasingly used to describe the diversity of organisms. Until the beginning of 1980s, when molecular techniques enabling direct analysis of genome variability developed, methods for identification and classification of microorganisms were based mainly on phenotypic traits [Paffetti *et al*, 1995]. Molecular techniques that have been applied for identification and taxonomic studies of fungi include cDNA/cDNA reassociation [Vaughan-Martini & Kurtzman, 1985], karyotyping with pulse field electrophoresis [Johnston & Mortimer, 1986; Casey *et al*, 1988; Bidonne *et al*, 1992; Naumov *et al*, 1992; Vaughan-Martini *et al*, 1993], restriction fragment length polymorphism (RFLP) [Pederson, 1986; Degrè *et al*, 1989], sequencing of ribosomal RNA [Kurtzman & Robnett, 1991], restriction enzyme analysis (REA) of genomic DNA [Barberio *et al*, 1994] and random amplification of polymorphic DNA [Welsh & McClelland, 1990; Williams *et al*, 1990].

4.1.1.1 RAPD

RAPD stands for random amplification of polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified are random. Variation within species can be assayed using this method [Welsh & McClelland, 1990; Williams *et al*, 1990]. In lower eucaryotes, RAPD has led to the distinction between species and /or strains in several fungi such as *Neurospora crassa* [Welsh & McClelland, 1990], *Candida albicans* [Bostock *et al*, 1993], *Metarhizium* and *Beauveria* [Bidochka *et al*, 1994].

In this technique, 'arbitrary' or 'random' short oligonucleotide primers, targeting unknown sequences in the genome are used to generate amplification products that often show size polymorphisms within species. RAPD analysis offers the possibility of creating polymorphisms without any prior knowledge of the DNA sequences of the organism investigated. The patterns produced are highly polymorphic, allowing discrimination between isolates of a species if sufficient numbers of primers are screened. The method is fast and economic for screening large number of isolates. However it has a big drawback of reproducibility. The reproducibility within a laboratory is usually satisfactory. However, inter-laboratory comparison of RAPD patterns may not always be applicable since the RAPD patterns can be influenced by many technical factors like thermocycler, the type of Taq polymerase, source of primers, etc.

4.1.1.2 RFLP

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to compare species and also strains.

Restriction endonucleases used in this, are the enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Enzyme recognition sites are usually 4 to 6 base pairs in length. Generally, the shorter the recognition sequence, the greater the number of fragments generated. If molecules

differ in nucleotide sequence, fragments of different sizes may be generated. The fragments can be separated by gel electrophoresis. Restriction enzymes are isolated from a wide variety of bacterial genera and are thought to be part of the cell's defense against invading bacterial viruses. These enzymes are named by using the first letter of the genus, the first two letters of the species, and the order of discovery.

4.1.1.3 PCR – RFLP

PCR-RFLP makes use of both the PCR as well as RFLP techniques. RFLP for the genomic DNA requires the isolation of large amounts of purified DNA. With PCR it becomes possible to analyze specific sequences from small amounts of biomass. PCR can be performed on crude DNA extracts with a pair of region-specific primers. Variation of the amplified fragment can be further analyzed by restriction enzyme digestion and electrophoretic separation. The regions most commonly examined by PCR-RFLP are the rDNA sequences. In fungi, as in any other eukaryotes, rRNA genes are repeated up to several hundred times in a clustered manner. In each rDNA repeat, two internal transcribed spacers (ITS) separate the 18s, 5.8s and 28s rRNA genes. The rDNA sequences encoding 18s and 28s RNAs show slow evolutionary change and thus can be used to compare distantly related organisms [Bruns, 1992; Berbee & Taylor, 1993, Simon *et al.*, 1993, Begerow *et al.*, 1997]. The ITS region and the intergenic spacer of the rDNA repeat evolve much faster and sequence differences in these regions frequently occur between closely-related species or even between populations of the same species [Buchko & Klassen, 1990, Bernier *et al.*, 1994, Erland *et al.*, 1994, Lovic *et al.*, 1995]. Thus, analysis of the rDNA region is very useful for

comparisons over a wide range of taxonomic levels and it has high resolving power, depending on which part of the rDNA repeat is analyzed.

4.2 Objectives

The main objective was to isolate fungi from the deep-sea sediments from around 5000 m by various conventional as well as new techniques selective for barophiles (barotolerant) and describe the diversity of fungi in this extreme environment using conventional and molecular taxonomy.

4.3 Methodology

4.3.1 Sampling

In order to achieve the first objective to isolate fungi from the deep-sea sediments, the area selected was Central Indian Basin with an average depth of more than 5000 m. (Fig.4.1) Eleven box core samples were collected during cruise # AAS 34 (10°00'264" - 10°10'364"S; 75°21'000" - 76°05'160" E), 7 during cruise # AAS 46 (10°00'237" - 10°02'661" S; 75°59'498" - 76°09'822" E) and 38 during the cruise AAS # 61 (9°59'861" - 16°00'047" S; 73°29'819" - 76°30'559" E). Sediment samples were collected on board the Russian research vessel AA Sidorenko, using an Usnel type box corer of 50 cm³ size (Fig. 4.2 a - c). Sampling with a box corer is possible in this area because of the more or less flat topography of the ocean floor in the sampling area. Sediment at the sampling sites was mainly radiolarian ooze, light to dark brown

Fig. 4.1 Map of the Central Indian Basin showing, within inset, location of the sampling sites (9 - 16°S and 73 - 76°E) during cruises AAS 34, AAS 46 and AAS 61

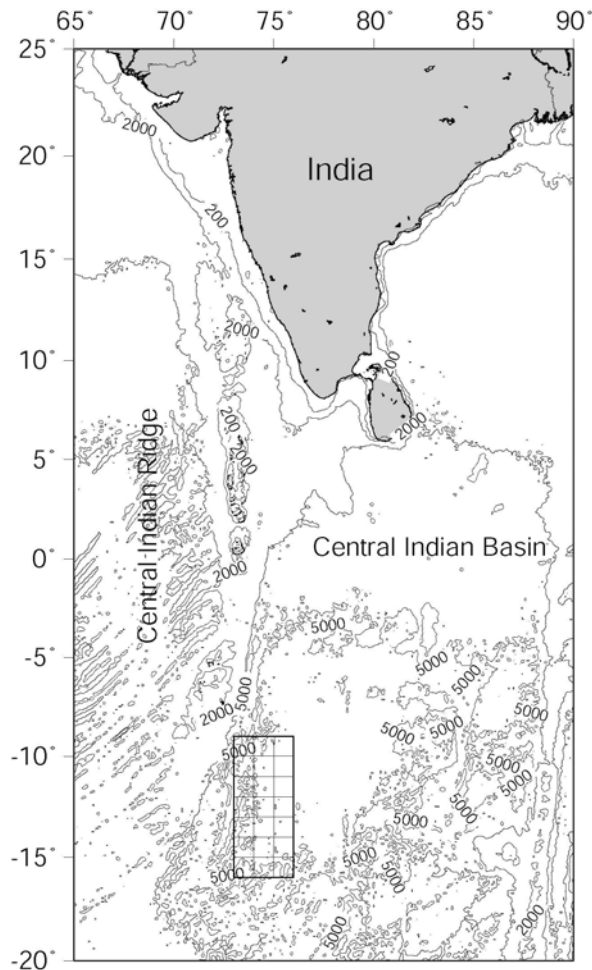
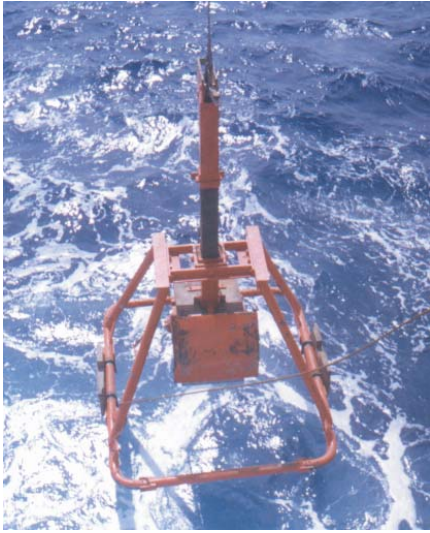


Fig.1

in color and intensely mottled indicating high bioturbation [Sharma *et al.*, 2001]. It was predominantly clayey-silt type with high water content and low shear strength and was loosely packed [Khadge, 2000].

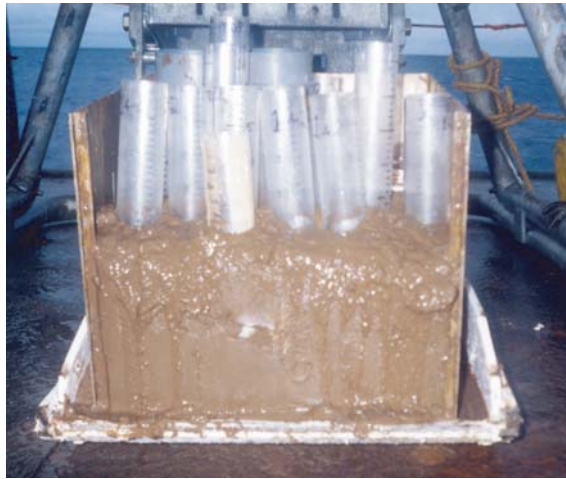
Subcores of sediments were collected from the center of the box corer with alcohol-sterilized PVC cylinders of 5 cm diameter. Subsections of 2 cm down to 10 cm depth and thereafter every 5 cm were extruded from these sediment cores of



(a) USNEL box cores being lowered for sampling



(b) The box corer retrieved with sediment



(c) Sediment cores being collected from the box corer



(d) Sediment core



(e) Subsectioning of the sediment core

Fig. 4.2 Sediment sampling being done onboard AA Sidorenko from Central Indian Basin

approximately 30 to 40 cm length (Fig. 4.2 d & e) directly into sterile plastic bags to avoid any aerial contaminants. The bags were closed with rubber bands and carried to the working area in microbiology laboratory on board.

4.3.2 Isolation of fungi

A portion of the sediment from the middle of each sub sample that had not been in contact the walls of the PVC cylinder was removed using an alcohol flame sterilized spatula and was used for the isolation of fungi [Raghukumar *et al.*, 2004]. The media used for isolations were malt extract agar (MEA), malt extract broth (MEB), corn meal agar (CMA), Sabouraud's dextrose agar (SDA), Czapek Dox agar (CDA) and Czapek Dox broth (CDB). All the media were used at 1:5 strength to simulate the low nutrient condition in the deep sea. They were prepared in seawater and fortified with streptomycin (0.1 g in 100 mL medium) and penicillin (40,000 Units in 100 mL medium) to inhibit bacterial growth. Fungi were isolated using the following techniques:

1. Dilution plating method, where approximately 0.1 g of sediment was suspended in sterile seawater, vortexed properly and 100 μ L aliquots were spread plated on different media (Fig. 4.3).
2. Particle plating technique [Bills and Polishook, 1994], where approximately 1 g of sediment was sieved successively through 200 and 100 μ m mesh. The particles that passed through 200 μ m but retained on the 100 μ m mesh were spread plated on different media (Fig. 4.3).

For both the above two techniques, the plates were incubated at 10°C for 15 - 20 days.

Fig. 4.3 Schematic representation of the two methods used for isolating fungi from the sediment samples

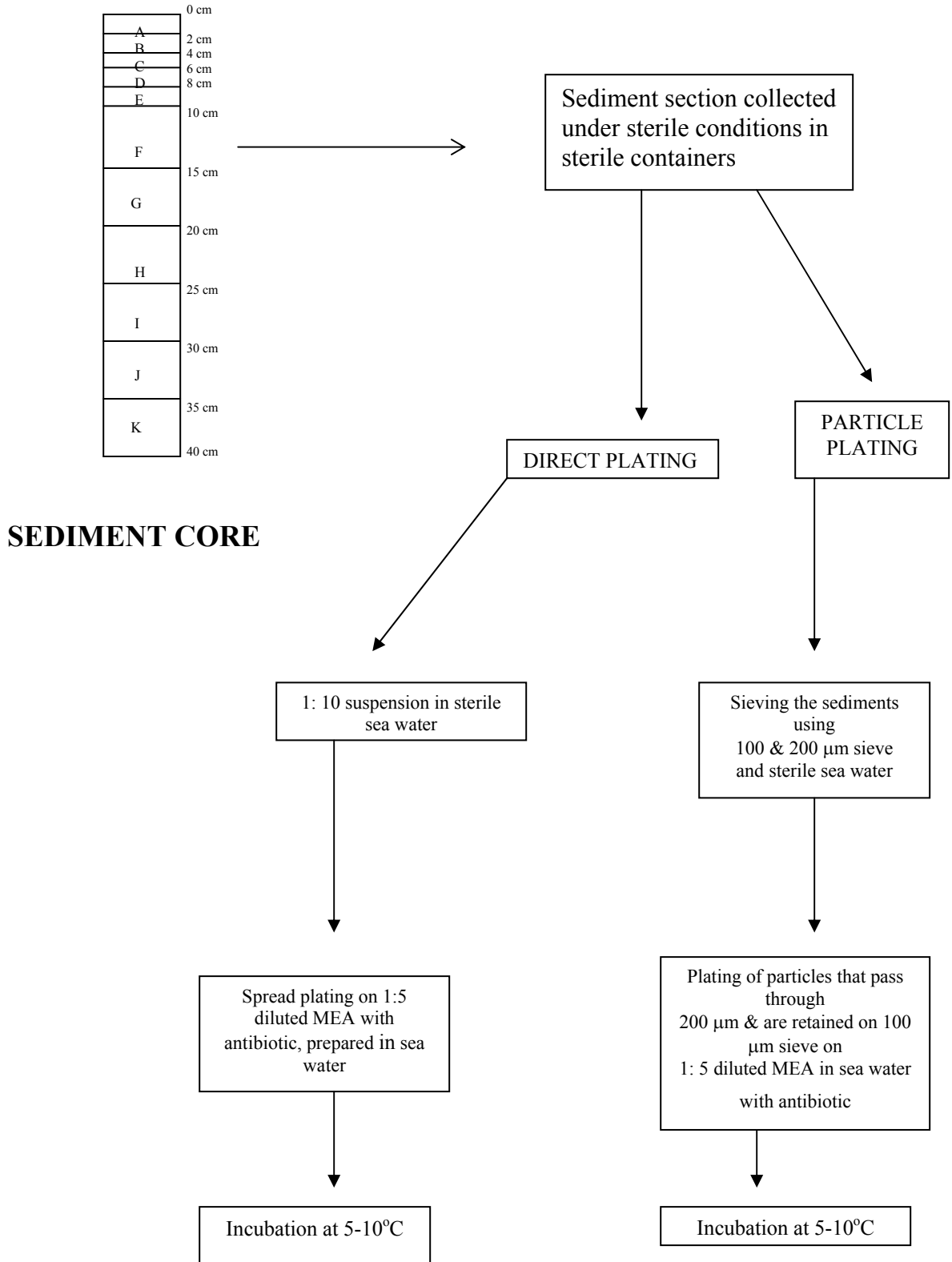
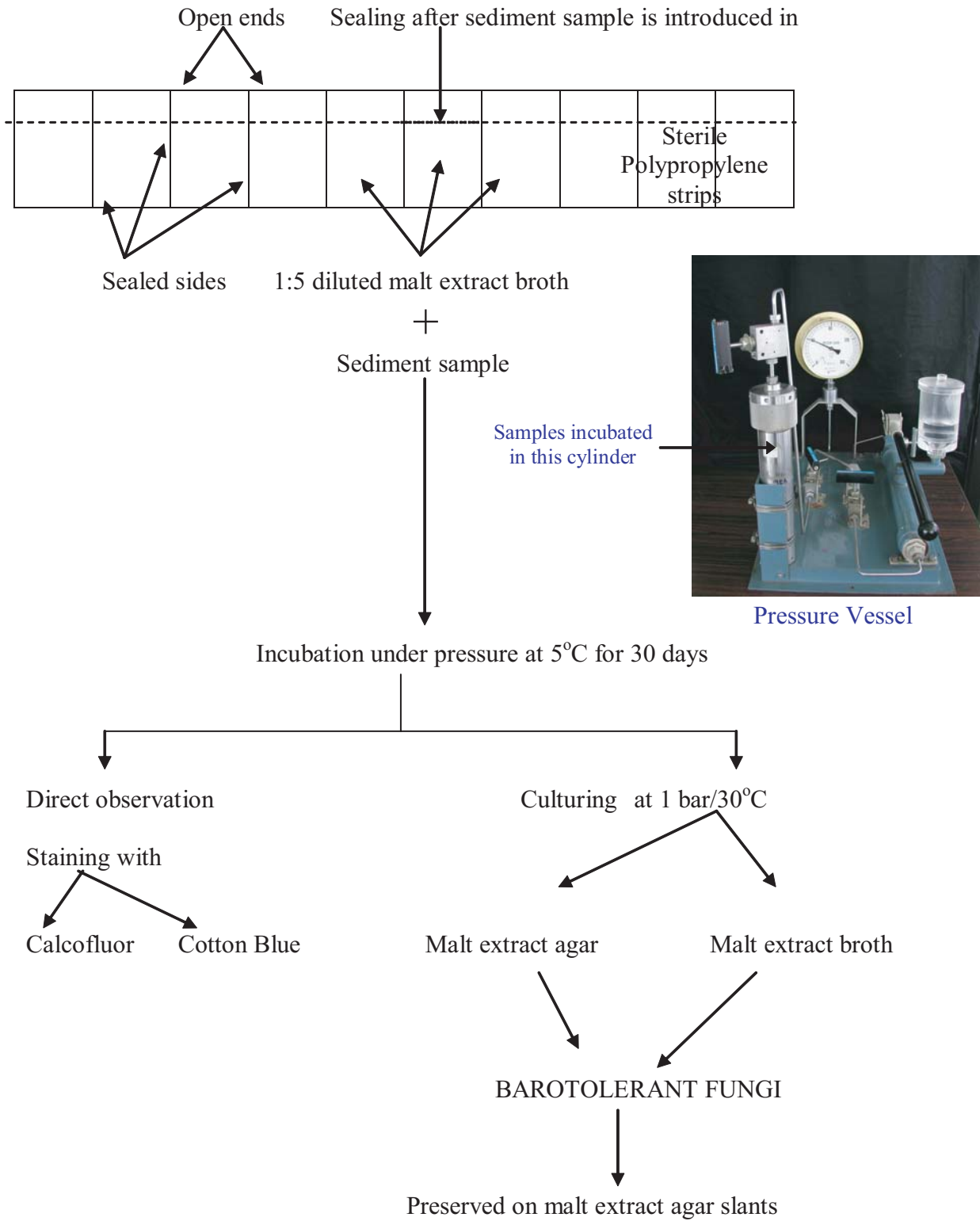


Fig. 4.4 Schematic representation of the pressure enrichment method used for isolation of fungi from the deep-sea sediments



3. Pressure incubation, in which approximately 0.5 g of sediments were placed in sterile plastic bags containing 2 mL of sterile MEB (Fig. 4.4) and the bags were sealed using an electrical sealing machine. Apart from MEB, different baits like agar discs, artemia larvae and eggs were used. The bags were then placed in pre-cooled (to 5°C) deep-sea culture vessels filled with sterile water and pressurized to 300 bar hydrostatic pressure. The pressure vessels were then immediately incubated at 5°C for 30 days. At the end of the incubation period, the pressure vessels were depressurized at a rate of 100 bar 30 min⁻¹. The bags were cut open and the contents transferred to sterile plastic vials. From this, 100 µL of the sediment mixture was spread plated, while another 100 µL was inoculated to 10 mL sterile MEB in a conical flask. The plates and flasks were incubated at 1 bar/30°C until fungal growth was seen (approximately 8 to 10 days). The cultures obtained by this method were called ‘barotolerant isolates’.

Duplicate plates were maintained for each sediment sample, medium and isolation technique. Media plates were exposed to air for 10 min on the deck of the research vessel where the cores were received, the microbiology laboratory and the inoculation hood in the microbiology laboratory to check for the presence of aerial fungal contaminants. These did not yield any fungal colonies, thus ruling out aerial fungal contaminants. This was repeated during every sampling station.

Fungi isolated from the deep-sea sediments using all these techniques were sub-cultured and maintained on MEA slants at 5°C.

Along with the sediments, fungi were also isolated from a few deep-sea macro – organisms like prawns, starfish which were hauled up along with the sediment

samples. These were surface sterilized, and then the cut pieces were placed on the surface of media and incubated at 10°C at 1 bar.

For comparison, fungi were also isolated from sediment samples collected from shallow coral reef slopes at a depth of 30 m off Lakshadweep Island Kavaratti (10°35' N and 72°39' E) in the Arabian Sea by the particle plating technique.

4.3.3 Detection of fungi in deep-sea sediments

About 0.5 g of each sediment sample in sterile vials were fixed in 5 % formalin solution and stored at 5°C for direct detection of fungi as per the method described by [Müller and Sengbusch \[1983\]](#). Aliquots of these fixed sediments were stained with 0.5 % solution of sterile-filtered Calcofluor, an optical brightener. The excess stain was washed by centrifugation with sterile seawater. Microscopic mounts of the sediment were then examined under ultraviolet light (excitation wave length 330-385 nm and barrier filter BA 420) of an epifluorescence microscope (Olympus BX 60, Japan) to detect fluorescing fungal hyphae. Fungal hyphae thus observed were recorded by photographing.

4.3.4 Detection of fungi in sediments by immunofluorescence techniques

The immunofluorescence technique, which has been widely used to detect specific fungi in terrestrial substrates [[Jellison & Goodell, 1988](#); [Friese & Allen, 1991](#); [Banks *et al.*, 1993](#)], was employed for the purpose. Antibodies were raised commercially for *A. terreus* (# A 4634), one of the most frequently isolated fungi from the deep-sea sediments of the Central Indian Basin. This isolate was obtained from core # BC 12 at the subsurface depth of 15–20 cm during the cruise # AAS 46 (10°10'10"S; 76°10'00"E at a depth of 5400 m). Antibodies were raised in New

Zealand male white rabbits by Genei India Pvt. Ltd., Bangalore, by standard protocols [Johnson & Thorpe, 1987]. Thus, about 2 mg of fungal pellet was crushed in 2 mL of 0.15 M NaCl and centrifuged, and 1 mL supernatant was emulsified with 1 mL Freund's adjuvant. Fifty mg of this antigen was injected subcutaneously at multiple sites on the backs of the rabbits followed by booster doses on days 30, 45, 55 and 65. The antibody titre was monitored by Dot ELISA and yielded a value of 1:10,000. The presence of *A. terreus* in natural sediment samples was studied by the following method: The antiserum containing the antibodies (as supplied by Genei India Pvt. Ltd., Bangalore) diluted to 1:10 with sterile phosphate buffered saline pH 7.0 (PBS) was the minimum concentration required for detection of fluorescence of the fungus in these samples. About 0.1 g of the deep-sea sediment sample from which the fungus was isolated was stained with the antiserum at 25°C for 60 min followed by 5 washes, each with 1000 µL PBS. The sediment was further incubated with 100 µL of 1:10 diluted secondary antibody (Genei India Pvt. Ltd.), namely the goat anti-rabbit anti-serum tagged with fluorescein isothiocyanate (FITC) for 60 min at 25°C. The excess stain was removed by washing the sediment 5 times with PBS. The sediment was spread evenly onto a slide and observed under an epifluorescence microscope (excitation wavelength 450–480 nm and barrier filter BA 515). The reactivity of the antiserum to *A. terreus* was confirmed by growing the fungus both at 1 bar and 200 bar pressure / 5° and 30°C and staining as described above. The antiserum containing the antibodies diluted to 1:100 with PBS was the minimum concentration required for detection of fluorescence of the fungus in culture. The absence of cross reactivity with other fungi was checked by staining an unidentified fungus, *Aspergillus* sp., a non-sporulating

fungus and a terrestrial *A. terreus* (MTCC # 279) with antiserum similarly and examining for the presence or absence of immunofluorescence.

4.3.5 Diversity of the isolated fungi

The isolated fungi were inoculated on MEA and CDA plates for the identification purpose. Slides were prepared with fungi that were just beginning to sporulate, mounted in lactophenol cotton blue and microscopically examined. The identification was done based on the taxonomic keys described by [Domsch *et al.* \[1980\]](#).

Type of fungi isolated using all different media and techniques were pooled for each individual subsection of the cores and diversity measurements for each of these samples were calculated using the software PRIMER v5 [[Clarke & Gorley, 2001](#)]. The results were expressed as species richness, Pielou's evenness index and Shannon Wiener diversity index. These in turn express the richness of biodiversity in each sample, the extent of even distribution of different species and proportion among total counts respectively, in different depths of sediment cores. A population is said to have high diversity if it has many species and their abundances are fairly even. Conversely, diversity is low when the species are few and their abundances uneven. Since diversity depends on two independent properties of a population, ambiguity is inevitable; thus a population with few species and high evenness could have the same diversity as another population with many species and low evenness [[Pielou, 1977](#)]

4.3.6 Comparison of the deep-sea fungi with terrestrial isolates

The cultures isolated from the deep-sea sediments by different methods of isolation were identified to be similar to terrestrial species of fungi or “geofungi” a term coined by [Miller & Whitney \[1981\]](#). To check the uniqueness of the cultures, these cultures were compared with similar terrestrial cultures obtained from culture collection at IMTECH, Chandigarh, India. The cultures were compared with respect to their growth, morphology and nutrient requirements. The ability of the cultures to grow in medium prepared with seawater and the amount of biomass produced by these at different salinities was compared. The growth morphology was compared by growing these on different solid media like malt extract agar, Czapek Dox agar and corn meal agar. The ability of cultures to grow at varying temperatures was also compared. The biochemical characters of these cultures were compared with respect to different C-source utilization.

4.3.7 Characterization of the yeast cultures

The yeast cultures were identified based on their biochemical and morphological characters using a software IDENT. For the biochemical characterization, all the cultures were not used. The ITS region of the eight similar looking cultures were amplified and subjected to restriction digestion (described in details later). Based on the restriction digestion patterns, only 3 of these and one filamentous yeast culture were used for studying the biochemical characters in detail.

4.3.8 Differentiation of cultures based on molecular methods

In this study, RFLP (Restriction Fragment Length Polymorphism) was used to describe the diversity of the isolated cultures and to compare with the terrestrial known cultures. REA (Restriction Enzyme Analysis) of ITS products was also carried out for differentiation of the isolates.

4.3.8.1 DNA extraction

DNA was extracted from the cultures in their log phase of growth. DNA was extracted from the frozen biomass using the method described by [van Burik *et al.* \[1998\]](#) ([Appendix A - I](#))

The DNA concentration was estimated spectrophotometrically by measuring absorbance at 260 nm and the concentration was calculated from the standard curve plotted using pure DNA (HiMedia, Cat. RM 511) ([Appendix A - II](#)). The purity of the extracted DNA was also determined by calculating the 260/280 ratios.

4.3.8.2 PCR-RFLP

Restriction digestion was carried out for the deep-sea isolates and the similar terrestrial species procured from IMTECH. The genomic DNA was used for amplification of the ITS 1 and ITS 2 regions flanking either sides of 5.8s rRNA.

Table 4.2 Deep sea and terrestrial cultures used for comparative studies

Culture	Deep-sea culture	Terrestrial IMTECH culture
<i>Aspergillus terreus</i>	# A4634	MTCC 279 MTCC 479
<i>Aspergillus ustus</i>	# NIOCC 20	MTCC 2200
<i>Aspergillus sydowii</i>		MTCC 635

Two primers used for the amplification were pITS1 and pITS4 [White *et al.*, 1990]. The reaction volume was 50 μ L containing 1.5 mM MgCl₂, 7.5 picomoles of primers, 20 mM of dNTP mix, 50 ng of genomic DNA and 3 U of Taq polymerase. The reaction mixture was subjected to amplification in MJ Research thermocycler (U.S.A.) using the following temperature profile:

Denaturation	-	94°C for 5 minutes
35 cycles of	-----	
Denaturation	-	94°C for 30 seconds
Primer annealing	-	57°C for 30 seconds
Amplification	-	72°C for 90 seconds
Final extension	-	72°C for 10 minutes.

The reaction was stopped by chilling the mixture at 4°C.

The amplified product was purified using Sigma PCR gel purification kit (Cat.NA 1111-1KT). The purified product was then subjected to restriction digestion using the restriction enzymes Hinf III and Msp I. Around 500 ng of purified PCR product was used along with 0.5 U of these two enzymes at 37°C for 60 min. The digestion was stopped by inactivating the enzymes at 80°C for 20 minutes. Five μ L of each of the digestion mixture was loaded on a 1.5 % agarose gel in 40 mM Tris-acetate, 1 mM EDTA pH 8.0 (TAE) electrophoresis buffer and containing 0.5 mg mL⁻¹ ethidium bromide. The restriction pattern obtained was scanned using Alpha Imager gel documentation system.

4.3.8.3 Molecular phylogeny

The gel purified ITS products of A 344, BC7E1, A 61P35 and Y8 were sequenced at Microsynth Inc., USA. Partial sequences for BC7E1, A 61P35 and Y8 were obtained, but the sequencing failed for A 344 in spite of repeating many times. With these partial sequences, phylogenetic tree was constructed using NJ Plot [Perrière & Gouy, 1996].

4.4 RESULTS

4.4.1 Isolation of fungi

The fungi were isolated from the deep-sea sediments by all the three techniques used. The particle plating method, being a selective method, yielded the lowest number of

Table 4.2 Number of fungi isolated by various techniques

Source		Particle plating	Dilution plating	Pressure incubation* (300 bar/5°C)
Deep sea	Total number of sediment samples used	416	72	224
	Number of fungi recovered	94	28	88
	% recovery	23	39	39
Shallow water	Total number of sediment samples used	16		Not done
	Number of fungi recovered	26		
	% recovery	163		

*The sediment immediately after retrieval from deep-sea was incubated in dilute nutrient at 300 bar pressure and 5°C for 20 days. Fungi were isolated from these sediments using particle plating technique at 1 bar pressure at 5-7°C.

fungi while dilution plating and pressure enrichment resulted in almost similar recovery of fungi (Table 4.2). The highest number of species was often obtained at 0 - 2 cm depth of deep-sea sediment cores, while the numbers were much less below 25 cm depth (Table 4.3). *Aspergillus* species were the dominant fungi isolated followed by non-sporulating and unidentified sporulating fungi. A one-way analysis of variance (ANOVA) comparing the number of species isolated from different subsections of each core showed that differences between the subsections were not significant (F value 0.34, P-value 0.97, d.f. 10,143). Species richness, Pielou's evenness and Shannon index values were more or less similar up to 20–25 cm depth, after which there was a reduction. Species richness is a measure related to the total number of species present. Hence a sample containing more species than another will be more diverse. Pielou's evenness index (J') gives the equitability of the species that expresses how evenly the individuals are distributed among the different species.

$$J' = H' \text{ (observed)} / H'_{\text{max}}$$

where H'_{max} is the maximum possible diversity, which would be achieved if all the species were equally abundant.

Shannon index takes into account both the species richness and evenness of the species.

Among the various media used for isolation of fungi, MEA and MEB followed by CMA were found to be better than the other media used. None of the media were selective for isolating specific fungi (Table 4.4). Details of the media used and the isolation for some of the fungi are shown in Table 4.5.

Table 4.3 Percentage distribution of fungi at different depths in the sediment core

Genera	Sub-surface depths (cm)										
	0-2 a	2-4 b	4-6 c	6-8 d	8-10 e	10-15 f	15-20 g	20-25 h	25-30 i	30-35 j	35-40 k
<i>Aspergillus sp.</i>	2 (8)	2 (11)	1 (5)	4 (25)	4 (29)	2 (12)	1 (6)	3 (14)	2 (18)	7 (54)	1 (13)
<i>Aspergillus terreus</i>	7 (28)	1 (5)	3 (15)	2 (13)	1 (7)	3 (18)	3 (19)	3 (14)	1 (9)	1 (8)	-
<i>Aspergillus restrictus</i>	3 (12)	5 (26)	2 (10)	3 (19)	3 (21)	2 (12)	2 (13)	2 (9)	-	-	-
<i>Aspergillus sydowii</i>	-	1 (5)	1 (5)	1 (6)	1 (7)	1 (6)	-	-	-	-	-
<i>Penicillium sp.</i>	2 (8)	1 (5)	2 (10)	-	1 (7)	1 (6)	2 (13)	2 (9)	-	-	-
<i>Cladosporium sp.</i>	1 (4)	-	3 (15)	-	1 (7)	1 (6)	2 (13)	1 (5)	-	2 (15)	-
<i>Curvularia sp.</i>	1 (4)	-	-	-	-	-	-	-	-	-	-
<i>Fusarium sp.</i>	-	-	-	-	-	-	-	-	-	-	1 (13)
Non-sporulating fungi	4 (16)	2 (11)	2 (10)	2 (13)	1 (7)	3 (18)	1 (6)	4 (18)	4 (36)	2 (15)	1 (13)
Unidentified sporulating fungi	4 (16)	6 (32)	5 (25)	3 (19)	2 (14)	4 (24)	5 (31)	5 (23)	4 (36)	1 (8)	5 (63)
Unidentified Ascomycetes	-	-	-	-	-	-	-	1 (5)	-	-	-
<i>Aureobasidium sp.</i>	1 (4)	-	-	-	-	-	-	-	-	-	-
Unidentified yeasts	-	1 (5)	1 (5)	1 (6)	-	-	-	1 (5)	-	-	-
Total species	25	19	20	16	14	17	16	22	11	13	8
Total sediment sections used	56	56	56	56	56	56	56	55	50	42	20
Shanon index	1.99	1.81	2.06	1.84	1.91	1.96	1.80	2.05	1.26	1.30	1.07
Pielou's evenness	0.90	0.87	0.94	0.95	0.92	0.94	0.93	0.93	0.91	0.81	0.77
Species richness	2.49	2.38	2.67	2.16	2.65	2.47	2.16	2.59	1.25	1.56	1.44

Cumulative frequency of fungi isolated from 3 cruises, AAS 34, AAS 46 and AAS 61 in the Central Indian Basin using various techniques. The numbers represent the frequency of occurrence of various genera in different subsections of sediment cores collected during all the 3 cruises. The numbers within brackets represent percentage frequency occurrence calculated as = total number of sediment showing presence of a particular fungus/ total number of sediment sections showing presence of fungi x 100.

1-way ANOVA between the numbers of species present in different sub-sections of the cores was not significant (F value 0.34, P-value 0.97, df= 10,143).

Shannon index, Pielou's evenness and species richness were calculated for different depths using the software PRIMER v5 (1994) of Plymouth Marine Laboratory, U.K. These values further show almost homogeneous distribution of fungi up to 20-25 cm depth, after which there is a marked reduction.

Table 4.4 Details of the media used for the isolation of fungi from the deep –sea sediments

	AAS 34		AAS 46			
	Dilution Plating		Dilution Plating	Particle plating	Pressure incubation	
	MEA	ZMA	MEA	MEA	MEB	Seawater MEA
<i>Aspergillus</i> sp.	5	2	1	4	2	-
<i>Aspergillus terreus</i>	-	-	1	1	5	4
<i>Aspergillus restrictus</i>	-	-	-	-	-	-
<i>Aspergillus sydowii</i>	-	-	-	-	2	1
<i>Penicillium</i> sp.	3	1	-	-	-	1
<i>Cladosporium</i> sp.	3	-	-	-	1	-
<i>Curvularia</i> sp.	-	1	-	-	-	-
<i>Fusarium</i> sp.	-	-	-	-	-	-
Non-sporulating fungi	2	3	-	4	-	-
Unidentified sporulating fungi	4	4	-	-	3	-
Unidentified Ascomycetes	-	-	-	-	-	-
<i>Aureobasidium</i> sp.	-	-	-	1	-	-
Unidentified yeasts	-	-	-	-	-	-

	AAS 61									
	Particle Plating					Pressure Incubation				
	ZMA	CMA	MEA	CDA	SA	MEB	Seawater			
						MEA	CMA	SA	Artlarvae	
<i>Aspergillus</i> sp.	1	2	5	-	4	3	-	-	-	-
<i>Aspergillus terreus</i>	-	1	1	1	1	4	-	4	-	2
<i>Aspergillus restrictus</i>	-	1	1	-	-	15	-	2	1	2
<i>Aspergillus sydowii</i>	-	-	-	-	-	-	-	1	-	1
<i>Penicillium</i> sp.	-	-	2	-	1	2	-	1	-	-
<i>Cladosporium</i> sp.	-	1	2	-	-	4	-	-	-	-
<i>Curvularia</i> sp.	-	-	-	-	-	-	-	-	-	-
<i>Fusarium</i> sp.	-	1	-	-	-	-	-	-	-	-
Non-sporulating fungi	-	2	4	-	4	4	-	1	-	2
Unidentified sporulating fungi	1	2	7	5	3	8	1	4	-	2
Unidentified Ascomycetes	-	-	1	-	-	-	-	-	-	-
<i>Aureobasidium</i> sp.	-	-	-	-	-	-	-	-	-	-
Unidentified yeasts	-	-	-	-	-	3	-	-	-	1

Table 4.5 Isolation details of some of the fungi

Isolate #	Fungi	Cruise #	Latitude (South)	Longitude (East)	Depth (m)	Sub surface depth (cm)	Core and section #	Method of isolation	Medium used
A 4637	<i>A. terreus</i>	AAS 46	10° 01'	75° 59'	5305	0 - 2	BC 4A	PI	MEB
A 4636	<i>A. terreus</i>	AAS 46	10° 01'	76° 00'	5400	0 - 2	BC 12A	PI	MEB
A 4634	<i>A. terreus</i>	AAS 46	10° 01'	76° 00'	5400	15 - 20	BC 12G	PI	MEB
A 4633	<i>A. terreus</i>	AAS 46	10° 01'	76° 00'	5400	10 - 15	BC 12F	PI	MEB
A 4630	<i>Aspergillus</i> sp.	AAS 46	10° 02'	76° 00'	5296	4 - 6	BC 7C	PI	MEB
A 4628	<i>A. terreus</i>	AAS 46	10° 01'	76° 00'	5400	8 - 10	BC 12E	PI	MEB
A 61 P10	<i>A. terreus</i>	AAS 61	14° 00'	75° 30'	5145	20 - 25	BC 14H	PI	MEB
A 61 P4	Unidentified	AAS 61	11° 59'	76° 29'	5280	0 - 2	BC 3A	PI	Artemia
A 4625	Unidentified	AAS 46	10° 02'	76° 00'	5296	8 - 10	BC 7E	PI	MEB
A 614	<i>A. terreus</i>	AAS 61	13° 00'	73° 30'	4810	10 - 15	BC 17F	PP	SDA
A 6137	Unidentified	AAS 61	10° 01'	76° 00'	5280	15 - 20	3MBC 5G	PP	CMA
A 3457	<i>Fusarium</i> sp.	AAS 34	10° 00'	76° 01'	5294	15 - 20	BC 3G	PP	MEA
A 3449	<i>Fusarium</i> sp.	AAS 34	10° 03'	76° 01'	5294	20 - 25	BC 5H	PP	MEA
A 3441	Unidentified	AAS 34	10° 03'	76° 01'	5294	8 - 10	BC 5E	PP	MEA
A 348	Non-sporulating	AAS 34	10° 10'	76° 05'	5250	8 - 10	BC A ₁ /B E ₁	DP	ZMA
A 3428	<i>Curvularia</i> sp.	AAS 34	10° 09'	75° 21'	5180	0 - 2	BC 8A	DP	ZMA
A 61 P63	Yeast	AAS 61	10° 59'	73° 29'	5100	20 - 25	BC 23H	PI	Artemia
A 3426	Unidentified	AAS 34	10° 02'	76° 00'	5280	8 - 10	BC 14E ₁	DP	MEA
A 6136	<i>Aspergillus</i> sp.	AAS 61	10° 02'	76° 01'	5320	20 - 25	3MBC 11H	PP	MEA
A 6139	Unidentified	AAS 61	10° 01'	76° 00'	5280	15 - 20	3MBC 12G	PP	CDA
A 61 P64	Yeast	AAS 61	15° 00'	74° 30'	5390	8 - 10	BC 12E	PI	MEB
A 6128	<i>Aspergillus</i> sp.	AAS 61	12° 59'	75° 29'	5070	30 - 35	BC 19J	PP	CMA
A 6126	<i>Aspergillus</i> sp.	AAS 61	12° 59'	75° 29'	5070	30 - 35	BC 19J	PP	SDA
A 3415	Unidentified	AAS 34	10° 09'	75° 21'	5180	25 - 30	BC 8I ₁	DP	ZMA
A 3412	<i>Aspergillus</i> sp.	AAS 34	10° 09'	75° 21'	5180	30 - 35	BC 8J ₂	DP	ZMA

PI - Pressure Incubation, PP - Particle Plating, DP - Dilution Plating, MEB - Malt Extract Broth, MEA - Malt Extract Agar, ZMA - Zobell Marine Agar, CDA - Czapek Dox Agar, SDA - Sabourauds Dextrose Agar, Artemia - Autoclaved *Artemia* larvae suspended in seawater

4.4.2 Morphology

Many of the cultures showed abnormal morphology immediately after isolation. These showed extremely long conidiophores with vesicles being covered by long hyphae, instead of phialides or metulae or conidia, as is typical of the genus *Aspergillus* (Fig 4.5 a - f). Most of these were later identified as belonging to *Aspergillus* species, when these abnormal features disappeared.

4.4.3 Direct detection

The observation of fungi under the microscope was made easy by the calcofluor staining. Fungal hyphae as well as spores (Fig. 4.6 a - e) were seen in both the enriched sediments from the pressure incubation and directly from the deep-sea sediments. A total of 35 and 13 out of 165 and 90 sediment samples collected during the cruises # AAS 61 and # AAS 46, respectively, showed presence of fungi by this method.

4.4.4 Immunofluorescent staining

Sediment samples of the core # BC 12 (subsection 15–20 cm, Table 4.5) stained with FITC-tagged antiserum against *A. terreus* # A 4634 revealed fluorescing hyphae (Fig. 4.7 a - d). An unidentified organic particle in these sediments was densely colonized by the fungus. These showed positive fluorescent reaction to the antiserum (Fig. 4.7 e). The antiserum did not react with other fungi thus showing specificity for the fungus against which the antibodies were raised in rabbits. The fungus showed positive fluorescence after growth at 1 and 200 bar / 5° and 30°C.

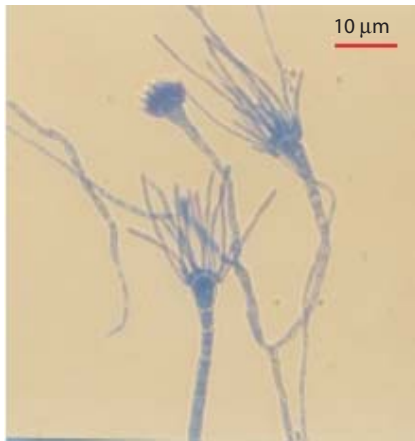
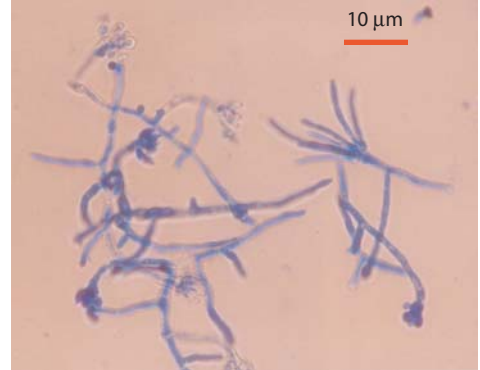
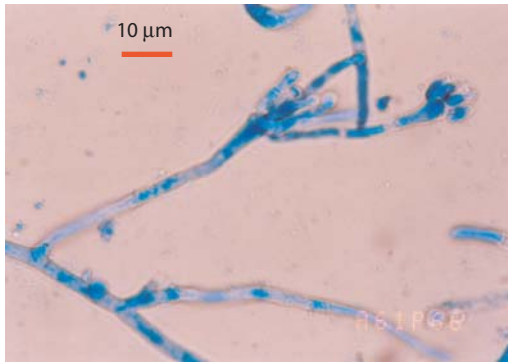
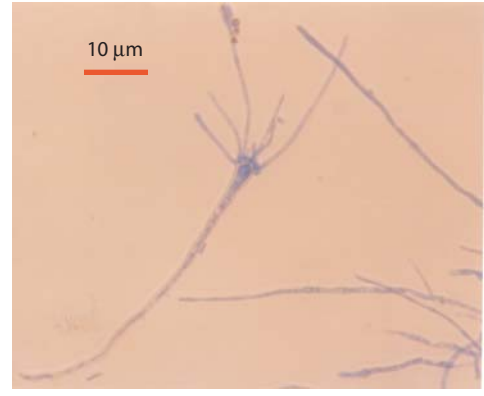
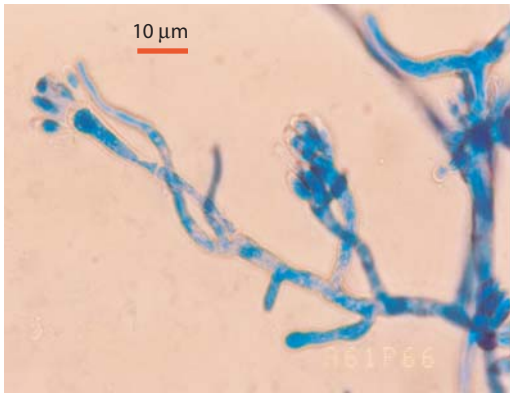
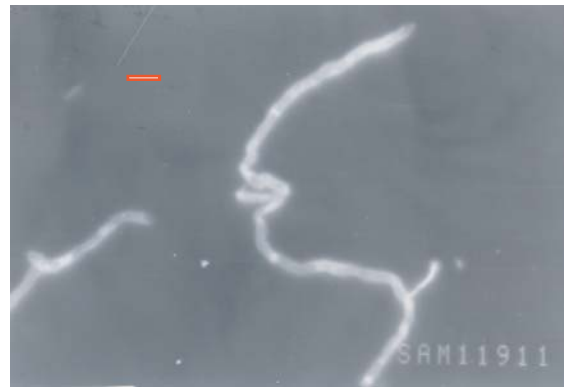


Fig. 4.5 Abnormal morphological features seen in the deep sea fungal cultures just after isolation from the deep sea sediments

(a)



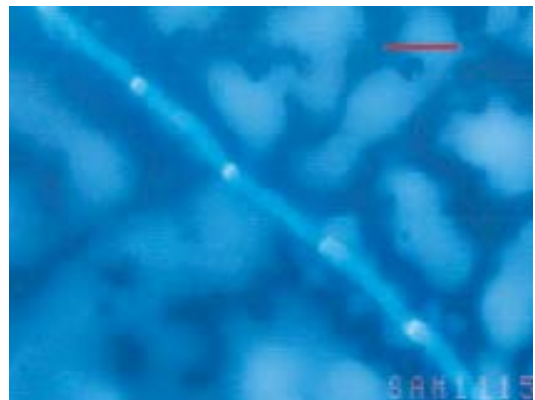
(b)



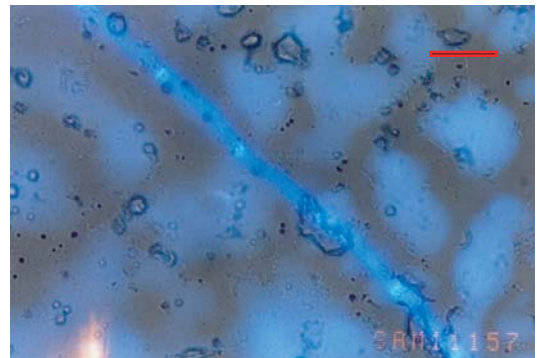
(a) & (b) Epifluorescence microscopy photograph of calcofluor stained hyphae from deep sea sediments. Bar equals 10 μm .



(c) An epifluorescence photomicrograph of a calcofluor-stained geminating spore from deep sea sediment incubated under 400 bar hydrostatic pressure and 5°C. Bar equals 10 μm .

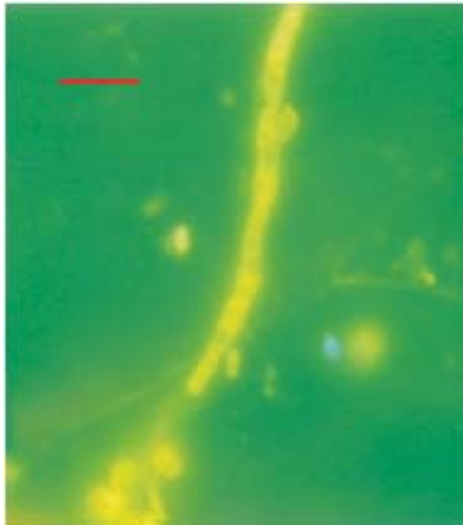


(d) Epifluorescence microscopy photograph of Calcofluor stained hyphae from deep sea sediments. Bar equals 10 μm .



(e) Photomicrograph of the same hypha as seen under partial bright field and fluorescence light. Bar equals 10 μm .

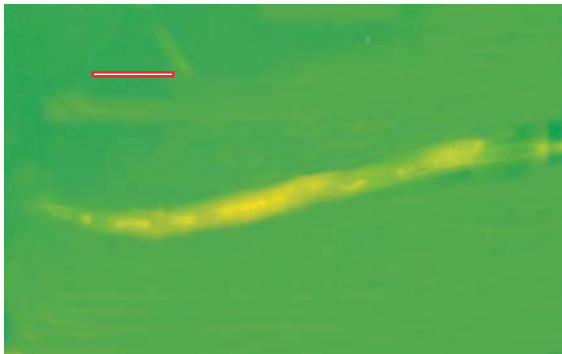
Fig. 4.6 Visualization of fungal hyphae in the deep-sea sediment stained with Calcofluor under an epifluorescence microscope.



(a) Hypha showing immunofluorescence
From the sediment. Bar is equal to 10 μm



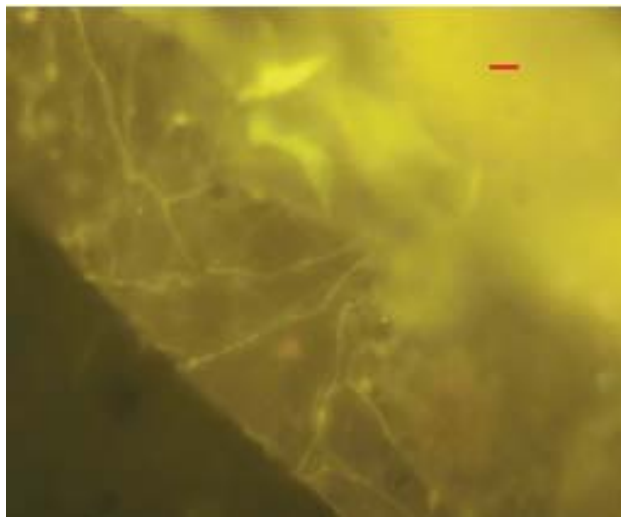
(b) Bright field photomicrograph of the same
Hypha as in (a). Bar is equal to 10 μm



(c) Hypha showing immunofluorescence
from the sediment. Bar is equal to 10 μm



(d) Bright field photomicrograph of the same
Hypha as in (c). Bar is equal to 10 μm



(e) Immunofluorescence detection of a dense cluster
of hyphae of *Aspergillus terreus* on an organic
matter from the deep sea sediment

Fig. 4.7 Detection of hyphae of *Aspergillus terreus* (isolate # A4634) from the deep sea sediments using immunofluorescence staining method

4.4.5 Comparison of deep sea and terrestrial cultures

The deep sea and the terrestrial isolates of *Aspergillus terreus* obtained from IMTECH were showing almost similar morphological characteristics on all the three solid media used. Initially the IMTECH cultures did not grow well in seawater medium, but after repeated subculturing, the growth was equally good as that of the deep-sea isolates.

Out of the 35 different C - sources used, both the deep-sea and IMTECH *Aspergillus terreus* strains showed a few differences. The two IMTECH *Aspergillus terreus* strains showed differences amongst themselves as well (Table 4.6).

Table 4.6 Carbon source utilization of the deep sea and terrestrial fungal isolates

S.No.	Carbon source	A 4634	MTCC 279	MTCC 479	NIOCC20	MTCC 2200	CH2	MTCC 635
1	Lactose	+	+	+	-	+	+	-
2	Xylose	+	+	+	D	+	+	+
3	Maltose	+	+	+	+	-	-	+
4	Fructose	+	+	+	D	-	-	-
5	Dextrose	+	+	+	+	+	-	+
6	Galactose	+	+	+	+	D	D	+
7	Raffinose	+	+	+	+	-	-	+
8	Trehalose	+	+	+	+	-	-	+
9	Melibiose	+	+	+	+	+	-	+
10	Sucrose	+	+	+	+	-	-	+
11	L-arabinose	+	+	+	-	+	+	+
12	Mannose	+	+	+	D	+	+	+
13	Inulin	-	-	-	D	-	-	-
14	Sodium gluconate	-	-	-	-	-	-	-
15	Glycerol	-	D	-	+	-	-	D
16	Salicin	-	-	-	-	-	-	-
17	Glucosamine	D	D	D	-	-	-	D
18	Dulcitol	-	-	-	-	-	-	-
19	Inositol	-	-	-	-	-	-	-
20	Sorbitol	-	-	-	-	-	-	-
21	Mannitol	-	-	+	-	-	-	-

22	Adonitol	-	-	-	-	-	-	-
23	α -methyl-D-glucoside	-	-	-	-	-	-	-
24	Ribose	+	+	+	-	+	+	D
25	Rhamnose	D	-	-	-	-	+	-
26	Cellobiose	+	-	+	-	-	-	D
27	Melezitose	-	-	-	-	-	-	-
28	α -methyl-D-mannoside	-	-	-	-	-	-	-
29	Xylitol	-	-	-	-	-	-	-
30	ONPG	+	-	+	+	+	-	+
31	Esculin	+	+	+	+	+	-	+
32	D – arabinose	-	-	-	-	-	D	-
33	Citrate	+	+	+	+	+	+	+
34	Malonate	+	+	+	+	+	+	+
35	Sorbose	-	-	-	-	-	-	-

Key : + positive result
 - negative result
 D doubtful result

4.4.6 Deep-sea Yeasts

RFLP of ITS region showed two clear groupings. The four isolates obtained directly from the deep-sea sediments showed similar restriction pattern and the remaining 4 isolates obtained from the sediment pore water a different pattern (Fig. 4.8 a, b). Based on these results, two yeast cultures from sediment and one from the pore water were studied for their biochemical characters. Out of these 4 isolates, three isolates showed almost similar biochemical characters (Table 4.7), based on which they were assigned to *Rhodotorula muciliginosa*. The filamentous yeast was identified to be species *Cryptococcus albidus* or *Cryptococcus curvatus*.

Fig. 4.8

(a) ITS products of all the 8 cultures

(b) Restriction digestion pattern of the ITS products

1,2 - uncut PCR product, 3 - A61P35, 4 - A61P63, 5 - A61P64, 6 - BC7E1, 7 - Y5, 8 - Y6, 9 - Y7, Y10 - Y8, M - 2 kb marker.

A61P35, A61P63, A61P64 & BC7E1 - isolates obtained from sediments

Y5, Y6, Y7 & Y8 - isolates obtained from sediment pore water

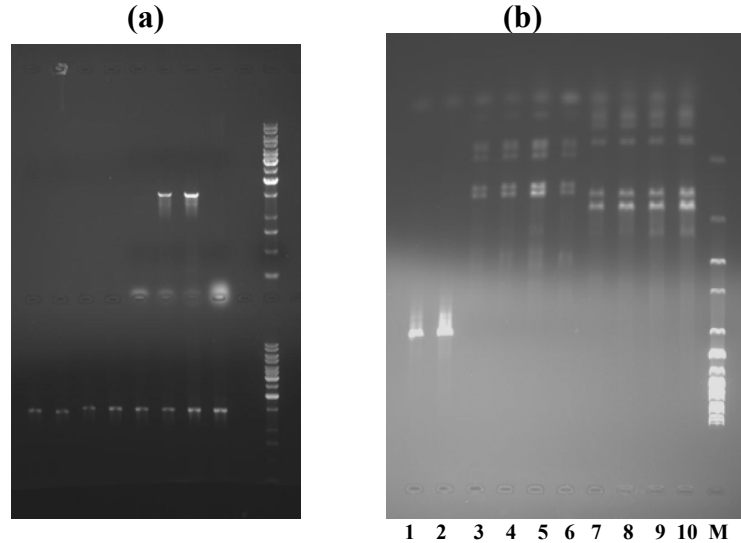


Table 4.7 Morphological and biochemical characters of the four yeast isolates

S. No.	Tests	A 344	BC 7 E1	A61P35	Y8
A. Fermentation					
0	YNB without glucose	-	-	-	-
1	D – glucose	+	-	-	-
2	D – galactose	-	-	-	-
3	Maltose	-	-	-	-
4	Me - α - D – glucoside	-	-	-	-
5	Sucrose	-	-	-	-
6	α, α - trehalose	-	-	-	-
7	Melibiose	-	-	-	-
8	Lactose	-	-	-	-
9	Cellobiose	-	-	-	-
10	Melizitose	-	-	-	-

11	Raffinose	-	-	-	-
12	Inulin	-	-	-	-
13	Starch	-	-	-	-
B. Carbon source utilization					
14	D – galactose	+	+	+	?
15	L – sorbose	+	?	+	-
16	D – glucosamine	?	-	?	-
17	D – ribose	?	+	+	?
18	D – xylose	+	+	+	+
19	L – arabinose	+	+	+	-
20	D – arabinose	-	+	+	?
21	L – rhamnose	-	-	-	-
22	Sucrose	+	+	+	+
23	Maltose	+	+	+	-
24	α,α - trehalose	-	-	-	-
25	Me - α - D- gluoside	-	-	-	-
26	Cellobiose	-	-	-	+
27	Salicin	?	-	-	+
28	Melibiose	?	-	-	-
29	Lactose	?	-	-	-
30	Raffinose	+	+	+	+
31	Melizitose	+	+	+	-
32	Inulin	?	-	-	?
33	Starch	?	-	-	-
34	Glycerol	+	?	-	?
35	Erythritol	+	-	-	-
36	Ribitol	+	-	-	-
37	Xylitol	+	+	-	+
38	L – arabinitol	?	-	-	+
39	D – glucitol	+	?	+	+
40	D – Mannitol	+	?	+	+
41	Galactitol	?	-	-	+
42	Myo – inositol	+	-	-	-
43	D - glucono - 1,5 -lactone	+	?	?	+
44	2 - keto - D - gluconate	?	?	?	?

45	3 - keto - D - gluconate	?	?	?	?
46	D – gluconate	+	+	+	+
47	D – glucoronate	+	+	+	+
48	DL – lactate	+	+	+	+
49	Succinate	+	-	-	-
50	Citrate	+	-	-	-
51	Methanol	-	?	?	-
52	Ethanol	+	?	?	+
53	YNB + glucose	+	+	+	+
54	YNB	+	-	-	?
C. Growth at					
55	25°C	+	+	+	+
56	30°C	+	+	+	+
57	37°C	+	?	?	?
D. Additional tests					
58	Starch formation	-	-	-	-
59	Acetic acid production	-	-	-	?
60	Urea hydrolysis	-	+	+	+
E. Morphology					
61	Pink colonies	-	+	+	+
62	Budding cells	+	+	+	+
63	Filamentous	+	-	-	-
64	Pseudohyphae	+	-	-	-
65	Septate hyphae	-	-	-	-
66	Arthroconidia	-	-	-	-
67	Ballistoconidia	-	-	-	-
68	Ascospores	-	+	+	+
69	Ascospores - round, oval, conical, reniform	-	+	+	+
70	Ascospores - cap, hat, Saturn, walnut-shaped	-	-	-	-
71	Ascospores - needle-shaped or whip-like	-	-	-	-

Key : + positive result
- negative result
? doubtful result, test not done (the software used for identification needs the input in the form of '?' symbol for any result other than '+' or '-')

4.4.6.1 Physiological and Phylogenetic relationships

The three yeast cultures, BC7E1, A 61P35 and Y8, assigned to *Rhodotorula minuta* as per biochemical characteristics showed variation, in the sense the first two cultures belonged to same species, while the third one was from different species. A BLAST search [Altschul *et al.*, 1997] using the partial ITS amplicons of the three cultures showed a 100 % match with *Rhodotorula mucilaginosa* for BC7E1 and A 61P35, and Y8 with *Rhodospiridium paludigenum*.

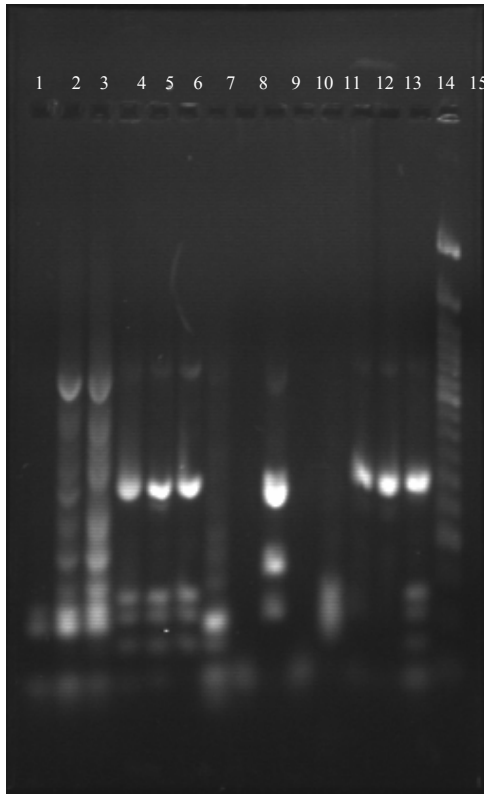
In the phylogenetic tree (Fig. 4.9) constructed using the partial ITS sequences of the three cultures, BC7E1 and A 61P35 were placed in a broad cluster comprising of all *Rhodotorula* and *Rhodospiridium* species supported by a high bootstrap value (99%), while Y8 was placed separately with a *Rhodotorula paludigenum* CBS 6567 AF444493 strain, separated by a boot strap value of 85 % from the first two.

4.4.7 RFLP – Genomic and PCR

The two restriction enzymes used were unable to cut the genomic DNA of all the cultures used, while the ITS product was cut by the enzymes at various positions to give distinct restriction patterns for each culture (Fig. 4.10). *Aspergillus terreus* strains, deep-sea and terrestrial, showed a different profile with Msp I while almost

similar profile with Hinf I for all the 3 strains (lanes 1-6). But, MTCC 479 and A 4634 showed almost same profile for both the restriction enzymes (lanes 2 & 3). *Aspergillus*

Fig. 4.10 Restriction digestion pattern of the ITS products of the deep-sea isolates and the terrestrial cultures by the enzymes Msp I and Hinf I



- | | |
|----|---------------------------|
| 1 | MTCC 279 – Msp I digest |
| 2 | MTCC 479 – Msp I digest |
| 3 | A 4634 – Msp I digest |
| 4 | MTCC 279 – Hinf I digest |
| 5 | MTCC 479 - Hinf I digest |
| 6 | A 4634 - Hinf I digest |
| 7 | MTCC 635 - Msp I digest |
| 8 | CH2 - Msp I digest |
| 9 | MTCC 635 - Hinf I digest |
| 10 | CH2 - Hinf I digest |
| 11 | NIOCC 20 - Msp I digest |
| 12 | MTCC 2200 - Msp I digest |
| 13 | NIOCC 20 - Hinf I digest |
| 14 | MTCC 2200 - Hinf I digest |
| 15 | Marker |

sydowii strains, MTCC 635 and CH2 showed completely different profiles with both the enzymes (lanes 7 – 10). Amongst the two *Aspergillus ustus* strains, MTCC 2200 did not seem to have any restrictions sites for Msp I (lane 12) while NIOCC 20 showed restriction sites for the enzyme (lane 11). In case of Hinf I the case was exactly the opposite, with NIOCC 20 not showing any restriction sites (lane 13) while MTCC 2200 showing almost 4 restrictions sites (lane 14).

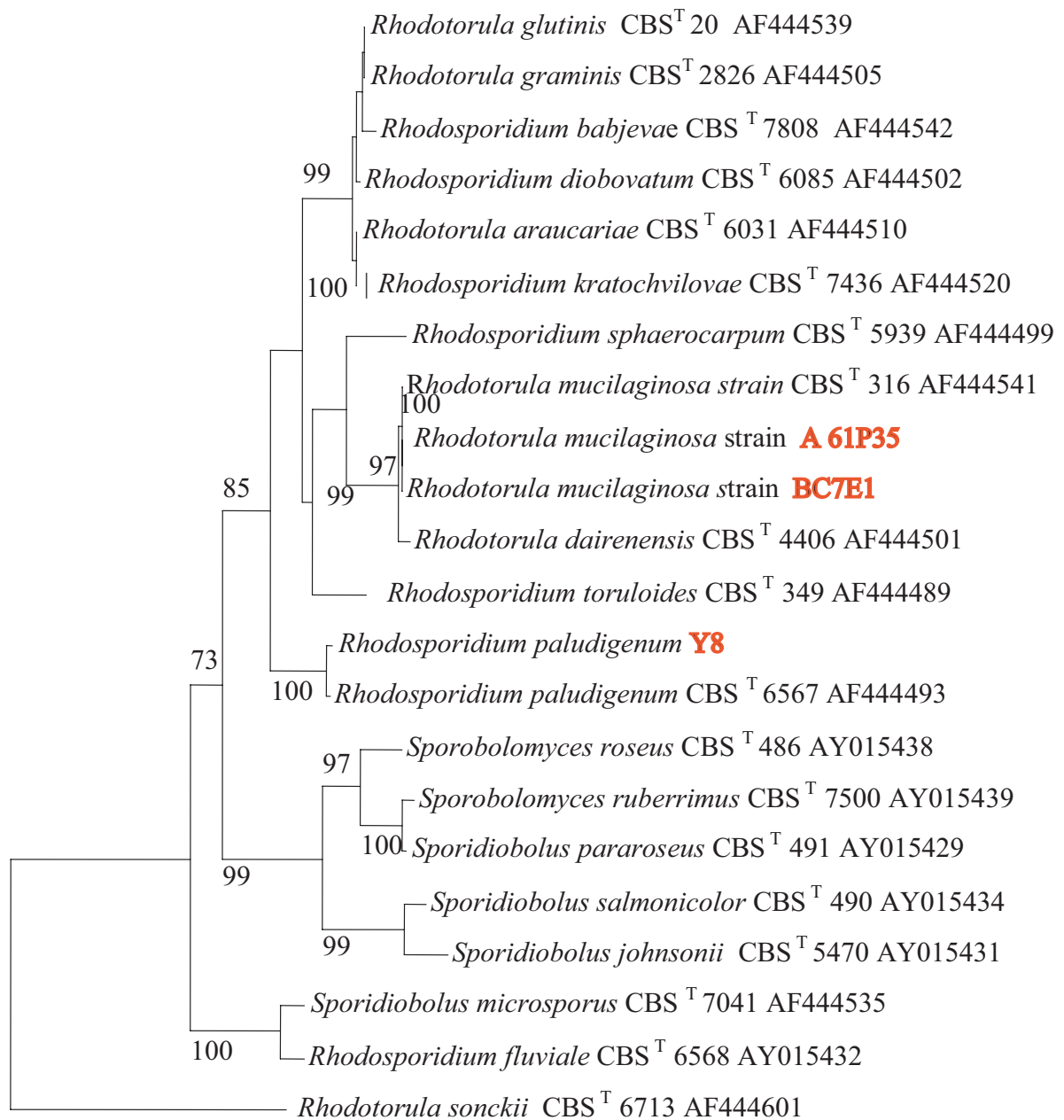


Fig. 4.9 Phylogenetic placement of A 61P35, BC7E1 & Y8 based on the ITS region of the large subunit rRNA gene. The tree was generated by NJPlot. Bootstrap values (Each expressed as a percentage of 1000 replications) greater than 70 % are given at nodes.

Rhodotorula sonckii is used as out group

4.5 Discussion

Studies on fungi in deep-sea sediments are fraught with the danger of contamination by fungal structures (both spores and mycelia) in the sampling devices, as well as from air on deck and in the laboratory. In this study, utmost caution has been exercised to reduce this risk. One of the culture techniques that were used for enhancing recovery of native fungi was the particle plating method, which employs culturing from particles in the 100–200 μm size range, thus considerably removing loose spores [Bills & Polishook, 1994]. Further, sediment samples were placed in a diluted nutrient medium under elevated hydrostatic pressure and low temperature immediately upon recovery of sediments on board and incubated for 30 days under hydrostatic pressure prior to isolating fungi from them. This would minimize possible ‘deep-sea non-adapted’ aerial contaminants. The most direct evidence for the occurrence of fungi in the deep-sea sediments from $\sim 5000\text{m}$ depth was obtained by staining sediment samples with the optical brightener Calcofluor, which enhances fluorescence of cellulose and chitin, the latter being a characteristic fungal cell wall component [Müller & Sengbusch, 1983]. The mycelial state of fungi represents an actively growing vegetative condition within or interspersed among organic particles. The presence of organic particles in the deep-sea sediment samples (Fig. 4.7 e) allows growth of fungal mycelia therein. The total organic matter (TOM) in these sediments was in the range of 4–12 mg g^{-1} dry sediment. Labile organic matter (LOM) comprising carbohydrate, protein and lipids varied from 0.5 to 1.5 mg g^{-1} dry sediment [Raghukumar *et al.*, 2001]. Evidence for the presence of several megafaunal species and faecal casts of benthic animals has been observed in the Central Indian Basin using deep-sea video-photography [Rodrigues *et al.*, 2001]. Hence, presence of fungi

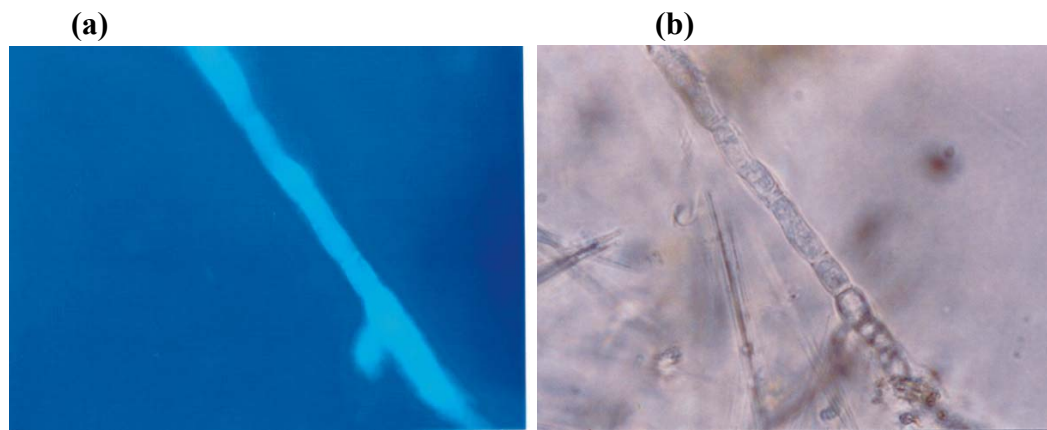
in the deep-sea sediments may be expected in view of the abundant organic material present therein. The frequency of fungal species recovered from various depths in the sediment core was not significantly different (Table 4.3), indicating the homogenous nature of the sediment in the area. Colonies of the terrestrial fungi in the isolations from the sediments might have resulted either from dormant spores or actively growing mycelia. Hence, presuming that the fungi that were found in the deep-sea sediments originated from land, the following sequence of events can be deduced from the experimental and observational approach to understand if such fungi were capable of an active mycelial growth under the elevated hydrostatic pressures and cold temperatures of the deep sea. Fungi may be transported to the sea, both in the form of hyphae growing on organic particles from land and in the form of spores transported to the sea by wind. There are several ways in which fungi can be transported in their mycelial forms to the deep sea. Large particulate organic matter, such as decaying leaves and wood, may be carried offshore and eventually sink. Turner [1973] has described the presence of 'islands of wood' in the deep-sea, which seem to be due to sinking of waterlogged wood washed offshore during monsoons in the tropics or spring runoff in high latitudes. She further speculated that such "persistent but constantly shifting 'islands' of wood might bring in saprophytic species that serve as dispersal centers and contribute to habitat diversity, niche specialization and enrichment". Even substrates of purely oceanic origin, such as marine aggregates in surface waters, may not be devoid of fungi. Fungal colonization of transparent exopolysaccharides (TEPS) collected from coastal Arabian Sea waters (Figs. 4.11 a, b) and also oceanic waters have been observed. [Raghukumar, unpublished data]. Many species of fungi, when transported to the deep-sea from land in the form of hyphae,

may be capable of growth under the prevalent high hydrostatic pressures and low temperature.

Fig 4.11

(a) Epifluorescence microscopy of a fungal hypha in a transparent exopolymeric particle (TEP) collected from 30 m depth in the Arabian Sea. TEP was stained with Calcofluor and photographed under blue light with an epifluorescence microscope. Scale 10 μm .

(b) The same hypha photographed under bright field. Scale 10 μm .



Many of the fungi that reach the sea floor as mycelia may initially be highly stressed by the extreme conditions existing therein. Thus, several fungi showed swellings in their hyphae and other abnormalities when grown at 200 bar/5°C. The abnormalities were much less at 200 bar/30°C, suggesting that the low temperature was more adverse than the high pressure itself. [Lorenz \[1993\]](#) has shown abnormal growth of *A. ustus* under elevated hydrostatic pressure. Those fungi that adapt themselves may eventually be able to grow normally under deep-sea conditions. Thus, the isolate # A 3415, an unidentified fungus, grew in the form of normal hyphae under simulated deep-sea conditions. Use of the immunofluorescence technique revealed normal hyphae of *A. terreus* # 4634 in the deep-sea sediment collected from a depth of 15–20 cm of a core ([Fig. 4.6 a - d](#)).

Aspergilli are physiologically very versatile and are some of the most successful fungi in colonizing a variety of substrates on land [Domsch *et al.*, 1980]. The fungi that were isolated in this study corresponded morphologically to known terrestrial species. However, deep-sea adaptations might have resulted in biochemical and genetic modifications as reported here. The biochemical differences cannot be assigned to specific reasons. This aspect of variability needs to be addressed in the future.

In contrast to the hypothesis of Turner as discussed earlier, based on their studies on *Escherichia coli*, Sato *et al.* [1995] hypothesized that since life might have originated in the deep-sea environment, a high-pressure gene expression system is conserved in living organisms, even if they are presently adapted to atmospheric pressure. Bartlett [2002] also reported that bacterial and archaeal piezophiles in culture are closely related to shallow-water microbes, which are not piezophilic. Thus it appears that “high-pressure selection has not required the evolution of dramatically different lineages of life”. These studies further support the present observation on growth of terrestrial fungi under elevated hydrostatic pressure.

In conclusion, in this study the active presence of fungi in deep-sea sediments by direct detection using Calcofluor stain and immunofluorescence detection using polyclonal antibodies has been observed. The present study confirms the earlier hypothesis put forth by Raghukumar & Raghukumar [1998] that terrestrial fungi blown to the sea surface and sinking to the deep-sea sediments have adapted to the alien environmental conditions. Besides, it also suggests that these are some of the hardest forms that can adapt and survive under the most extreme conditions.