Fungi in deep-sea sediments of the Central Indian Basin

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Abstract: Although a great amount of information is available on bacteria inhabiting deep-sea sediments, the occurrence of fungi in this environment has been poorly studied and documented. We report here the occurrence of fungi in deep-sea sediments from ~5000 m depth in the Central Indian Basin (9-16^{o}S and 73-76^{o}E). A total of 181 cultures of fungi, most of which belong to terrestrial sporulating species, were isolated by a variety of isolation techniques. Species of *Aspergillus* and non-sporulating fungi were the most common. Several yeasts were also isolated. Maximum species diversity was observed in 0-2 cm sections of the sediment cores. Direct staining of the sediments with Calcofluor, a fluorescent optical brightener, revealed the presence of fungal hyphae in the sediments. Immunofluorescence using polyclonal antibodies raised against a deep-sea isolate of *Aspergillus terreus* (# A 4634) confirmed its presence in the form of hyphae in the sub-section from which it was isolated. A total of 25 representative species of fungi produced substantial biomass at 200 bar pressure at 30^{o} as well as at 5^{o}C. Many fungi showed abnormal morphology at 200 bar/5^{o}C. A comparison of terrestrial isolates with several deep-sea isolates indicated that the former could grow at 200 bar pressure when growth was initiated with mycelial inocula. However, spores of a deep-sea isolate *Aspergillus terreus* (# A 4634), but not the terrestrial ones, showed germination at 200 bar pressure and 30^{o}C. Our results suggest that terrestrial species of fungi transported to the deep sea are initially stressed but may gradually adapt themselves for growth under these conditions.

Key words: Deep-sea fungi, hydrostatic pressure, diversity, Central Indian Basin

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1. Introduction

Fungi play a crucial role as saprotrophs in the ecology of terrestrial sediments. They occupy a wide variety of niches on land by virtue of their highly versatile physiological adaptations. One of the least studied habitats of fungi is the deep sea, an environment characterized by low temperature, high hydrostatic pressure and a 'feast and famine' nutrient condition (Morita, 1982; Herbert and Codd, 1986). The presence and ecological importance of deep-sea bacteria has been well recognized ever since Zobell and Morita (1957) isolated bacteria specifically adapted to grow under high pressures and termed them 'barophiles'. Yayanos (1979) obtained barophilic bacteria for the first time in pure culture. Much progress has been made with deep-sea bacteria since then with respect to their diversity (cultured and uncultured), molecular phylogeny, growth and enzyme profiles and pressure-adaptations. In contrast, one of the major groups of eukaryotic microorganisms, the fungi, has largely remained neglected. Roth et al. (1964) isolated marine fungi for the first time from oceanic waters of northwestern subtropical Atlantic Ocean down to a depth of 4450 m. Kohlmeyer and Kohlmeyer (1979) reported obligate marine fungi from wooden panels immersed at depths of 500-3000 m. However, these fungi were not cultured. One of the first reports of fungi in deep-sea sediments was provided by Raghukumar et al. (1992), who isolated fungi from calcareous sediments of the Bay of Bengal at a depth of 965 m and demonstrated germination of spores of *Aspergillus ustus* under simulated deep-sea conditions. Subsequently, cultivation of marine yeasts (Lorenz and Molitoris, 1992) and filamentous fungi and germination of fungal spores (Zaunstőck and Molitoris, 1995) under simulated deep-sea conditions of low temperature and elevated hydrostatic pressure were reported. Takami et al. (1997) showed the presence of fungi and yeasts in sediment samples obtained from the Mariana Trench at a depth of 10,500 m in the Pacific Ocean. These were later identified to be *Penicillium lagena* and *Rhodotorula mucilaginosa*, respectively (Takami, 1999). The presence of fungi based on direct detection and isolation techniques in a 4.7 m long sediment core from the Chagos Trench in the Indian Ocean at a depth of ~5000 m was reported recently (Raghukumar et al., 2004). However, these have been sporadic reports and not comprehensive enough to prove the existence of fungi in deep-sea sediments. We have used

the following approach to address the occurrence and diversity of fungi from deep-sea sediments at an average depth of 5000 m in the Central Indian Basin (CIB): (1) Isolation of fungi by different culturing techniques and their identification; (2) direct detection of fungal hyphae in deep-sea sediments in order to confirm their growth and (3) experiments to study their growth under simulated deep-sea conditions.

2. Methods

2.1. Sampling site and collection of deep-sea sediments

Sediment samples were obtained from depths of 4900 to 5390 m in the Central Indian Basin (9-16^{o}S and 73-76^{o}E) (Fig. 1) on board the Russian research vessel AA Sidorenko during 3 cruises. Samples were collected with an USNEL-type box corer of 50 cm^{3} size. Sampling with a box corer was possible because of the more or less flat topography of the ocean floor in the sampling area. Eleven box core samples were collected during cruise # AAS 34 in April 2001 (10°00'264" to 10°10'364" S; 75°21'000" to 76°05'160" E), 7 during Cruise # AAS 46 in June 2002 (10°00'237" to 10°02'661" S; 75°59'498" to 76°09'822" E) and 38 during cruise # AAS 61 in March 2003 (9°59'861" to 16°00'047" S; 73°29'819" to 76°30'559" E). Sediment at the sampling sites was mainly radiolarian ooze, light to dark brown 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 length were extruded from these sediment cores of ~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 laminar flow hood in the microbiology laboratory on board.

2.2. Isolation of fungi

A portion of the sediment from the middle of each sub-section that had not been in contact with the walls of the PVC cylinder was removed with a flame - sterilized spatula and placed in sterile vials for 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), Sabaurauds dextrose agar (SDA), Czapek Dox agar (CDA) and Czapek Dox broth (HiMedia Pvt. Ltd., India). 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 by the following methods: 1) Dilution plating method, where ~0.1 g of sediment was suspended in sterile seawater, vortexed for 1 min and 100 µl aliquots spread-plated. 2) Particle plating technique (Bills and Polishook, 1994), where approximately 1 g of sediment slurry was sieved successively through a mesh size of 200 µm and 100 µm screens. The particles that passed through 200 µm mesh but were retained on the 100 µm mesh were spread-plated. For both the above techniques, the plates were incubated at 5^{o}C at 1 bar pressure for 15-20 days. 3) Pressure incubation, in which approximately 0.5 g of sediments were placed in sterile plastic bags containing 2 ml of sterile malt extract broth (MEB) and the open ends of the bags sealed with an electrical sealing machine (Quickseal, Sevana, India). The bags were placed in a deep-sea culture vessel (Tsurumi & Seiki Co., Japan) filled with sterile water and pressurized to 300 bar pressure. The pressure vessels were immediately placed at 5^{o}C and incubated for 30 days. At the end of this incubation period, 100 µl of the sediment was spread-plated on nutrient media and the plates were incubated at 1 bar pressure and 30^{o}C until fungal colonies appeared (within 8-10 days). Three replicate plates were maintained for each sediment sample, medium and isolation technique. Media plates were exposed to air for 10 minutes on the deck of the research vessel where the cores were received, the microbiology laboratory on board and the laminar flow inoculation hood to check for the presence of aerial contaminants. This was repeated during every sampling station.

Fungi isolated from the deep-sea sediments were subcultured and maintained on MEA slants at 5^{o}C. Slides of fungi were prepared in lactophenol cotton blue and microscopically examined prior to photomicrography and identification using the taxonomic keys (Domsch et al., 1980). Species 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 was calculated using the software PRIMER v5 (Clarke and Gorley, 2001). The results are expressed as species richness, Pielou's evenness index and Shanon Wiener diversity index (log2). 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.

For comparison, fungi were also isolated from sediments collected from shallow coral reef slopes at a depth of 30 m off Lakshadweep Island Kavaratti ($10^{o}35'$ N and $72^{o}39'$ E) in the Arabian Sea by the particle plating technique. Two isolates of *Aspergillus terreus* (MTCC 279 & MTCC 479) and one *Aspergillus sydowii* (MTCC 635) culture isolated from terrestrial environments obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India) were included in this study for comparison.

2.3. Direct detection of fungi in deep-sea sediments

About 0.5 g of each sediment sample in sterile vials were fixed with 5% formalin solution and stored at $5^{o}C$ for direct detection of fungi according to the method described by Mueller and Sengbusch (1983). Aliquots of these fixed sediments were stained with 0.5% solution of sterile-filtered Calcofluor, an optical brightener (Sigma Chemicals, USA). The excess stain was washed off by centrifugation with sterile seawater. Microscopic mounts of the sediment were then examined under ultraviolet light filter (excitation wave length 330 to 385 nm and barrier filter BA 420) of an epifluorescence microscope (Olympus BX 60, Tokyo, Japan) to detect fluorescing fungal hyphae. Fungal hyphae and spores were photographed with a digital camera (Olympus 4.1 Mp, Tokyo, Japan). Several sediment samples were scanned for the presence of fungi after each cruise. In addition, sediments with 0.5 ml sterile seawater were vortexed after addition of a drop of sterile detergent solution. The foam formed on the surface of trapped sediment material was pipetted out onto a sterile glass slide, stained and examined microscopically as described above.

2.4. Detection of Aspergillus terreus Thom (isolate # A4634) in deep-sea sediments by immunofluorescence:

The immunofluorescence technique, which has been widely used to detect specific fungi in terrestrial substrates (Jellison and Goodell, 1988; Friese and Allen, 1991; Banks et al., 1993), was employed for the purpose. Antibodies were raised commercially for *Aspergillus 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 to 20 cm during the cruise AAS 46 (10°01' S; 76°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 and 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 µg 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^{o}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^{o}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.

2.5. Growth of fungi under elevated hydrostatic pressure

Representative fungi isolated by different techniques and with different nutrient media were examined for spore germination and mycelial growth under elevated hydrostatic pressures by the following methods: 1) The selected fungi were grown in MEA plates at 1 bar pressure and 30^{o}C temperature, and the spores were collected by gently flooding the plates with sterile sea water. The spore suspension was appropriately diluted after haemocytometer counts, inoculated in malt extract broth (MEB) fortified with 1% glucose and 0.1% Tween 80 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 filled with sterile water and pressurized to 200 bar pressure and incubated at 30^{o} and 5^{o}C. Similarly prepared pouches were incubated at 1 bar pressure at 30^{o} and 5^{o}C for comparison. After 3 days of incubation, the deep-sea culture vessels were decompressed gradually (at the rate of 50 bar/15 min), and the percentage of germinating conidia was by counting in 20 microscope fields. For comparison, two isolates of *Aspergillus terreus* (MTCC 279 & MTCC 479) and one *Aspergillus sydowii* (MTCC 635) culture isolated from terrestrial environments obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India) were included in this study. 2) For raising mycelial biomass, cultures of 25 deep-sea fungi were grown in MEB for 3 days at 1 bar and 30^{o}C. Vegetative mycelium prior to the onset of sporulation was homogenized with sterile glass beads. A known weight of the finely broken mycelial suspension was inoculated in 20 ml MEB and incubated at 30^{o} and 5^{o}C/200 bar pressure as described above. 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). A similar experiment

was carried out to compare growth of 8 deep-sea, 3 terrestrial and 3 shallow water fungi. The experiments were carried out with 5 ml of MEB medium.

Fungi grown under high hydrostatic pressure at 5^{o} and 30^{o}C were stained with Calcofluor and lactophenol cotton blue, and their morphology under different culture conditions was recorded by photomicrography.

2.6. Gradual adaptation to growth under elevated hydrostatic pressure:

A total of 109 of the 181 isolates obtained in this study failed to grow at 300 bar pressure. An experiment was carried out to examine if these could be gradually acclimatized to growth at elevated pressures. The cultures were initially grown at 50 bar/30°C for 20 days, after which the pressure vessel was decompressed and the bags brought back to 1 bar pressure and checked for growth and viability upon culturing. Cultures that had grown at 50 bar pressure were transferred to fresh pouches under sterile conditions and incubated at 100 bar pressure. This process was continued at 200, 300 and 400 bar pressure. Viability of fungi after exposure to each pressure was tested by growing in MEA at 30° C/1 bar pressure.

3. Results

3.1. Abundance and diversity

The percentages of culturable fungi obtained by dilution plating and direct incubation of deep-sea sediments under elevated pressure were similar (Table 1). Particle plating yielded a smaller number of fungi. Sediments from shallow coral reef waters yielded much higher numbers of fungi (Table 1). 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 2). *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). Shanon index, Pielou's evenness and species richness values were similar up to 20 – 25 cm depth, after which there

was a marked reduction. 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 (data not shown). Details of the media used for some of the fungi are shown in Table 3.

Many of the aspergilli 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. 2).

3.2. Direct detection of fungi in sediments

An actively germinating fungal spore was detected in sediment samples placed in dilute nutrient medium and incubated at 300 bar pressure and 5^{o}C (Fig. 3). Fungal hyphae were directly detected by staining the deep-sea sediments with Calcofluor (Fig. 4). 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.

3.3. Detection of fungi in sediments by Immunofluorescence

Sediment samples of the core # BC 12 (subsection 15-20 cm, Table 3) stained with FITC-tagged antiserum against *A. terreus* # A 4634 revealed fluorescing hyphae (Figs. 5a & 5b). An unidentified organic particle in these sediments was densely colonized by the fungus. These showed positive fluorescent reaction to the antiserum (Fig. 5c). 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^{o} and 30^{o}C.

3.4. Growth under simulated deep-sea conditions

Out of a total of 181 fungi isolated from deep-sea sediments, a representative 25, which showed a capability for growth at 300 bar upon initial isolation, were selected for growth under different pressures and temperatures. Details of their isolation are given in Table 3. All the fungi showed growth at 200

bar pressure, both at 5° and 30°C (Table 4). Thirteen fungi grew better at 200 bar/5°C, than at 200 bar/30°C. Five of these belong to *Aspergillus* species. All the fungi grew best at 1 bar and 30°C, growth being 3-4 times greater than under elevated hydrostatic pressure (200 bar) at 5° and 30°C. .

Several fungi showed abnormal features when grown under elevated pressure either at 5° or 30°C. For example, *A. terreus*, # A 4634 showed normal mycelial growth at 1 bar pressure at 30° and 5°C (Fig. 6a), while hyphal swellings and constrictions occurred at 30° and 5°C/200 bar pressure (Fig. 6b). An orange-pigmented yeast showed equally good growth at 30°C/1 bar pressure and 5° C/200 bar pressure as well (Figs. 7a, 7b). The conidia of *Penicillium* sp. (isolate # 4615) showed regular germination at 30°C/100 bar pressure, while those of *Aspergillus* sp. (isolate # 3454) showed swollen conidia (Figs. 8, 9). The conidia of these species showed normal germination at 30°C/1 bar pressure.

3.5. Comparison of the growth of deep-sea fungi with that of terrestrial isolates under elevated hydrostatic pressure

Spores of the terrestrial isolates *Aspergillus terreus* and *Aspergillus sydowii* totally failed to germinate at 200 bar/30°C (Table 5). However, 66% of the spores of the deep-sea isolate *A. terreus* (isolate # A 4634) germinated under these conditions. None of these showed germination at 5°C/200 bar (Table 5).

Contrary to spores, both terrestrial and deep-sea isolates of fungi showed growth at 5°C/200 bar and 30°C/200 bar when mycelia were used as inocula (Table 6). The deep-sea isolates *A. terreus* (# A 4634), *Aspergillus* sp. (# A 61 P4) and the unidentified isolate # 3415 did not show significant difference in biomass production at 30°C/1 bar or 200 bar pressure. The orange yeast (# A61P63) showed almost similar growth under all combinations of temperature and pressure. Several of the filamentous fungi showed equally good growth at 5°C/1 bar or 200 bar pressure (Table 6).

2.5. Gradual adaptation of fungi to increasing hydrostatic pressure:

A large number of the 109 fungi that did not grow at 300 bar pressure grew at 50 bar pressure (Table 7). Decreasing numbers of fungi showed viability

and growth when they were gradually subjected to higher pressure. Only 2 strains of *A. terreus* and the yeast # A344 grew at 300 bar and only the latter grew at 400 bar pressure.

4. 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. We have attempted to reduce this risk by exercising utmost caution, as detailed under Methods. One of the culture techniques that we 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 and 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 prior to isolating fungi from them. We believe that 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 ~5000 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 (Mueller and Sengbusch, 1983). The mycelial state of fungi represents an actively growing vegetative condition within or interspersed among organic particles. We believe that the presence of organic particles in the deep-sea sediment samples (Fig. 5c) allows growth of fungal mycelia therein. The total organic matter (TOM) in these sediments was in the range of 4 to 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 have 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 2), indicating the homogenous nature of the sediment in the areas sampled. Raghukumar et al. (2001) have earlier reported the homogenous distribution of total organic matter and labile organic matter in these sediments. This might be the result of high bioturbation by sediment in-fauna (Ingole et al., 2001). The homogeneous nature of sediments at different depths was further indicated by the similar Shanon index and species richness values (Table 2).

The identity of fungi present in the sediments cannot be determined without culturing. All the different culture techniques that we employed yielded filamentous fungi, belonging to the genera such as *Aspergillus, Penicillium, Cladosporium, Curvularia, Fusarium* and several non-sporulating forms which are known from terrestrial habitats. *Aspergillus terreus* was one of the most common fungi isolated. Colonies of the terrestrial fungi in our isolations from the sediments might have resulted either from dormant spores or actively growing mycelia. Hence, presuming that the fungi that we found in the deep-sea sediments originated from land, the following sequence of events can be deduced from our 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. We have

observed fungal colonization of transparent exopolysaccharides (TEPS) collected from coastal Arabian Sea waters (Figs. 10a, 10b) and also oceanic waters. 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. Thus, in one experiment, all the fungi that we tested, irrespective of whether they were isolated from deep-sea sediments, shallow coral reef lagoon waters or terrestrial sources, grew and produced substantial biomass when mycelial inocula were used (Table 6). Contrary to hyphae, spores may be poor candidates for propagation of fungi in the deep-sea sediments. None of the four fungi that we tested, including 3 terrestrial and 1 deep-sea isolate, germinated at 5^{o}C at 1 or 200 bar. The terrestrial isolates germinated very poorly at 200 bar 30^{o}C, while the marine isolate showed substantial germination (Table 5). Thus, pressure appears to have different effects on mycelium and spores of fungi. Alternatively, their germination under deep-sea conditions may be substantially delayed, as observed by Zaunstőck and Molitoris (1995).

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 (Fig. 2). 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 15-20 cm core (Fig. 4). 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 we isolated corresponded morphologically to known terrestrial species. However, deep-sea adaptations might have resulted in genetic modifications. This aspect needs to be addressed in the future.

Based on studies on *Escherichia coli*, Sato et al. (1995) hypothesized that since life 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 our observation on growth of terrestrial fungi under elevated hydrostatic pressure.

The effect of hydrostatic pressure varies according to the species being considered. While the biomass of *A. terreus* (#A 4634) was not significantly reduced after cultivation under elevated hydrostatic pressure (Table 6), the yeast # A344 showed reduction in biomass under such conditions. Morphologically, the yeast cells showed pseudomycelium formation under the simulated deep-sea conditions and *A. terreus* showed abnormal hyphal swellings (Fig. 6B). Nagahama et al. (2001) have reported 99 yeast strains from the deep-sea floor in the northwest Pacific Ocean, but pressure tolerance and growth of these under elevated hydrostatic pressure is not known. Lorenz and Molitoris (1997) have shown growth of the basidiomycetous yeast *Rhodosporidium sphaerocarpum* at 400 bar pressure with some abnormalities.

We also found that numerous isolates obtained from the deep sea did not grow at 200 bar/5^{o}C, even with mycelial inocula. Hence, it is possible that not all fungi that reach the deep sea show growth under these conditions. However, a few of them may still be capable of gradual adaptation to the deep-sea conditions. Thus, out of the 109 fungi that did not grow at 300 bar pressure, a yeast could be gradually be adapted to grow at 400 bar pressure and three fungi were able to grow at 300 bar pressure (Table 7). These were once again a strain of *A. terreus* and a yeast. It has been shown that yeast cells show increased barotolerance after heat-shock treatment, and it has been proposed that the effect of hydrostatic pressure on yeast is analogous to high temperatures and that a heat-shock pretreatment is able to induce barotolerance (Iwahashi et al., 1991).

In conclusion, we demonstrate in this study the active presence of fungi in deep-sea sediments by direct detection using Calcofluor stain, immunofluorescence detection using polyclonal antibodies, culturing and experimentation under simulated deep-sea conditions. Some of the cultures obtained, grew under elevated hydrostatic pressure, while some did so following a period of adaptation. A few others grew under elevated pressure but showed abnormalities in their morphology. The present study confirms the earlier hypothesis (Raghukumar and 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 hardiest forms that can adapt and survive under the most extreme conditions.

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Table1. Number of fungi isolated by various techniques

Source		Particle plating	Dilution plating	Isolation following pressure incubation at 300 bar/5°C
Deep sea	Total number of sediment samples used	376	72	224
	Number of fungi isolated	65	28	88
	% frequency of occurrence	17	39	39
Shallow water	Total number of sediment samples used	16	Not done	
	Number of fungi isolated	26		
	% frequency of occurrence	163		

Table 2. Percentage distribution of fungi at different depths in the sediment core

Genera	**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 isolated	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
Aspergillus sp.	8	11	5	25	29	12	6	14	18	54	13
Aspergillus terreus	28	5	15	13	7	18	19	14	9	8	-
Aspergillus restrictus	12	26	10	19	21	12	13	9	-	-	-
Aspergillus sydowii	-	5	5	6	7	6	-	-	-	-	-
Penicillium sp.	8	5	10	-	7	6	13	9	-	-	-
Cladosporium sp.	4	-	15	-	7	6	13	5	-	15	-
Curvularia sp.	4	-	-	-	-	-	-		-	-	-
Fusarium sp.	-	-	-	-	-	-	-	-	-	-	13
Non-sporulating fungi	16	11	10	13	7	18	6	18	36	15	13
Unidentified sporulating fungi	16	32	25	19	14	24	31	23	36	8	63
Unidentfied Ascomycetes	-	-	-	-	-	-	-	5	-	-	-
Aureobasidium sp.	4	-	-	-	-	-	-	-	-	-	-
Unidentified yeasts	-	5	5	6	-	-	-	5	-	-	-
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 eveness	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

Table 3. Isolation details of deep-sea fungi used for experiments.

Isolate #	Fungi	Cruise #	Core #	Latitude (South)	Longitude (East)	Depth (m)	Section of the core (cm)	Method of isolation	Medium used
A 4637	*A. terreus*	AAS 46	BC 4	10° 01'	75° 59'	5305	0 - 2	PI	MEB
A 4636	*A. terreus*	AAS 46	BC 12	10° 01'	76° 00'	5400	0 - 2	PI	MEB
A 4634	*A. terreus*	AAS 46	BC 12	10° 01'	76° 00'	5400	15 - 20	PI	MEB
A 4633	*A. terreus*	AAS 46	BC 12	10° 01'	76° 00'	5400	10 - 15	PI	MEB
A 4630	*Aspergillus* sp.	AAS 46	BC 7	10° 02'	76° 00'	5296	4 - 6	PI	MEB
A 4628	*A. terreus*	AAS 46	BC 12	10° 01'	76° 00'	5400	8 - 10	PI	MEB
A 61 P10	*A. terreus*	AAS 61	BC 14	14° 00'	75° 30'	5145	20 - 25	PI	MEB
A 61 P4	Unidentified	AAS 61	BC 3	11° 59'	76° 29'	5280	0 - 2	PI	Artemia
A 4625	Unidentified	AAS 46	BC 7	10° 02'	76° 00'	5296	8 - 10	PI	MEB
A 614	*A. terreus*	AAS 61	BC 17	13° 00'	73° 30'	4810	10 - 15	PP	SDA
A 6137	Unidentified	AAS 61	3MBC 5	10° 01'	76° 00'	5280	15 - 20	PP	CMA
A 3457	*Fusarium* sp.	AAS 34	BC 3	10° 00'	76° 01'	5294	15 - 20	PP	MEA
A 3449	*Fusarium* sp.	AAS 34	BC 5	10° 03'	76° 01'	5294	20 - 25	PP	MEA
A 3441	Unidentified	AAS 34	BC 5	10° 03'	76° 01'	5294	8 - 10	PP	MEA
A 348	Non-sporulating	AAS 34	BC A_1/B	10° 10'	76° 05'	5250	8 - 10	DP	ZMA
A 3428	*Curvularia* sp.	AAS 34	BC 8	10° 09'	75° 21'	5180	0 - 2	DP	ZMA
A 61 P63	Yeast	AAS 61	BC 23	10° 59'	73° 29'	5100	20 - 25	PI	Artemia
A 3426	Unidentified	AAS 34	BC 14	10° 02'	76° 00'	5280	8 - 10	DP	MEA
A 6136	*Aspergillus* sp.	AAS 61	3MBC 11	10° 02'	76° 01'	5320	20 - 25	PP	MEA
A 6139	Unidentified	AAS 61	3MBC 12	10° 01'	76° 00'	5280	15 - 20	PP	CDA
A 61 P64	Yeast	AAS 61	BC 12	15° 00'	74° 30'	5390	8 - 10	PI	MEB
A 6128	*Aspergillus* sp.	AAS 61	BC 19	12° 59'	75° 29'	5070	30 - 35	PP	CMA
A 6126	*Aspergillus* sp.	AAS 61	BC 19	12° 59'	75° 29'	5070	30 - 35	PP	SDA
A 3415	Unidentified	AAS 34	BC 8	10° 09'	75° 21'	5180	25 - 30	DP	ZMA
A 3412	*Aspergillus* sp.	AAS 34	BC 8	10° 09'	75° 21'	5180	30 - 35	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 A*rtemia* larvae suspended in seawater.

Table 4. Biomass produced by various deep-sea fungi under different pressure and temperature conditions.

Isolate #	Fungi	Biomass produced (mg dry wt.)		
		200 bar / 30°C	200 bar /5°C	1 bar / 30°C
A 4637	*A. terreus*	30.0	31.2*	134.5
A 4636	*A. terreus*	38.4	7.7	132.7
A 4634	*A. terreus*	18.3	19.6	156.3
A 4633	*A. terreus*	20.8	19.8	121.3
A 4630	*Aspergillus* sp.	25.6	9.0	126.7
A 4628	*A. terreus*	10.0	13.7*	118.3
A 61 P10	*A. terreus*	20.6	12.9	125.7
A 61 P4	Unidentified	17.8	27.1*	128.2
A 4625	Unidentified	65.2	13.8	140.2
A 614	*A. terreus*	8.1	2.9	59.6
A 6137	Unidentified	15.4	6.5	71.6
A 3457	*Fusarium* sp.	21.1	29.6*	228.0
A 3449	*Fusarium* sp.	14.2	10.1	125.8
A 3441	Unidentified	31.5	38.9*	178.0
A 348	Non-sporulating	4.7	5.4*	64.3
A 3428	*Curvularia* sp.	4.5	19.3*	175.7
A 61 P63	Yeast	23.4	9.1	249.2
A 3426	Unidentified	21.0	22.2*	234.6
A 6136	*Aspergillus* sp.	20.0	9.1	169.2
A 6139	Unidentified	17.5	15.4	171.4
A 61 P64	Yeast	12.2	16.8*	150.7
A 6128	*Aspergillus* sp.	9.3	9.3	165.7
A 6126	*Aspergillus* sp.	9.8	8.9	203.4
A 3415	Unidentified	1.7	11.3*	105.3
A 3412	*Aspergillus* sp.	3.1	1.7	140.0

* Fungi showing better growth at 5°C than at 30°C under 200 bar pressure

Table 5. Germination of spores of the deep-sea isolate *Aspergillus terreus* (# A4634) and the terrestrial *Aspergillus* species from Microbial Type Culture Collection, Chandigarh, India.

Culture	**% germination**			
	200 bar/30°C	**200 bar/5°C**	**1 bar/30°C**	**1 bar/5°C**
Aspergillus terreus MTCC 279	1 ± 3.1	No germination	92 ± 7.2	No Germination
Aspergillus terreus MTCC 479	0.5 ± 5.6		94 ± 6.3	
Aspergillus sydowii MTCC 635	0		91 ± 6.7	
Aspergillus terreus - deep sea isolate A 4634	66 ± 26.7		94 ± 8.1	

Table 6. Comparison of biomass produced by the deep-sea isolates and the terrestrial species obtained from Microbial Type Culture Collection, Chandigarh, India and some shallow water cultures isolated from Lakshadweep coral reef slope. Mycelial inocula were used.

Culture	Biomass produced by the fungi (mg)			
	1 bar / 30°C	200 bar / 30°C	1 bar / 5°C	200 bar / 5°C
	Deep-sea isolates			
A. terreus **(# A 4634)**	11.2	8.9	5	9.6
Aspergillus **sp (#A 61 P4)**	4.6	3.0	2.5	2.0
Unidentified (#A 3415)	10.5	8.0	6.2	5.0
Aspergillus **sp (# A 6128)**	10.0	3.3	1.9	1.6
Cladosporium **sp (#A 6136)**	3.7	0.9	1.4	1.5
Non-sporulating (# A 3428)	1.9	0.4	0.7	0.6
Orange yeast (#A 61P63)	1.9	1.3	1.5	1.3
Off-white yeast (#A 344)	6.6	1.0	3.3	1.7
Terrestrial and shallow-water isolates				
Aspergillus terreus **MTCC # 279**	12.7	6.2	4.7	6.6
Aspergillus terreus **MTCC # 479**	10.6	7.7	4.3	10.4
Aspergillus sydowii **MTCC # 635**	12.6	7.6	9.3	6.3
Shallow water non-sporulating form 1.	16.1	0	5.0	9.3
Shallow water non-sporulating form 2.	17.1	7.7	10.1	5.7
Shallow water non-sporulating form 3.	13.3	4.9	6.7	3.4

Table 7. Number of fungi surviving and showing active growth after exposure to sequential increase of hydrostatic pressure. All experiments were carried out at 30°C.

	Total fungi tested - 109	
Incubation pressure	**Viable, but not growing***	**Actively Growing**
50 bar	32	74
100 bar	39	42
200 bar	6	16
300 bar	8	3
400 bar	2	1

*Viability tested by plating on MEA medium.

Legends to Figures

Fig. 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. 2. *Aspergillus* sp. isolated from deep-sea sediments with abnormal morphology, showing hyphae in place of metulae and conidia-bearing phialides on the surface of the vesicle. Bar represents 10 µm.

Fig. 3. An epifluorescence microscopy photograph of a Calcofluor-stained germinating spore from deep-sea sediments (0-2 cm subsection of a core) incubated under 400 bar pressure and 5^{o}C. Bar represents 10 µm.

Fig. 4. An epifluorescence microscopy photograph of a fungal hypha from a deep-sea sediment sample stained with Calcofluor. Bar represents 10 µm.

Fig. 5a. Immunofluorescence detection of a hypha of *Aspergillus terreus* (isolate # A 4634) from 15-10 cm depth of a core of the deep-sea sediment treated with FITC-tagged polyclonal antibodies raised against the fungus. Bar represents 10 µm.

Fig. 5b. Bright-field photomicrograph of the same hypha as in Fig. 5a. Bar represents 10 µm.

Fig. 5c. Immunofluorescence detection of a dense cluster of hyphe of *Aspergillus terreus* on an organic particle from 15-20 cm core of a deep-sea sediment treated with FITC-tagged polyclonal antibodies raised against the fungus. Bar represents 10 µm.

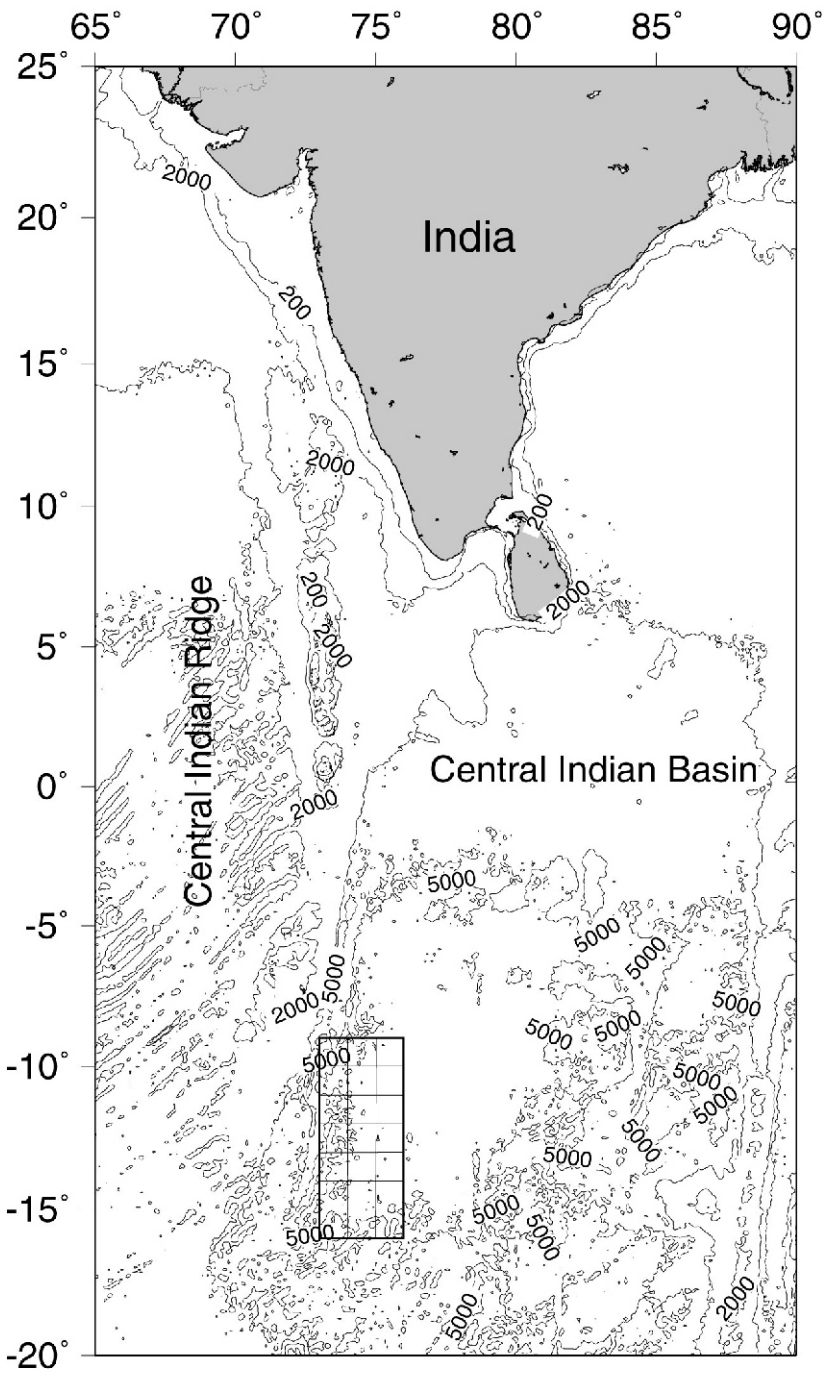
Fig. 6. Photomicrographs of *Aspergillus terreus* (isolate # A 4634) showing a) normal hyphae during growth at 1 bar/30^{o}C, and b) Abnormal hyphae with swellings during growth 200 bar/5^{o}C. Bars represents 10 µm. b) was photographed under phase contrast.

Fig. 7. Nomarski Differential Interference Contrast photomicroagraphs of the yeast (isolate # A 344) showing a) normal growth at 1 bar/30^{o}C and b) normal growth with abundant pseudomycelia at 200 bar/5^{o}C (arrow). Scale 10 µm.

Fig. 8. Spores of *Penicillium* sp. (isolate # 4615) showing normal germination at 300 bar/ 30^{o}C. Scale 10 µm.

Fig. 9. Spores of *Aspergillus sp.* (isolate # 3454) germinating at 300 bar/30^{o}C. Note the swollen conidia (arrow). Scale 10 µm.

Fig.10. 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.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70°
75°
80°
85°
90°
25°
20°
15°
10°
5°
0°
-5°
-10°
-15°
-20°
India
Central Indian Ridge
Central Indian Basin
2000
200
5000

Fig.1

Fig 2

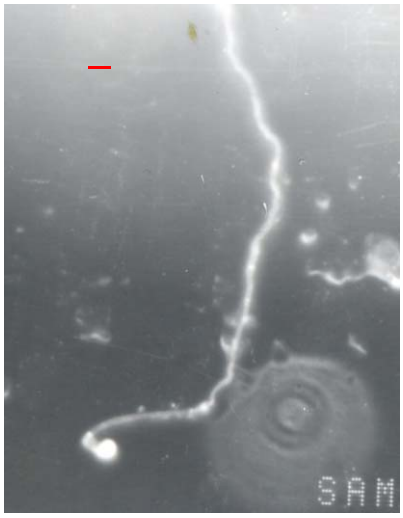


Fig 3

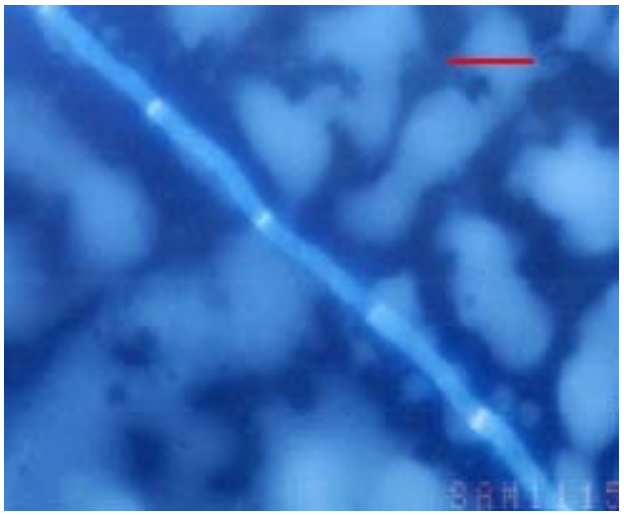
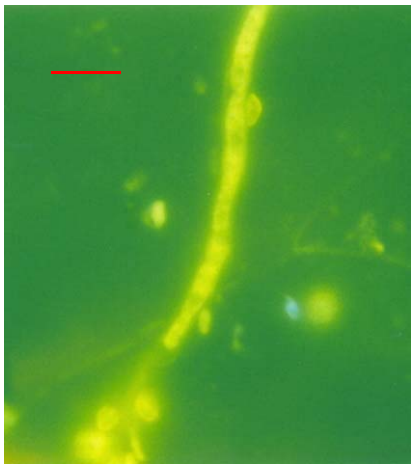
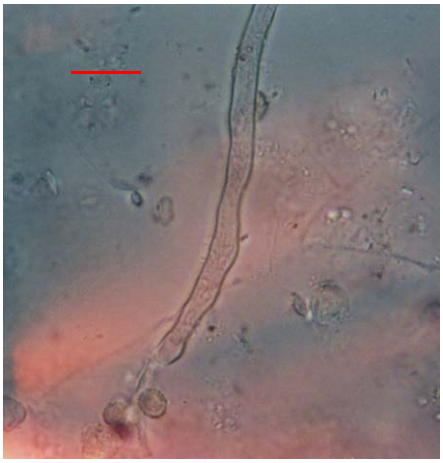


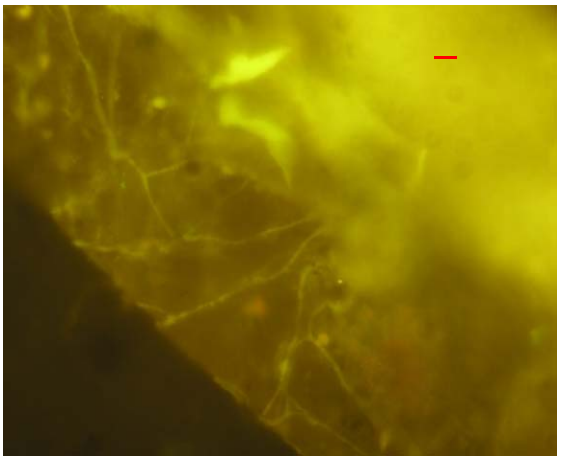
Fig 4



a

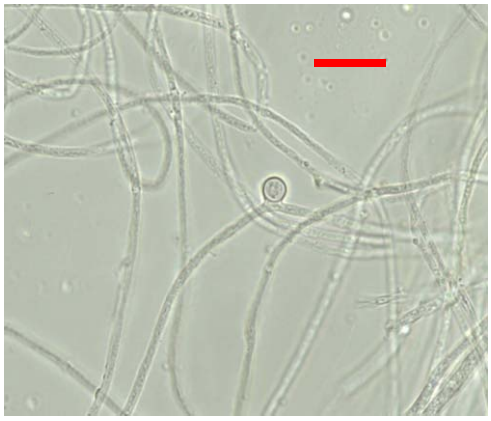


b



c

Fig. 5



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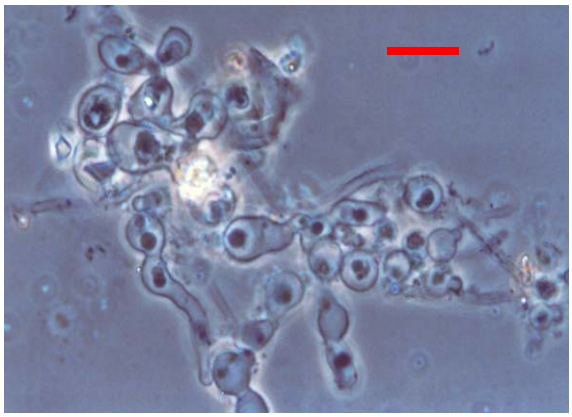
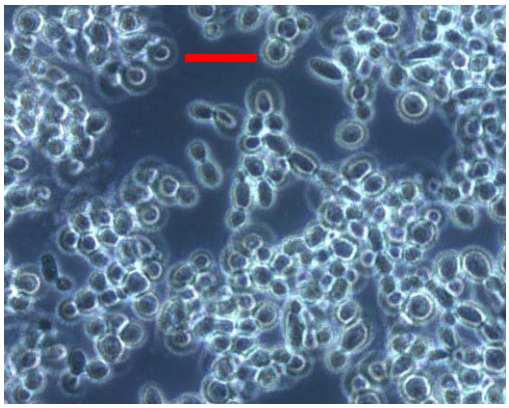


b.

Fig.6



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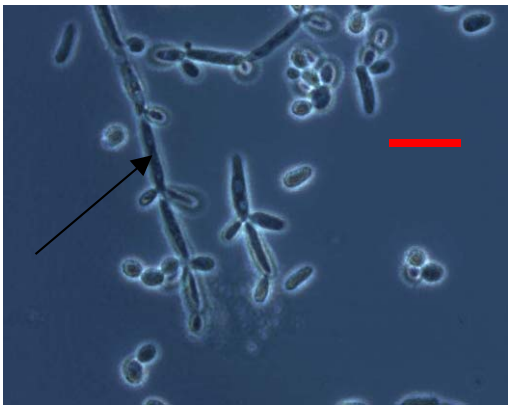

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Fig 7

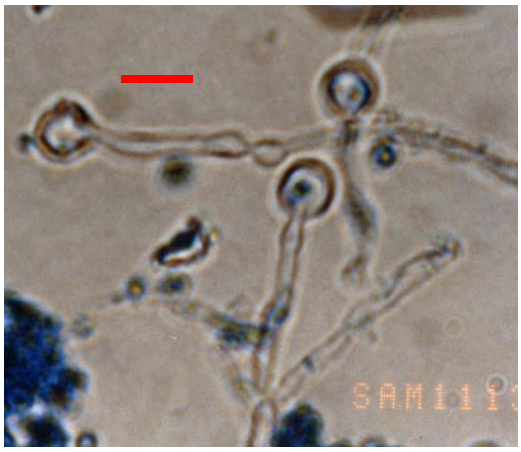


Fig 8

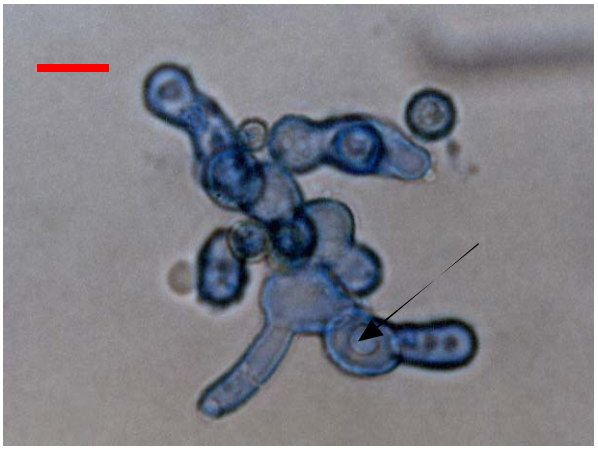
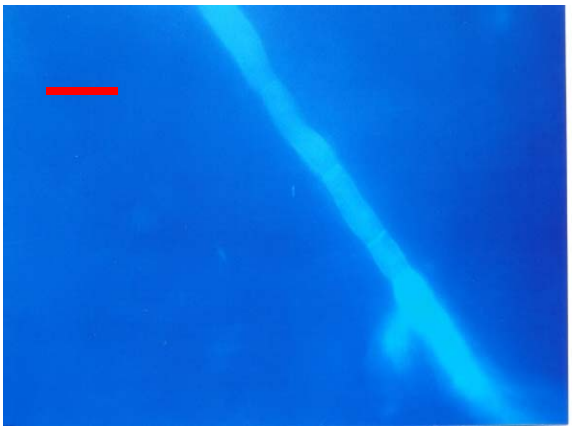


Fig 9



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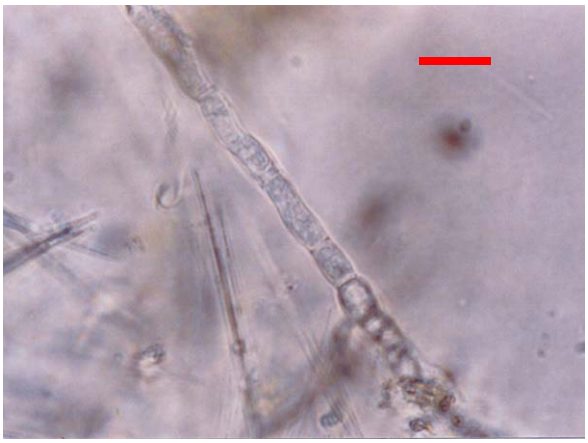

b.

Fig 10