Deep - Sea Fungi: Occurrence and Adaptations

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by

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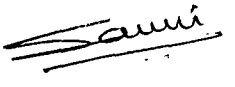
Statement

As required under the University Ordinance 0.19.8 (vi), I state that the present thesis entitled "**Deep - sea fungi: occurrence and adaptations**" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

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



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Certificate

This is to certify that the thesis entitled " **Deep-sea fungi: occurrence and adaptations**" submitted by **Samir R. Damare** for the award of the degree of Doctor of Philosophy in Department of Marine Sciences is based on his original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any degree or diploma in any University or Institution.

Place: Dona Paula

Date: 20/3/07

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All corrections incorporated.
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DEDICATED TO ALL THOSE WHO MADE THIS POSSIBLE

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Chapter 1

Deep - sea Environment

The earth is uniquely favoured among the planets – it has rain, rivers and seas. Ocean basins are a primary feature of the earth's surface. Until recently, the ocean floor was hidden from human observation by miles of ocean water. Now it is possible to map the ocean bottom in detail using satellites and acoustic techniques and to observe ocean-bottom processes from the submersible. With these new techniques, major new discoveries come virtually every year. Our improved knowledge of the ocean basins is changing our view of the earth as much as the exploration of the New World changed human perspectives in the 15^{th} and 16^{th} centuries.

The earth is home to many different forms of life, living in many different environments. Until the 20th century, most life was found in the "normal" environment, where humans live. In the past century, scientists have found organisms that survive in conditions that are very different from the normal. Such organisms are said to live in "extreme environments." High or low temperatures, pressures, salinities, acidities, etc characterize extreme environments.

The deep sea, the largest single ecosystem on earth is an example of such an extreme environment. The sea surface occupies ~70 % of the surface of the earth, and 50 % of the surface of the earth is covered by more than 3000 m^2 of ocean, with a mean depth of ~ 3800 m. It is the very remoteness of the deep sea and the difficulties encountered in its exploration that have resulted in it being one of the least understood environments on earth. At present, detailed information about specific areas of the deep sea are available, but these are mere 'pinpricks' in the vastness of this environment. The understanding of the deep-sea ecosystem is entwined with some of the most exciting aspects of scientific exploration and with the development of

technologies for sampling and penetrating this environment, the information is increasing [Tyler, 2003].

Explorers and commercial interests have used the sea as a means of transport for millennia. However, it was only in the latter part of the 19th century that scientists went to the sea with the specific aim of looking downwards into impenetrable depths. One of the first was Forbes [1844], who sampled down to a depth of 600 m in the Aegean. Today one would consider this choice of sampling station as unfortunate, since now it is known that this region of the Mediterranean deep sea is faunistically poor, and the lack of animals in Forbes's samples led to the 'azoic theory' that little or no life existed below 600 m. The establishment of such a paradigm was in direct opposition to the observations of the ophiuroid *Astrophyton* being brought up on a sounding line from a depth of 1800 m in Baffin Bay [Tyler, 1980], and the pioneering work of Sars in Norwegian fjords [1864 & 1868]. Establishing the presence of a fauna in the deep sea presented irresistible challenges to a small group of scientists led by Charles Thomson. Thomson used HMS *Porcupine* to sample the ocean to the northwest of Scotland and to the west of Ireland in the late 1860s, and found fauna at depths exceeding 4000 m [Thomson, 1873]. The results of the *Porcupine* sampling programme led directly to the HMS Challenger expedition of 1872 to 1876. This expedition traversed the oceans of the globe and demonstrated a widespread and varied fauna in the deep sea, as well as taking numerous physical and chemical measurements. The results of this cruise are now considered the forerunner of modern oceanography. The Challenger expedition led directly to the 'heroic' age of deep-sea exploration, with expeditions sampling many areas of the world's oceans [Menzies *et*

al., 1973; Mills, 1983]. The heroic age culminated in the Danish *Galathea* expedition of 1950 to 1952, which demonstrated that life could be found in the deepest of all the oceans, in the ocean trenches. Taking stock of deep-sea ecology at this point in time would have led to the establishment of the following paradigms:

1. The deep sea was species poor.
2. It was a tranquil environment.
3. There was a slow rain of material from surface to the deep sea [Moseley, 1880].
4. No primary production occurred within deep sea.

The 1960s heralded a new approach to deep-sea ecology, driven by technology. Quantification became the name of the game. With the information gathered, the concept of high biodiversity in the deep sea was established, although the absolute biodiversity is still very much subject to debate, but it is now believed that the deep oceans are as diverse as tropical rain forests. Although known to be diverse, it was assumed that the deep-sea system was heterotrophic, relying on the slow sinking of material from surface waters to provide an energy source for the inhabitants. The 1970s and 1980s provided evidence that this environment was more dynamic than originally thought. The first example was the discovery of hydrothermal vents along the Galapagos Ridge in 1977.

The technology has allowed humans to penetrate the 'remote' environment. SCUBA diving is limited to the top water column; but the development of submersibles has allowed scientists to dive to the deep-sea bed. Current knowledge of hydrothermal vents and cold seeps would be insignificant if it were not for the

submersible. Submersibles are still used today; but the Remote Operated Vehicle (ROV) allows similar access from the comfort of the surface tender without the potential damage of manned submersibles.

Today one may summarize the paradigms for the deep-sea environment as:

1. High species diversity.
2. Periods of benthic storms perturbing an apparently gentle environment
3. Seasonal input of surface derived energy for heterotrophic organisms
4. Primary production at vents and cold seeps.

The change in understanding of the deep sea has been a function of increase in the ability of scientists to gain knowledge from this environment. Despite recent recognition of the above paradigms, all are natural phenomenon. As yet deep sea is exploited only to a very limited extent, but this may change in the future. The deep sea has also been suggested as a repository for the excess CO_2, causing the so-called 'greenhouse effect'. The vastness of the deep-ocean aids its stability.

The deep sea is usually defined as beginning at the shelf break, because this physiographic feature coincides with the transition from the basically shallow water fauna of the shelf to the deep-sea fauna [Sanders *et al.*, 1965, Hessler, 1974, Merrett, 1989]. The shelf break is at about 200 m depth in many parts of the ocean, so the deep sea is said to begin at 200 m. The deep-sea floor is therefore a vast habitat, covering more than 65 % of the Earth's surface [Svendrup *et al.*, 1942]. Much of it is covered by sediment.

The deep-sea floor is an extreme environment; pressure is high, temperature is low, and food input is small. It has been characterized as a physically stable environment [Sanders, 1968].

1. Pressure: The pressure increases by one atmosphere for every 10 m increase in water depth. Water pressure at the surface of the ocean is 1 bar. The ocean, with an average depth of 3800 m and therefore a pressure of 380 bar, comprises approximately 70% of biosphere. Water pressure in the ocean is as high as 1100 bar the Mariana Trench (in the West Pacific, 400 km SW of Guam). It has been suggested that life originated in the deep sea some 3.5 to 4 billion years ago. Therefore hydrostatic pressure would have been very important stimulus for the early stages of life. Recently, it has been suggested that life might have originated in deep-sea hydrothermal vents, and thus it seems possible that high pressure-adapted mechanisms of gene expression, protein synthesis, or metabolism could represent features present in early forms of life [Thistle, 2003].

2. Temperature: The temperature generally decreases with increasing depth, reaching ~2°C on the abyssal plain, but the pattern varies with latitude and region [Mantyla & Reid, 1983]. Above 500 m in mid-latitude, temperature varies seasonally, but with diminishing amplitude with increasing depth. At high latitudes, the vertical gradient in bottom-water temperature is small [Svendrup *et al.*, 1942]. A small vertical temperature gradient also occurs in regions where the bottom water is warm (e.g. the Mediterranean Sea and the Red Sea). Most of the water overlying the deep-sea floor is cold compared to that over most shallow-water habitats, the typical temperatures at the

ocean floor being just a little above the freezing point. At depths below ~ 800 m, temperature is remarkably constant at around 3-4°C.

3. Salinity: The salinity at most locations in the deep sea varies little with time. In most of the deep sea, the salinity of the bottom water is fully marine (35 ppt). Exceptions include the Mediterranean and Red Sea (> 39 ppt) and hypersaline basins such as the Orca Basin in the Gulf of Mexico (300 ppt) [Shokes *et al.*, 1976].

4. Oxygen: Oxygen enters the ocean by exchange with the atmosphere and as by-product of photosynthesis by marine plants in the euphotic zone. The dissolved gas is carried to the deep-sea floor by the descent of surface waters. The water overlying most of the deep-sea floor is saturated with oxygen or nearly so (5-6 ml L^{-1}). Oxygen concentration also varies with depth in the sediment. Oxygen enters the pore water of deep-sea sediments by diffusion and by the activities of organisms that pump or mix water into the sediment. The depth of oxygen penetration into the sediment limits the vertical distribution of organisms. Oxygen is consumed by animal and microbial respiration and by chemical reactions in the sediment. Where the deposition rate of labile organic matter is relatively high and the oxygen concentration in the bottom water is low, as in the basins of the California Continental Borderland, free oxygen disappears within the first centimeter [Reimers, 1987]. Where organic deposition rates are low and bottom water is well oxygenated, as beneath the oligotrophic waters of the North Pacific, abundant free oxygen is present several centimeters into the seabed [Reimers, 1987].

5. Light: Light intensity decreases exponentially with the depth in the water column because incident photons are absorbed or scattered. Particles suspended in the water increase both the absorption and scattering, but even in the clearest ocean water no photosynthetically useful light reaches the sea floor below about 250 m. Therefore, the deep-sea floor differs from more familiar ecosystems as the plant primary production does not occur. Except for hydrothermal vent and cold-seep communities, the food of deep-sea floor organisms must be imported.

Benthic storms

In much of the deep sea, the near bottom water moves slowly as compared to that in shallow-water environments. The flow does move some material, in particular phytodetritus, which accumulates down. The water is never still, because tidal forces move water at all ocean depths. On the continental slope intense currents may be generated by internal tides and water column instability causing breaking at internal waves. Theoretical considerations reveal that prevailing synoptic weather systems can create strong (20 cm s^{-1}) near-bottom currents in the deep ocean. The maximum speed of these oscillating bottom currents depends on both atmospheric parameters (magnitude of the wind-stress curl anamoly, spatial scale of weather system and speed of large-scale background wind) and oceanic parameters (total water depth and combined effects of horizontal diffusion and bottom friction). Benthic storms are characterized by periods of daily averaged flow of more than 15 cm s^{-1} maintained for two or more days. During intermittently strong peaks in flow of more than 40 cm s^{-1},

the top few millimeters of sediment may be completely stripped [Hollister *et al.*, 1984; Hollister & McCave, 1984]. Such events occur due to vorticity propogated from upper water column in areas of strong surface flow, such as Gulf Stream [Weatherly & Kelley, 1985]. However, similar peaks in eddy energy may arise from atmospheric storm-driven motions in areas far from the continental margin [Gardner & Sullivan, 1981], from disturbances associated with intermittent flow over sills [Dickinson *et al.*, 1982] and from bottom-trapped topographic waves [Grant *et al.*, 1985].

Deep-sea sediments

Deep-sea sediment deposits are accumulations of minerals and rock fragments from the land mixed with insoluble shells and bones of marine organisms and some particles formed through chemical processes occurring in sea water. Much of the information about Earth history comes from study of such deposits. Particles in sediment deposits come from different sources like:

1. Terrigenous (derived from land)
2. Biogenic (derived from plants and animals)
3. Authigenic (formed on or in the sediments on the sea floor)
4. Volcanogenic (particles from volcanic eruptions)
5. Cosmogenous (particles from outer space)

Table 1.1 Major sediment input to the oceans

Source	Estimated amount (10^9 tons / year)
Rivers	18.3
Glaciers and ice sheets	2.0
Wind blown dust	0.6
Coastal erosion	0.25
Volcanic debris	0.15
Groundwater	< 0.48

1. Terrigenous sediments: These are derived from the erosion of the continents and are transported into the ocean as particles of gravel, sand or mud. Their mineral composition varies and reflects the source rock and weathering process (climate). Most of the world's largest rivers are located in the wet tropic regions where there is also high relief and intense chemical weathering so these are areas of high mud input into the oceans. The Ganges River discharges the most sediment per year – about 1500 million tonnes. Sediment is also blown off the continents into the ocean, particularly on the west coast of continents adjacent to the major deserts. Usually only very small particles (less than 20 μm) are carried long distances. The Abyssal clay or "red clay" that covers much of the deep ocean floor is largely of aeolian origin. While aeolian dust is deposited everywhere, it only dominates on the abyssal regions where low biological productivity and the dissolution of calcium carbonate prevent dilution. Melting of ice sheets and icebergs has been, and continues to be, a major provider of sediment to the sea floor in high latitudes. Ice is indiscriminate in what it carries: giant boulders to finely ground clay.

2. Biogenic sediment: This contains organically produced particles and is defined as any deposit which has more than 30% biogenous constituents by weight. There are three main groups of organic sediments – calcareous, siliceous and phosphatic.

The distribution of **calcareous biogenous sediments** is largely determined by what is known as the calcite compensation depth (CCD; sometimes erroneously referred to as the carbonate compensation depth). This is the depth, usually several km, below which calcite does not accumulate because it is a level on the sea floor where the rate of carbonate supply is equal to the rate of carbonate dissolution. It is analogous to the snow line on land. The depth, at which dissolution starts, is called the lysocline, (generally located 500-1000 m above the CCD). The CCD exists because the carbon dioxide rich deep water of the oceans is undersaturated with respect to calcite. Calcite also becomes more soluble with increasing pressure and lower temperatures. The warm tropical surface waters are supersaturated with calcium carbonate. Thus, the level of the CCD rises in high latitudes where the cold CO_2 rich water is at the surface and is depressed in low latitudes where the supply of calcite raining to the sea floor is higher. The dissolution of calcite recycles Ca as organisms precipitate more $CaCO_3$ than can be supported by the flux of calcium to the oceans from rivers. It is estimated that approximately 90% of the calcium carbonate precipitated by organisms in the upper layers of the oceans is dissolved in the deep ocean. Examples of carbonate sediments are – foraminefera, coccoliths and pteropods.

Foraminefera are single-celled protozoans that are both benthic, which live in sediments on the sea floor, and planktonic, which live in the upper 100 m or so of the ocean. Of the estimated 4000 species living today, 40 are planktonic but because of their great abundance they secrete more calcite than all other foraminifers. Foraminiferal shells (called 'tests') of both groups occur in a variety of shapes, and typically range from 0.1 mm to 1 mm in size (but have been found up to 18cm!). The shells of all planktonic and most benthic species are composed of calcite. The fossil record of benthic foraminifera dates back to more than 550 million years. Planktonic species have been around for the last 200 million years but really got going about 100 million years ago. Because of the large number of species (it is estimated that there are over 40,000 in the rock record), their wide distribution and environmental sensitivity, they can be used to determine past climate conditions. In addition, because of their rapidly changing form (species generally exists for about ~5-15 million years), they can be used to determine the age of sediments in which they occur.

Coccolithophores are a common group of phytoplankton - single cell algae (plants). They are unique in that the single cell is surrounded by armour of at least 30 calcite plates (called coccoliths) to form a sphere only 30 μm in diameter. Scientists estimate that the organisms deposit more than 1.5 million tons (1.4 billion kilograms) of calcite a year, making them the leading calcite producers in the ocean and together with the forams they deposit more calcite on the floor of the deep ocean than all the shells and corals on the continental shelves. Consequently they are responsible for many thick chalk and limestone beds. They first appear in the fossil record in the Jurassic and were particularly common in the Cretaceous, when they produced many

chalk deposits, like the White Cliffs of Dover. They were almost wiped out at the Cretaceous -Tertiary boundary but have persisted to the modern day. A related, but now extinct, group is the Discoasters, which secreted microscopic star-shaped calcite crystals. Together these microfossils are important in micropaleontology for evolutionary and environmental studies. Because of their small size they are often called nannofossils. Today Coccolithophores live mostly in subpolar regions. They are often found in nutrient poor water that cannot support other types of plankton. They form blooms, which because of the structure of their plates are visible from space. They appear as milky white or turquoise patches. Blooms are a regular occurrence off the north coast of Australia.

Pteropods are small gastropod mollusks, basically floating snails whose foot is modified for swimming. They produce large mucus feeding webs for trapping phytoplankton. They have a coiled shell composed of aragonite into which they can retreat if threatened (some species do not have the shell and are just a gelatinous blob). They favour tropical and warm-temperate seas. In some equatorial areas of the Indian Ocean, pteropod shells dominate the sedimentation, resulting in a subset of the carbonate ooze - the pteropod ooze.

Siliceous sediment is composed of siliceous shells or skeletons of opaline silica, a form of hydrated silicon dioxide. The principal silica producers are the radiolarians (animals) and diatoms (plants). On the whole the ocean is undersaturated with silica, therefore you might expect biogenic silica to dissolve and not be present in sediments. The solubility of silica decreases with increasing pressure and decreasing

temperature – that means that there is more of it in the deep ocean (the opposite to carbonate). Siliceous organisms are generally found in nutrient rich waters (areas of upwelling) that have high silica content. The shells do dissolve (pretty slowly), but because of high productivity there is a lot of them and they get buried before they get destroyed. The siliceous content of sediment is highest in deep water where calcareous sediment is absent (because of the CCD). Radiolarians are protozoans that construct beautifully complex silica exoskeletons that often have many spines extending outwards. They form oozes on the sea floor that over time can evolve into hard sedimentary rocks called radiolarian cherts or, if mixed in with calcareous ooze they form individual flint nodules in chalk. They have been around for the last 540 million years and are useful for dating rocks, because their skeletons are very well preserved in the sediment. During the Cretaceous-Tertiary mass extinctions, radiolarians did well in comparison to other planktonic life forms. Radiolarians absorb dissolved silica from seawater to construct their beautiful skeletons. Some sponges produce calcareous spicules that are common in some shelf and slope sediments. Diatoms are single cell algae (plants) that incorporate silica into their cell wall to form 'frustules'. Diatoms occur in both benthic and planktonic forms. The benthic ones are restricted to water depths of less than 100 meters. Diatom blooms are common in rivers and upwelling zones (due to the high nutrient content) and can be toxic to other organisms because of oxygen depletion or the biotoxins produced by some species.

3. Authigenic (sometimes called hydrogenous) deposits: These are deposits precipitated from seawater as a result of chemical reactions, (some of which may be assisted by bacteria). They include ferromanganese nodules (often called manganese

nodules) phosphorites, glauconites and evaporites. **Manganese nodules** form in the deep ocean and are particularly common in the Pacific where they are estimated to cover 30-50% of the sea floor. They are made up predominantly of manganese oxide (MnO_2) and iron oxide (Fe_2O_3) - average contents of 30% manganese and 20% iron. They are dark brown in colour, slightly flattened rough spheres, 5 to 10 cm in diameter and are generally found in water depths of 4000 to 6000 meters. In cross section, the nodules show concentric layers, or growth rings around a core - like tree rings. The core can be a fragment of anything, a bit of basalt, skeletal material etc. The growth rate of the nodules is very slow - nodules in the Pacific Ocean are estimated to be 2 to 3 millions years old. Nobody is quite certain about how manganese nodules form, but it seems likely that bacteria are involved. It is thought that the major sources of manganese in seawater are leaching of sea floor basalts and of hydrothermal activity along mid-ocean ridges.

4. Volcanogenic sediments: Three things come out of volcanoes: lava, tephra and gas. Tephra is all ejecta blown through the air or water by explosive volcanic eruptions. Tephra comes in different sizes classified as - blocks, bombs, lapilli, cinders and ash. Large-sized tephra generally falls close to the volcano. Smaller fragments are carried away by the wind. Volcanic ash can travel hundreds to thousands of kilometers downwind from a volcano. As the cloud of ash and gas moves away from the volcano, it loses altitude and ash falls to the ground forming a layer of sediment. Volcanic ash in deep-sea sediments may be in discrete layers or dispersed through other sediments. Size sorting by the wind may occur with distance from the source.

5. **Cosmogenous sediments**: These are extraterrestrial in origin. These are the least abundant sediment type and are generally found diluted by other sediments. There are two main sources: **Cosmic dust** - silt and sand-sized particles and **Comets & asteroids**. Research by the Ocean Drilling Program and others has revealed a thin and distinctive band of clay present in sediments around the world. This band is highly enriched in Iridium (Ir) and corresponds to the Cretaceous-Tertiary (KT) boundary. Iridium is a rare-earth element that is found at very low concentrations in the earth's crust but is common in meteorites. The source of the iridium in this clay band is thought to be a comet that hit the earth 65 million years ago. The impact produced a layer of sediment that can contain up to 20% cosmogenous material. Comets and asteroids are also capable of producing particles called tektites. They are dark-coloured, rounded silicate glass particles that can be less than a millimetre (microtektites) to several cm in size. They are found concentrated in areas around the world that are referred to as strewn fields. The tektites are formed by impact melting of surficial sediments. Microtektites are found in deep-sea sediments within the Australasian strew field (a large area which is thought to have resulted from an impact on the Indochina Peninsula). Ocean Drilling Program researchers examining cores from the Ninety East Ridge (Eastern Indian Ocean) and the Sulu Sea (both located in the Australasian strewn field) have found increased levels of Ir. The Ir concentrations and microtektites distribution have lead them to propose that the Australasian impact could have excavated a crater between 15 and 19 km in diameter.

Much of the deep-ocean floor is covered by such deposits, which accumulate slowly, particle by particle. Typical accumulation rates are between 0.1 and 1 cm per

thousand years. Since deep-ocean sediments accumulate slowly, particles may spend years suspended in seawater or exposed to the overlying waters while being slowly buried in the bottom.

Special habitats in deep sea

Apart from the above mentioned parameters of the deep-sea environment, there are a few deep-sea habitats which need a special mention. Ex.: Cold-seeps, hydrothermal events.

Cold seeps: A cold seep, sometimes called a cold vent, is an area of the ocean floor where hydrogen sulphide, methane and other hydrocarbon-rich fluid seepage occurs. Cold seeps are distinct from hydrothermal vents that the former's emissions are of the same temperature as the surrounding seawater, whereas the latter's emissions are super-heated. Cold seeps constitute a biome supporting several endemic species.

Cold seeps were first discovered in 1984 by Dr. Charles Paull in the Monterey Canyon, just off Monterey Bay, California, at a depth of 3,200 m. Since then, seeps have been discovered in the other parts of the world's oceans, including the Gulf of Mexico, the Sea of Japan, and in the waters off the coast of Alaska. The deepest seep community known is distributed between the Kuril and Japan trenches and the Kashima seamount in the Sea of Japan, at a depth of 5,000 to 6,500 m.

Unlike hydrothermal vents, which are volatile and ephemeral environments, cold seeps emit at a slow and dependable rate. Owing to the differing temperatures and stability, cold seep organisms are much longer-lived than those

inhabiting hydrothermal vents. Recent research has revealed seep tubeworms to be the longest living noncolonial invertebrates known, with a minimum lifespan of between 170 and 250 years.

Entire communities of light independent organisms develop in and around cold seeps, most relying on a symbiotic relationship with chemoautotrophic bacteria. These bacteria, both archaea and eubacteria, process sulphides and methane through chemosysnthesis into chemical energy. Higher organisms, namely vesicomyid clams and siboglinid tube worms use this energy for their life processes, and in exchange provide both safely and reliable source of food for the bacteria. Other bacteria form mats, blanketing sizable areas in the process.

Cold seeps develop unique topography over time, where reactions between methane and seawater create carbonate rock formations and reefs. These reactions may also be dependent on bacterial activity.

Hydrothermal vents: The discovery of hydrothermal vents in 1977 was certainly the most important event for the marine biologists because it changed the perception of the deep sea as a cold, dark, high-pressure and nutrient poor environment inhabited by psychrophilic, oligotrophic to barophilic microbial communities. By contrast, deep-sea vent areas are hot to warm and inhabited by animal communities whose densities may reach 50 kg m^{-2}. Invertebrates living in these warm biotopes are in endosymbiotic relationships with autotrophic sulphur-oxidisng bacteria. In the hot areas of the ecosystem, temperatures often reach 350°C, and precipitation produces mineral structures (black smokers) that contain thermophilic microorganisms.

Vents are known to exist in the Pacific and Atlantic oceans. Most are found at an average depth of about 2,100 meters (7,000 ft) in areas of seafloor spreading along the Mid-Ocean Ridge system — the underwater mountain chain that snakes its way around the globe. In some areas along the Mid-Ocean Ridge, the gigantic plates that form the Earth's crust are moving apart, creating cracks and crevices in the ocean floor. Seawater seeps into these openings and is heated by the molten rock, or magma, that lies beneath the Earth's crust. As the water is heated, it rises and seeks a path back out into the ocean through an opening in the seafloor. As the vent water bursts out into the ocean, its temperature may be as high as 400°C. Yet this water does not boil because it is under so much pressure from the tremendous weight of the ocean above. When the pressure on a liquid is increased, its boiling point goes up. Chimneys top some hydrothermal vents. These smokestacks are formed from dissolved metals that precipitate out (form into particles) when the super-hot vent water meets the surrounding deep ocean water, which is only a few degrees above freezing. So-called "black smokers" are the hottest of the vents. They spew mostly iron and sulfide, which combine to form iron monosulfide. This compound gives the smoker its black colour. Geologists are intrigued by how rapidly vent chimneys grow - up to 9 meters (30 ft) in 18 months. A scientist at the University of Washington has been monitoring the growth of "Godzilla," a vent chimney in the Pacific Ocean off the coast of Oregon. It reached the height of a 15-story building before it toppled. It is now actively rebuilding. These underwater geysers are believed to play an important role in the ocean's temperature, chemistry, and circulation patterns.

Chapter 2

Microbial Life in Deep-sea Sediments

As oceanic crust moves away from spreading centres, it becomes covered with increasingly thick layers of slowly accumulating sediments, which can accumulate to depths of several kilometers. Sediment layers can be much thicker at continental margins, where they accumulate from weathering of continental platforms and accretion of sediment scrapped off of subducting tectonic plates. The study of biodiversity of subsurface microorganisms from the sediments is only just beginning. A case can be made that the subsurface is both the largest portion of Earth's biosphere and the most poorly catalogued. In most subsurface studies to date, bacterial diversity has been evaluated through cultivation and enumeration of various metabolic functional groups. In most cases, only aerobic heterotrophs were characterized. In recent years, other functional groups have been routinely enumerated in the subsurface, including denitrifying bacteria, dissimilatory metal reducing bacteria, and methanogenic archaea. Relatively few of these organisms have been characterized mostly because of late surge in this field of study and lack of facilities for the studies.

As mentioned in the previous chapter, the deep sea was thought to be devoid of life till mid 19th century. This assumption was refuted in 1860 by the discovery of corals and sponges attached to a transatlantic cable that was hauled up from the seafloor for repair. From 1868 to 1870, Thomson led multiple cruises of HMS *Lightning* and HMS *Porcupine* to dredge the Atlantic seafloor at depths as great as about 4,600 meters. By the end of his project, he had discovered diverse life on much of the ocean floor. Since then, marine biologists have come to accept the existence of life on the seafloor and in the first few meters of deep-sea sediments. The existence of

high-pressure life was first documented by the oceanographic surveys of the *HMS Challenger,* during the 1870s. This pioneering expedition for the first time made extensive collections of deep-sea biota, with more than 100 dredgings at depths of more than 1 km. These collections revealed an unexpected abundance of life in the abyssal depths and thus started the fantastic journey of human beings to look into the life in deep sea.

As discussed earlier, conditions like low temperature, high pressure, and low nutrient levels combine to make the deep sea a potentially hostile environment. Most of the deep-sea bottom is stable, cold and dark; therefore it is possible that very ancient life forms may be present in a state of suspended animation in the world's largest refrigerator. In 1968, the research submarine Alvin accidentally filled with seawater and sank to a depth of 1540 m in the North Atlantic, where it remained for about one year. Upon recovery, it was observed that a boxed lunch consisting of bouillon, a bologna sandwich, and apples were all remarkably well preserved. This famous "bologna sandwich experiment" and its successors initiated a fascinating and controversial chapter in modern marine microbiology.

Zobell & Johnson [1949] first coined the term barophile and Zobell & Morita [1957] obtained the first evidence of peizophilic growth in mixed microbial cultures recovered from the deep sea. Yayanos [1979] reported the first isolate of pressure-adapted bacteria in 1979. Subsequently, many psychrophilic peizophiles with various optimal growth pressures have been isolated and characterized physiologically and genetically. Deep-sea hydrothermal vents are interesting sources of novel isolates, many of which were discovered in the course of investigation into the origin of life.

Thermophilic microorganisms have also been examined physiologically under high-pressure conditions. Thus, studies on the effects of pressure on microorganisms have been mainly performed using two types of microorganisms, psychrophilic peizophiles and thermophilic peizophiles.

As subsurface strata may be effectively isolated from each other, as well as from the subsurface, it is possible that microbial diversity may vary as a function of depth. Certainly, the abundance of broad functional groups of microorganisms has been reported to vary according to geochemical controls, such as the availability of electron acceptors.

Although most studies on diversity discuss the number of species, other aspects are at least as important, for example, there is cladistic or phylogenetic diversity, morphological diversity, ecological diversity and genetic diversity. All four are harder to study and to quantify than species diversity, and all are independent of it [Williamson, 1997]. Phylogenetic diversity gives the relationship of cellular organisms in the form of diagrammatic trees. The higher plants, animals and fungi occupy three small, closely related branches. At a molecular level, multicellular organisms may be very uniform, and this also reminds us that any one measure of biodiversity is insufficient. Morphological and ecological diversity are perhaps more relevant to the preservation of biodiversity, but there are no well-defined guidelines available about how either should be measured. Measuring ecological diversity is even harder, but probably nearer to what is needed for conservation decisions. So it is not surprising that quantitative studies of biodiversity have, apart from a nod to phyletic diversity,

been based almost entirely on species count. It is well known that most species have not been described.

High species richness of deep-sea sediments was first given prominence by Howard Sanders at the Woods Hole Oceanographic Institution in the 1960s. With his co-workers, he found that by washing the mud from anchor-dredge samples of the deep-sea ooze off New England through fine screens, a surprisingly large number of clearly differing species were revealed [Sanders *et al.*, 1965]. He focused his attention on the macrobenthos, a size fraction of the sediment-dwelling community of mainly invertebrate animals that are retained on screens with meshes of 0.25-0.5 mm and have an upper size limit, arbitrarily determined by visibility in sea bed photographs, separating them from the megabenthos.

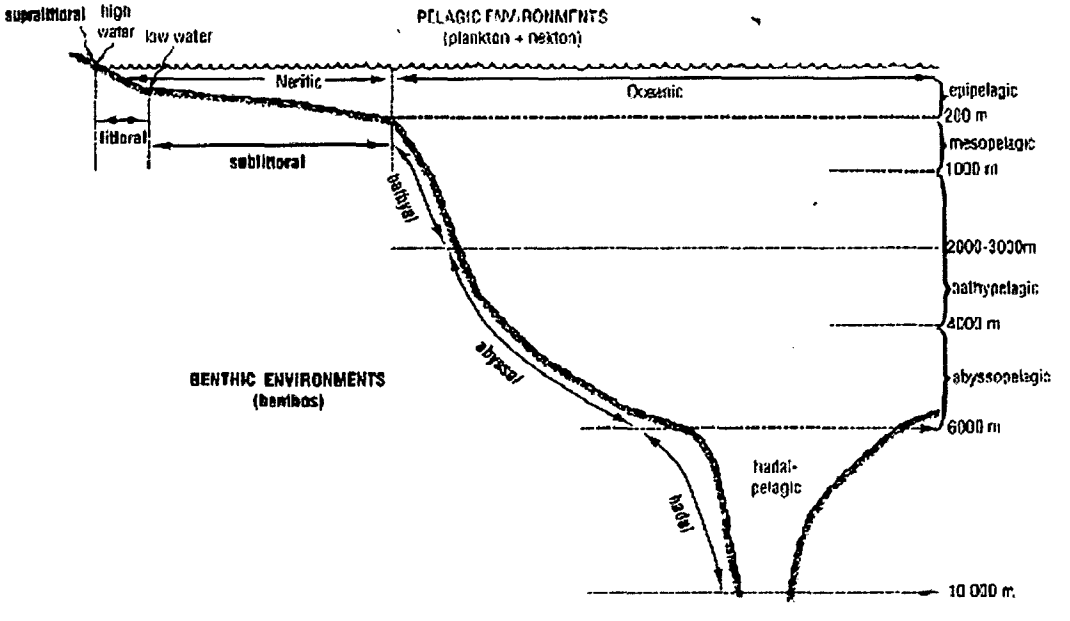
Marine sediments overlay two-thirds of the earth's surface and harbour diverse and abundant fauna. It was estimated earlier that of the 3.8×10^{30} prokaryotes calculated to be in the unconsolidated subsurface sediments, 97 % or 3.7×10^{30} occur at depths shallower than 600 m. The estimated number of prokaryotes for deeper sediments is only 0.13×10^{30} cells. This value was uncertain because it was based on extrapolation, but it still represents considerable microbial biomass. One would then wonder about their function, metabolism and biogeochemical role at these depths. In other words, what do they eat, how do they respire and what roles do they play?

Thus the study of microorganisms isolated from the deep sea promises to provide new information about the origin of life and its evolution, contributing to the overall marine biodiversity, which is poorly described so far. The study of these

extremophiles also gives an opportunity to investigate how life processes work at some of the extreme temperatures (both high and low) and pressures of the biosphere.

Marine organisms can be classified according to the marine environments they inhabit. Thus, there are oceanic species and neritic species depending upon whether the organisms are found in offshore or coastal waters, respectively. Similarly, plants or animals that live in association with seafloor are collectively called benthos. The benthos includes attached seaweeds, sessile animals like sponges and barnacles, and those animals that crawl on or burrow into the substrate.

Fig. 2.1 The basic ecological divisions of the ocean. The neritic (inshore) pelagic zone is separated from the oceanic (or offshore) pelagic zone by the edge of the continental shelf, which is generally at about 200 m depth. Benthic habitats are in black font whereas pelagic divisions in blue (figure not to scale)



Source: Biological Oceanography An Introduction, 2nd Edition (Lalli CM, Parsons TR, Eds.) p. 3, Butterworth-Heinemann Publications

In spite of the vast majority of the seafloor permanently submerged below tidal levels, relative to the intertidal regions, comparatively less is known about life in the bathyl, abyssal and hadal zones (Fig. 2.1). This is mainly due to their relative inaccessibility. Although it is possible to dive to several thousand metres in submersibles or to employ remote-controlled cameras, the number of hours of direct observations in the deep sea are extremely less. Most of the information on deep-sea ecology comes from indirect inferences based on animals contained in benthic samples obtained from ships. Whatever the method, expense is the limiting factor in the deep-sea research. Compounding this expense problem is the fact that animal life is just not very abundant in many deep-sea areas, so that it is desirable to have large numbers of samples. But now with new techniques for collection and observation, combined with accumulating numbers of analyzed deep-sea samples, assessment of benthic life in deeper water, is improving.

Most animal phyla are represented in this dark environment of low temperatures, high pressures and predominantly soft substrates of the deep sea. Some deep-sea residents have a cosmopolitan distribution and are found in all the major oceans; other species are restricted to relatively small areas. In general, species become more limited in geographic range as water depth increases. Only about 20 % of the species present below 2000 m in the Atlantic Ocean are also found in the Pacific or Indian Oceans. Many species found in areas deeper than 6000 m are endemic to the hadal region, and many are restricted to a particular trench.

The benthic animals are separated in to infaunal and epifaunal species, depending upon whether they live within sediments or on the surface of the seafloor respectively.

The benthic animals can also be separated in different categories based on the size (relative to the mesh size of sieves used to separate animals from sediments) as follows –

Macrofauna (or macrobenthos) are those animals that are retained by a 1.0 mm mesh sieve. These are the largest benthic animals, including starfish, mussels, most clams, corals, etc.

Meiofauna (or meiobenthos) are those animals retained by a 0.1 to 1.0 mm mesh sieve. These are small animals commonly found in sand or mud. The group includes very small molluscs, tiny worms, several small crustacean groups (including benthic copepods), as well as less familiar invertebrates.

Microfauna (or microbenthos) are those animals that are smaller than 0.1 mm in dimension. This smallest size category is largely made up of protozoans, especially ciliates.

Bacterial abundance in deep sea sediments exceeds 100000 cells/cm^3, even at a depth of few kilometers below the seafloor [Raghukumar *et al.*, 2001]. Bacteria play a key role in all major biogeochemical cycling processes in deep-sea sediments, where they contribute up to 90% to the benthic biomass [Pfannkuche, 1992].

Apart from bacteria, recognition of viral influence on pelagic processes has in the past few years led to an increased focus on the role of viruses in benthic environments [Danovaro *et al.*, 2001; Hewson *et al.*, 2001; Middelboe *et al.*, 2003;

Mei & Danovaro, 2004; Breitbart *et al.*, 2004]. Such studies have verified that viruses are abundant, diverse and dynamic members of benthic communities. There are 10^7–10^9 viruses cm^3 of surface sediment, which is 10–100 times higher than densities usually found in the overlying water column of the same area [Middelboe *et al.*, 2003], and their abundance and activity have been shown to be positively correlated with benthic microbial activity [Middelboe *et al.*, 2003; Glud & Middelboe, 2004]. Due to such high numbers, viruses can therefore potentially affect the bacterial population and biogeochemical processes in the marine sediments.

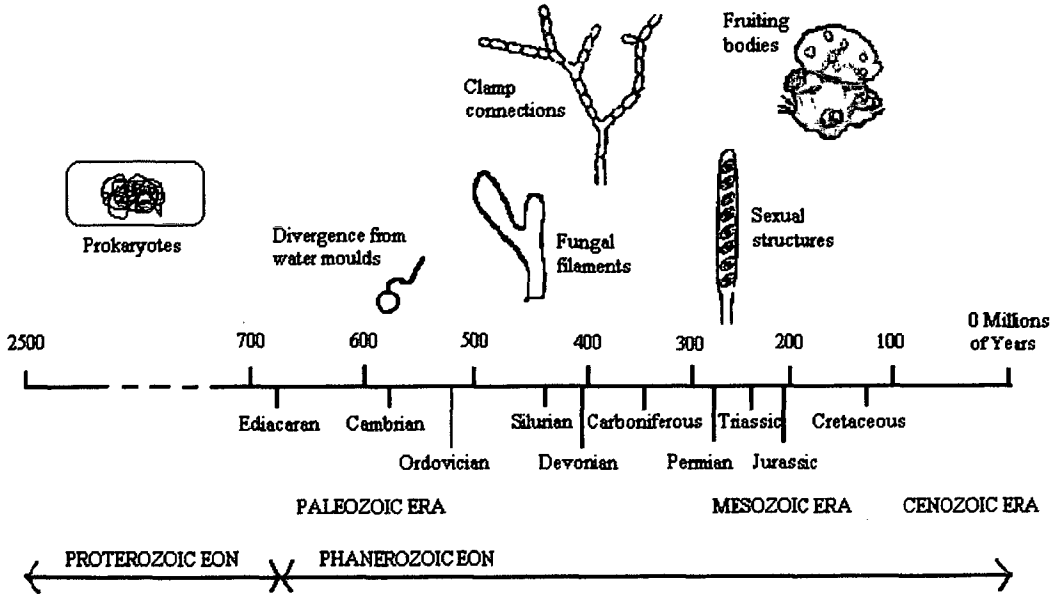
In the next chapter, we will see the distribution and occurrence of fungi in the marine environment.

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 doest 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 it's 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 brightner 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 *Rhodosporidium 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
Abyssomyces hydrozoicus	Hydrozoa chitin	631-641, Atlantic	Kohlmeyer & Kohlmeyer, 1974	Only preserved specimens
Bathyascus vermisporus	Wood	1615 & 1720, Pacific		
Oceanitis scuticella		3975, Atlantic		
Allescheriella bathygena		1720		
Periconia abyssa		3975 & 5315, Atlantic		
Aureobasidium pullulans	Water	1000-4500, subtropical Atlantic	Roth *et al.*, 1964	Colony Forming Units (CFU)
Cladosporium spp.				
Alternaria spp.				
Aspergillus sydowii				
Nigrospora spp.				
Penicillium solitum				

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

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 ($10^{o}00'264"$ - $10^{o}10'364"$S; $75^{o}21'000"$ - $76^{o}05'160"$ E), 7 during cruise # AAS 46 ($10^{o}00'237"$ - $10^{o}02'661"$ S; $75^{o}59'498"$ - $76^{o}09'822"$ E) and 38 during the cruise AAS # 61 ($9^{o}59'861"$ - $16^{o}00'047"$ S; $73^{o}29'819"$ - $76^{o}30'559"$ E). Sediment samples were collected on board the Russian research vessel AA Sidorenko, using an Usnel type box corer of 50 cm^3 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

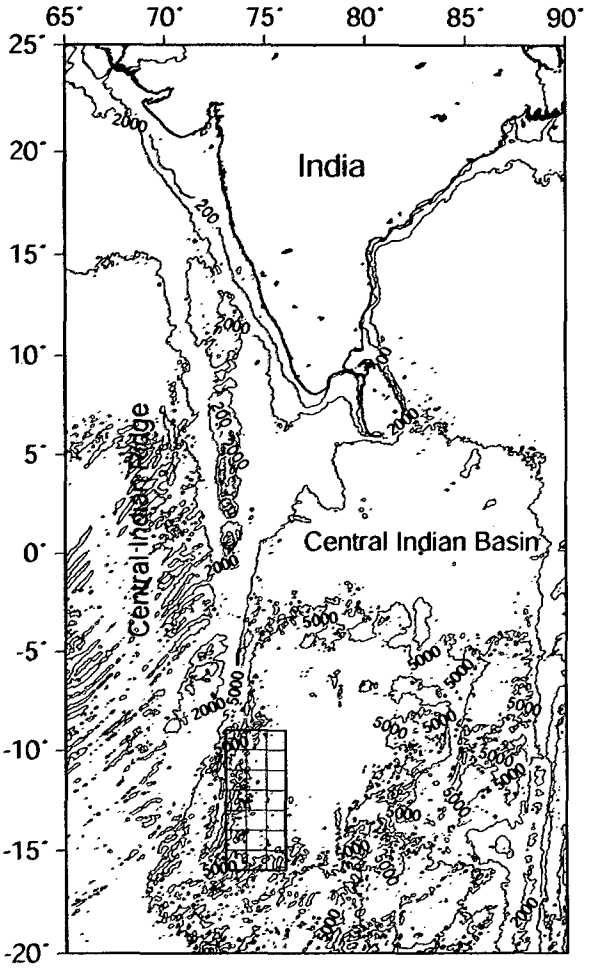
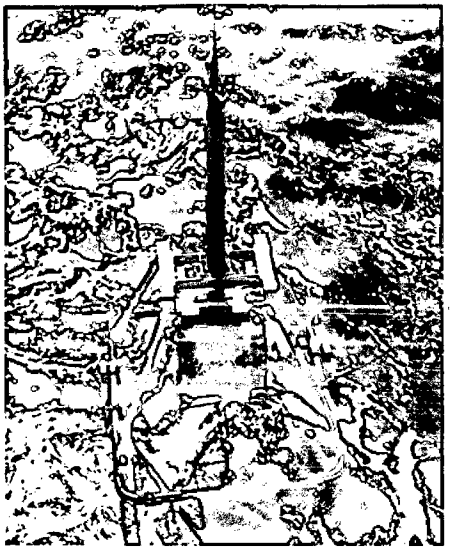


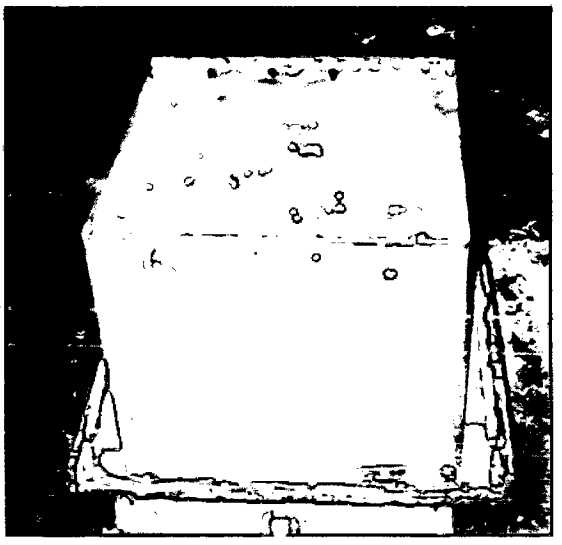
Fig.1

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

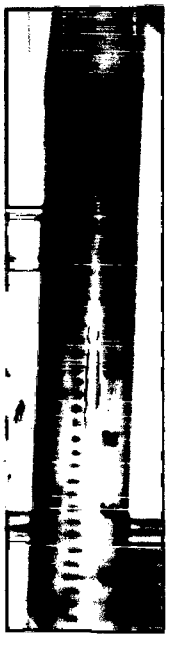


(a) USNEL box cores being lowered for sampling



(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), Sabauraud'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 µL 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 µm mesh. The particles that passed through 200 µm but retained on the 100 µm 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

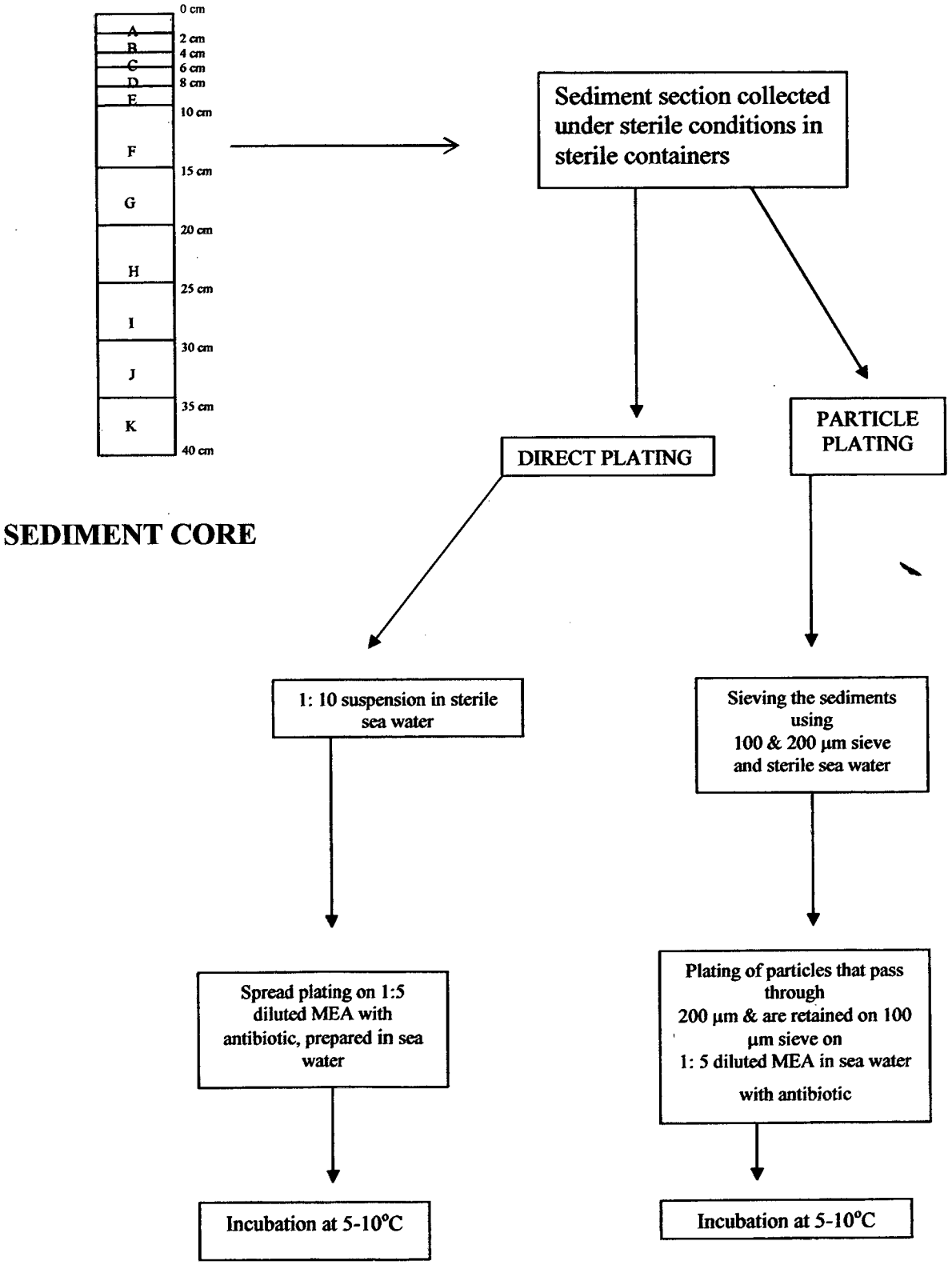
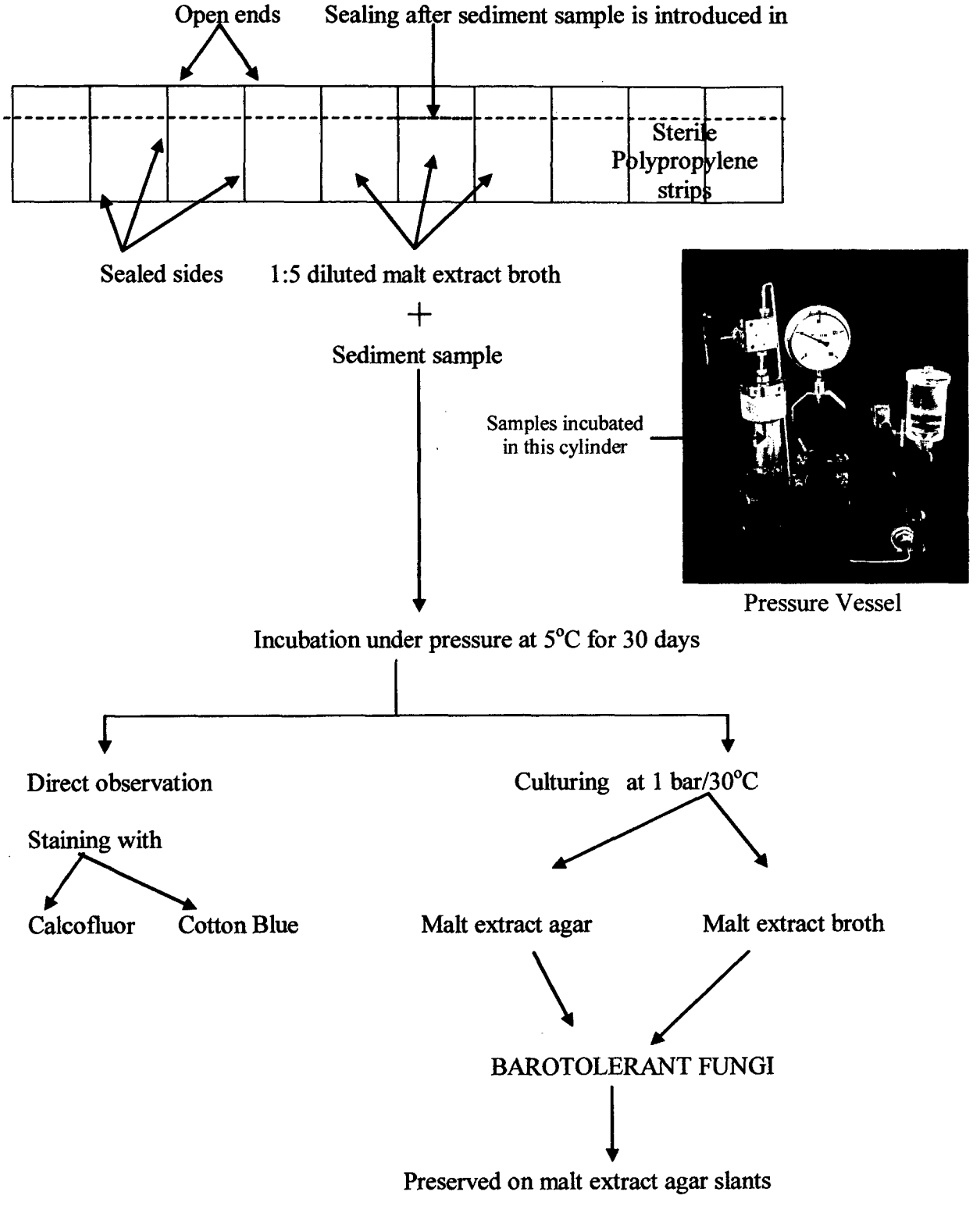


Fig. 4.4 Schematic representation of the pressure enrichment method used for isolation of fungi from the deep-sea sediments



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 Müeller 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
Aspergillus terreus	# A4634	MTCC 279
		MTCC 479
Aspergillus ustus	# NIOCC 20	MTCC 2200
Aspergillus sydowii		MTCC 635

Two primers used for the amplification were pITS1 and pITS4 [White *et al.*, 1990]. The reaction volume was 50 µL containing 1.5 mM $MgCl_2$, 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 µL 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.

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

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**
Aspergillus sp.	2 (8)	2 (11)	1 (5)	4 (25)	4 (29)	2 (12)	1 (6)	3 (14)	2 (18)	7 (54)	1 (13)
Aspergillus terreus	7 (28)	1 (5)	3 (15)	2 (13)	1 (7)	3 (18)	3 (19)	3 (14)	1 (9)	1 (8)	-
Aspergillus restrictus	3 (12)	5 (26)	2 (10)	3 (19)	3 (21)	2 (12)	2 (13)	2 (9)	-	-	-
Aspergillus sydowii	-	1 (5)	1 (5)	1 (6)	1 (7)	1 (6)	-	-	-	-	-
Penicillium sp.	2 (8)	1 (5)	2 (10)	-	1 (7)	1 (6)	2 (13)	2 (9)	-	-	-
Cladosporium sp.	1 (4)	-	3 (15)	-	1 (7)	1 (6)	2 (13)	1 (5)	-	2 (15)	-
Curvularia sp.	1 (4)	-	-	-	-	-	-		-	-	-
Fusarium sp.	-	-	-	-	-	-	-	-	-	-	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)
Unidentfied Ascomycetes	-	-	-	-	-	-	-	1 (5)	-	-	-
Aureobasidium sp.	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
Aspergillus sp.	5	2	1	4	2	-
Aspergillus terreus	-	-	1	1	5	4
Aspergillus restrictus	-	-	-	-	-	-
Aspergillus sydowii	-	-	-	-	2	1
Penicillium sp.	3	1	-	-	-	1
Cladosporium sp.	3	-	-	-	1	-
Curvularia sp.	-	1	-	-	-	-
Fusarium sp.	-	-	-	-	-	-
Non-sporulating fungi	2	3	-	4	-	-
Unidentified sporulating fungi	4	4	-	-	3	-
Unidentified Ascomycetes	-	-	-	-	-	-
Aureobasidium sp.	-	-	-	1	-	-
Unidentified yeasts	-	-	-	-	-	-

	AAS 61									
	Particle Plating					Pressure Incubation				
	ZMA	CMA	MEA	CDA	SA	MEB	Seawater MEA	Seawater CMA	Seawater SA	Seawater Artlarvae
Aspergillus sp.	1	2	5	-	4	3	-	-	-	-
Aspergillus terreus	-	1	1	1	1	4	-	4	-	2
Aspergillus restrictus	-	1	1	-	-	15	-	2	1	2
Aspergillus sydowii	-	-	-	-	-	-	-	1	-	1
Penicillium sp.	-	-	2	-	1	2	-	1	-	-
Cladosporium sp.	-	1	2	-	-	4	-	-	-	-
Curvularia sp.	-	-	-	-	-	-	-	-	-	-
Fusarium 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	-	-	-	-	-	-	-
Aureobasidium 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	*A. terreus*	AAS 46	10° 01'	75° 59'	5305	0 - 2	BC 4A	PI	MEB
A 4636	*A. terreus*	AAS 46	10° 01'	76° 00'	5400	0 - 2	BC 12A	PI	MEB
A 4634	*A. terreus*	AAS 46	10° 01'	76° 00'	5400	15 - 20	BC 12G	PI	MEB
A 4633	*A. terreus*	AAS 46	10° 01'	76° 00'	5400	10 - 15	BC 12F	PI	MEB
A 4630	*Aspergillus* sp.	AAS 46	10° 02'	76° 00'	5296	4 - 6	BC 7C	PI	MEB
A 4628	*A. terreus*	AAS 46	10° 01'	76° 00'	5400	8 - 10	BC 12E	PI	MEB
A 61 P10	*A. terreus*	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	*A. terreus*	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	*Fusarium* sp.	AAS 34	10° 00'	76° 01'	5294	15 - 20	BC 3G	PP	MEA
A 3449	*Fusarium* 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	*Curvularia* 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	*Aspergillus* 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	*Aspergillus* sp.	AAS 61	12° 59'	75° 29'	5070	30 - 35	BC 19J	PP	CMA
A 6126	*Aspergillus* 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	*Aspergillus* 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 A*rtemia* 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 of 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.

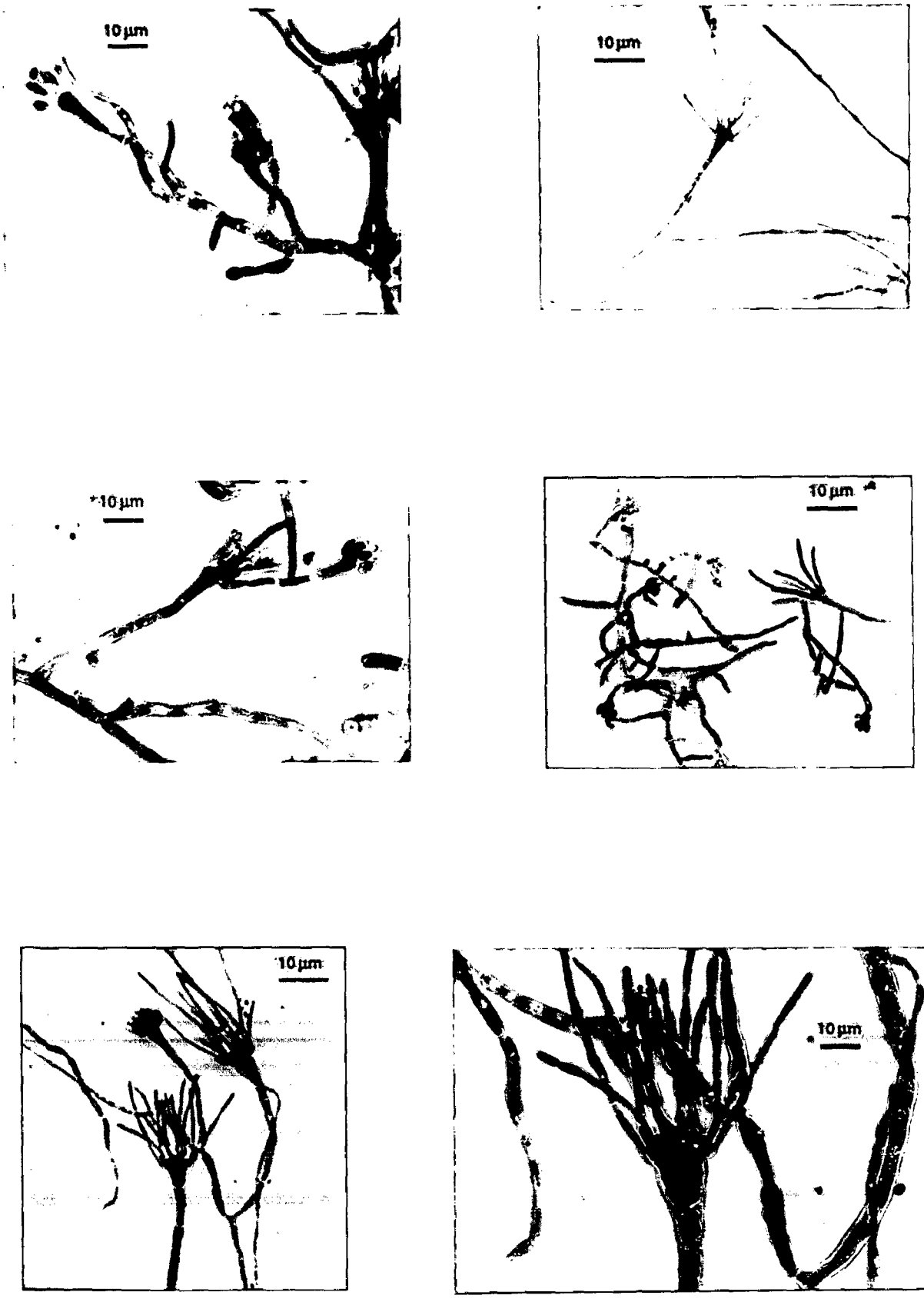
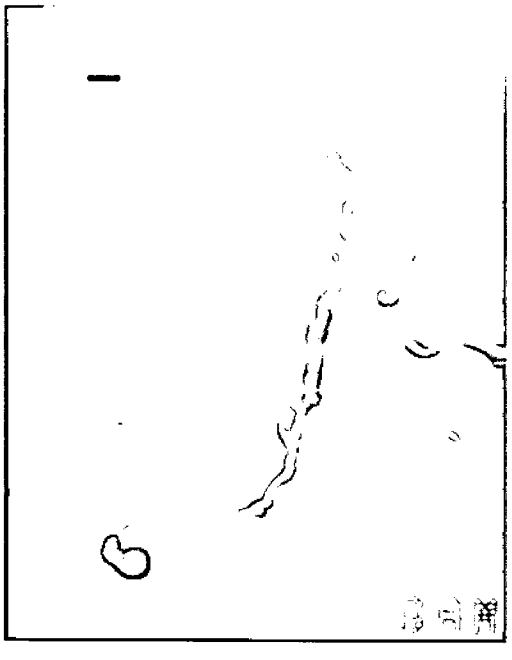


Fig. 4.5 Abnormal morphological features seen in the deep sea fungal cultures just after isolation from the deep sea sediments

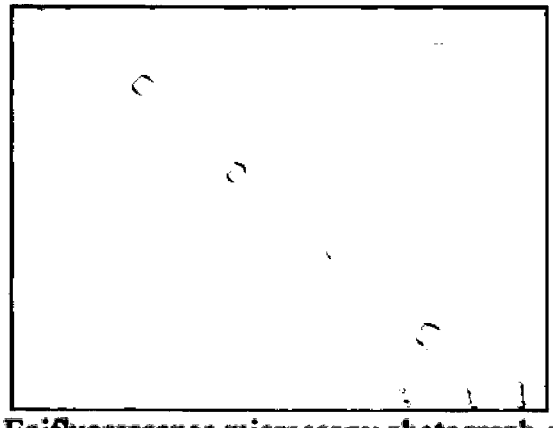
(a)

(b)

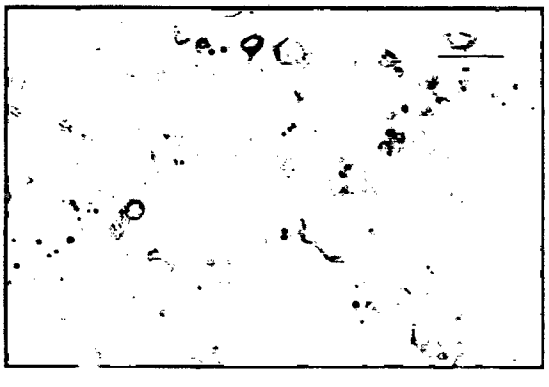
(a) & (b) Epifluorescence microscopy photograph of calcofluor stained hyphae from deep sea sediments. Bar equals 10 µm.



(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.

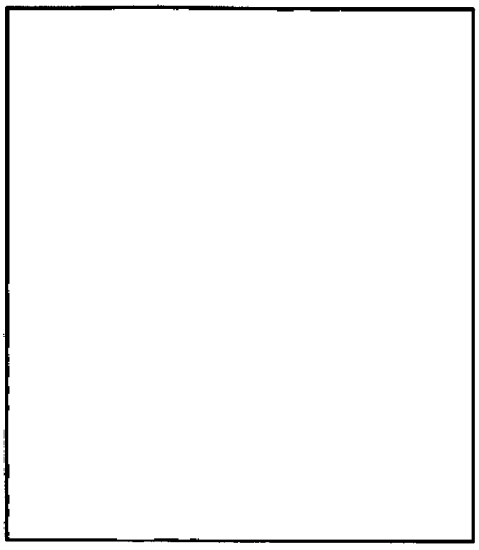


(d) Epifluorescence microscopy photograph of Calcofluor stained hyphae from deep sea sediments. Bar equals 10 µm.

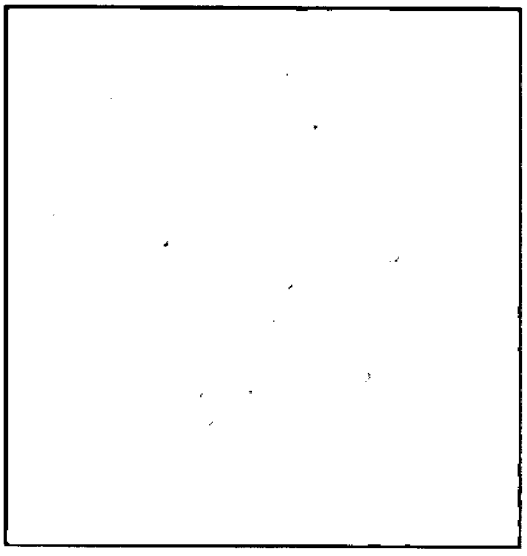


(e) Photomicrograph of the same hypha as seen under partial bright field and fluorescence light Bar equals 10 µm.

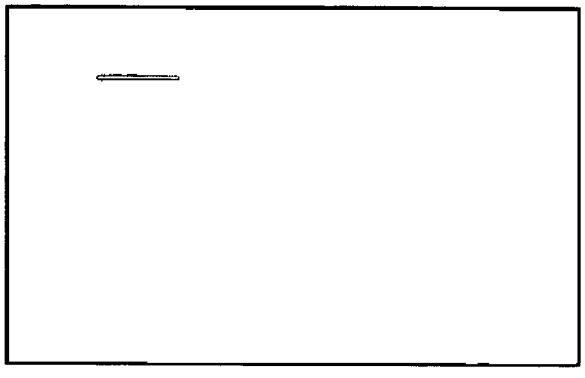
Fig. 4.6 Visualization of fungal hyphae in the deep-sea sediment stained with Calcofluor under an epifluorescence microscope.



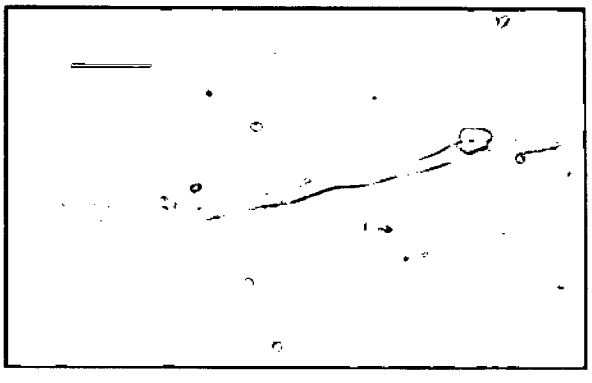
(a) Hypha showing immunofluorescence From the sediment. Bar is equal to 10 µm



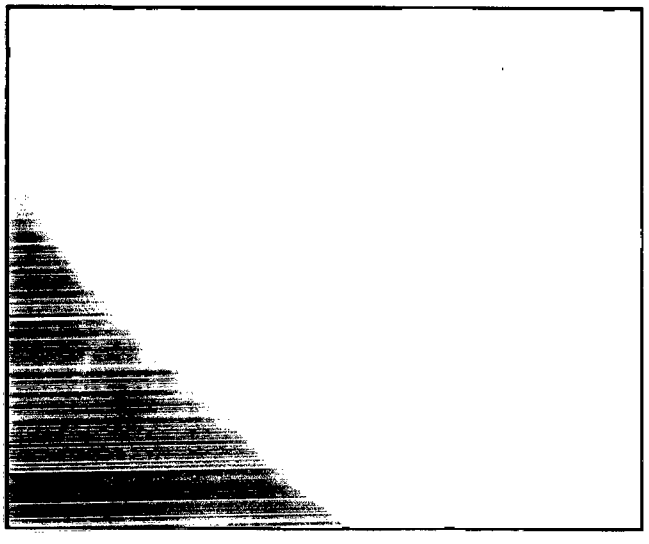
(b) Bright field photomicrograph of the same Hypha as in (a). Bar is equal to 10 µm



(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

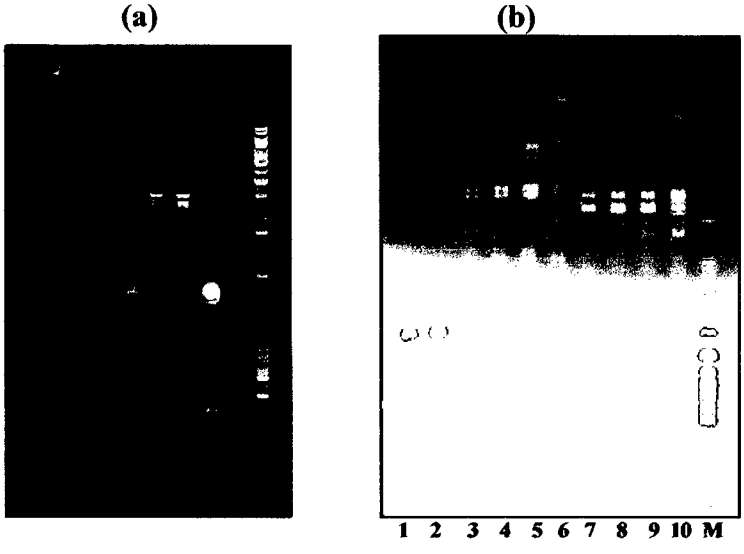


Table 4.7 Morphological and biochemical characters of the four yeast isolates

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	Ascopsores - needle-shaped or whip-like	-	-	-	-

Kev : + 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 *Rhodosporidium 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 *Rhodosporidium* 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

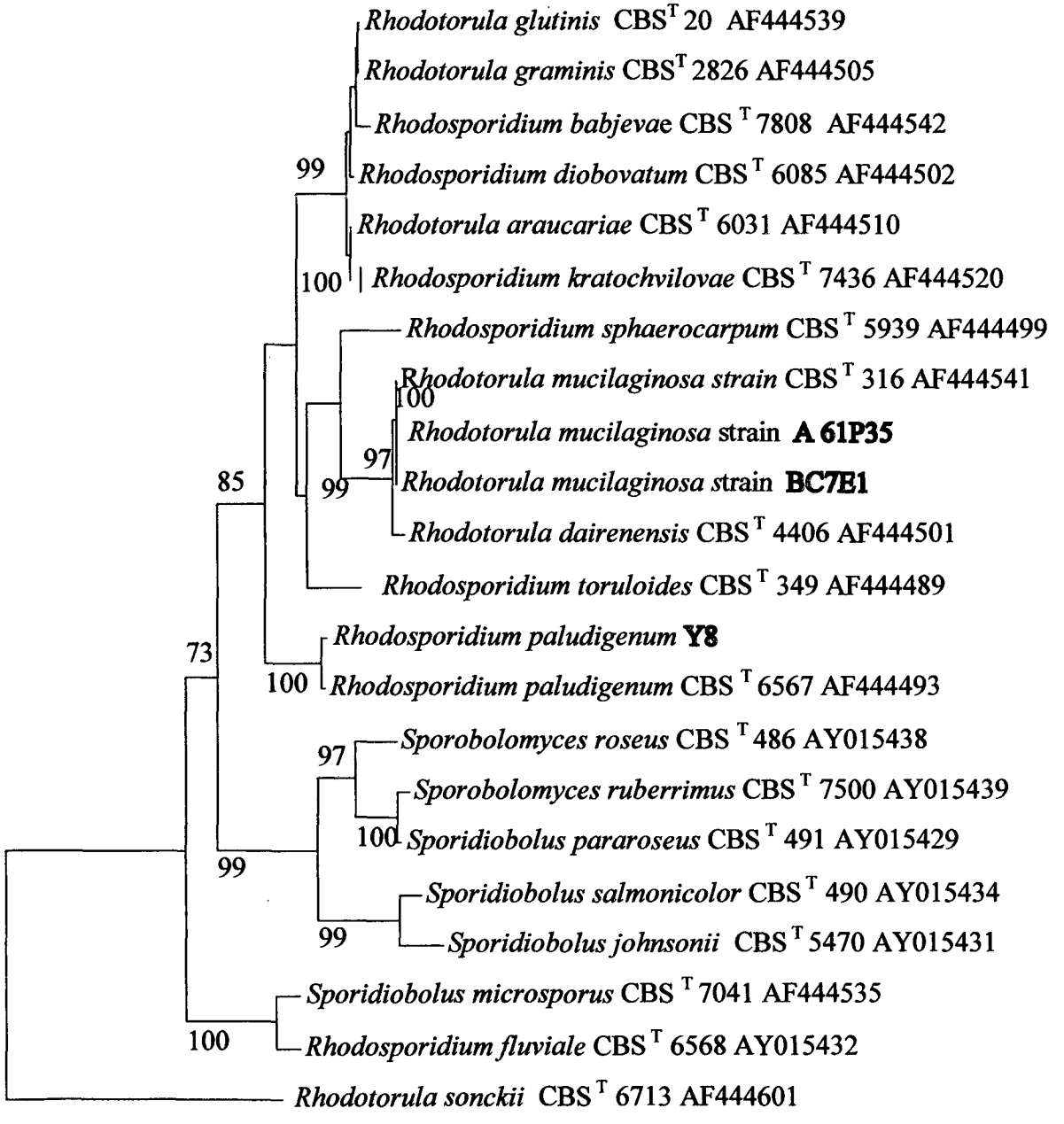
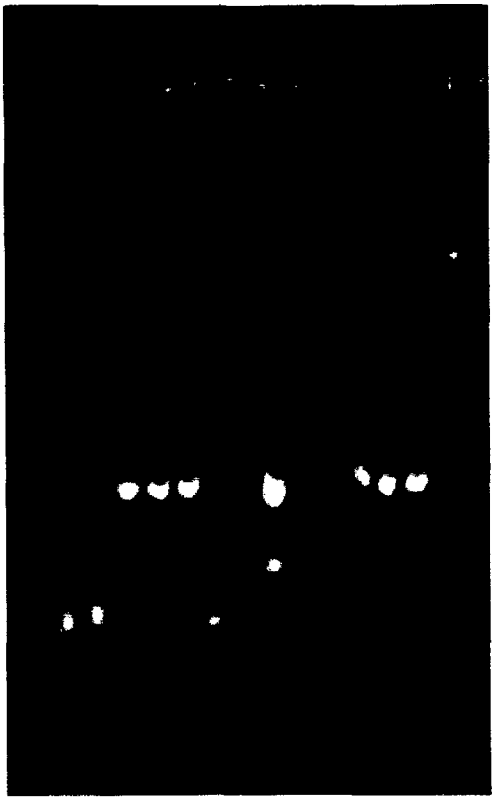


Fig. 4.9 Phylogenetic placement of A 61P35, BC7E1 & Y8 based on the ITS region of the large subunit rRNA gene. The tree was generated by NJPlot. Bootstrap values (Each expressed as a percentage of 1000 replications) greater than 70 % are given at nodes.

Rhodotorula sonckii is used as out group

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).

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 ~ 5000m 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 [Müeller & 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

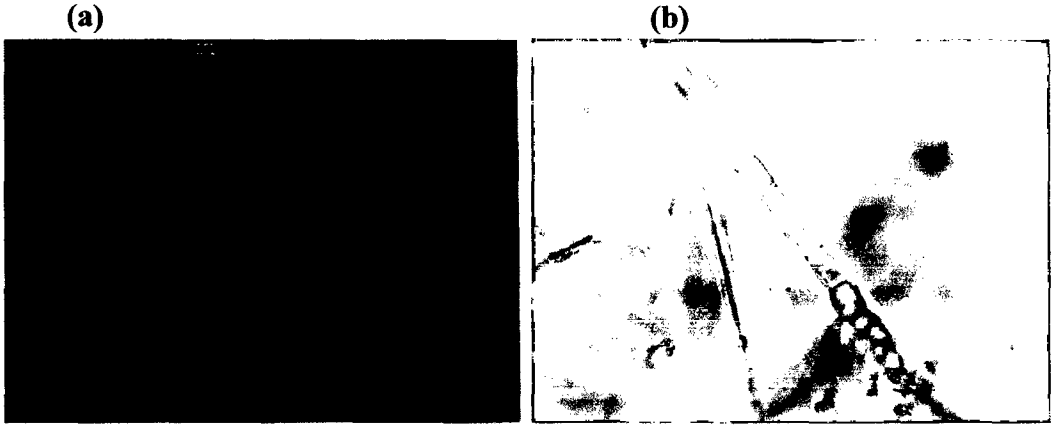
in the deep-sea sediments may be expected in view of the abundant organic material present therein. The frequency of fungal species recovered from various depths in the sediment core was not significantly different (Table 4.3), indicating the homogenous nature of the sediment in the area. Colonies of 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 hardiest forms that can adapt and survive under the most extreme conditions.

Chapter 5

Fungal Growth Under Simulated Deep-sea Conditions

5.1 Introduction

Hydrostatic pressure, as distinct from high hydrostatic pressure, is present in all biological environments. In air, the pressure is typically close to normal atmospheric pressure at sea level but it decreases with increase in altitude, simultaneously changing the partial pressure of constituent gases. The aquatic environments present the full range of hydrostatic pressure, from micro-pressures generated by a few centimeters of water column to approximately 1100 bar in the deepest ocean trenches. Microbial activity under elevated pressure, compared with that at 1 atmosphere (1 bar) is inhibited. All aspects of activity, including growth, respiration, and specific biochemical processes, appear to be affected at elevated pressures. Since the report of presence of barophilic bacteria by Yayanos in 1979, the research on these organisms started in full vigour.

The adaptation of animals and microorganisms to the high hydrostatic pressure of deep water has been studied at the cellular, kinetic and molecular levels, using orthodox biochemical and physiological preparations and methods, in conjunction with less familiar high pressure techniques and thermodynamic analysis [Somero, 1992].

Most of the physical parameters for the deep sea, such as salinity (34.8 ± 0.3 ppt), pH (7.8 – 8.0), oxygen concentration (3 - 5 mL O_2 L^{-1}) and temperature (> 50 % of the marine water masses have temperatures of less than 2 - 4°C in the deep sea) are not growth limiting in the deep-sea habitat [Zobell, 1968; Dietrich, 1978; Gage & Tyler, 1991]. The effect of hydrostatic pressure on growth of fungi is the least

investigated physical parameter but results of Zobell & Johnson [1949] and Yamasato *et al.* [1974] indicate a general and broad tolerance, at least in most yeast species.

It is a surprising fact that although the susceptibility of animals in shallow water to high (i.e. deep sea) pressures was established in 1870s, it was only in the 1970s that comparable pressure experiments were carried out on deep water animals to ascertain their short term tolerance to decompression and to high pressure [MacDonald *et al.*, 1972; MacDonald & Teal, 1975; Menzies *et al.*, 1974]. The basic requirement for these studies was an instrument, which would be able to stimulate the elevated pressure conditions existing in the deep-sea environments. Different types of instruments were used for this, but the majority of the work was carried on bacteria. Lorenz and Molitoris developed one such equipment, modified from an equipment developed for bacteria [Jannasch & Wirsen, 1977 a, b; Helmke, 1979; Schade *et al.*, 1980, Bernhardt *et al.*, 1987], to cultivate fungi, especially yeasts, under simulated deep sea condition of elevated hydrostatic pressure. With the help of these instruments developed, a lot of studies for bacteria have been carried out, but fungi have remained neglected.

Elevated pressure either inhibits or favours those biochemical processes which occur with an expansion or reduction in system volume, respectively [Somero, 1992a]. The upper pressure limit for life is yet unknown. Pressure-inducible genes, which aid in pressure acclimatization, have been proposed to exist in marine bacteria, which experience large vertical changes in the water column [Bartlett, 1991].

In case of bacteria, the pressure effects on gene expression, membranes, membrane proteins, DNA structure & function, cell division, protein & enzyme

functions have been studied in detail (Table 5.1); while in case of fungi the studies have remained restricted only up to their detection in the deep-sea sediments and ability to grow under elevated pressure and produce extracellular enzymes active under elevated hydrostatic pressure [Raghukumar & Raghukumar, 1998].

Despite the fact that the basis for all pressure effects arise from a single influence, namely the change in system volume that accompanies a physiological or biochemical process, high pressure exerts many effects on living organisms, making it difficult to indicate exact pressure - points in cell growth and viability. In addition, pressure effects also depend on other physicochemical characters, like the nutrients [Marquis, 1982], temperature & pH [Marquis, 1994]. Several workers have studied the 'wonder microorganism', *Escherichia coli* extensively for the pressure-sensitive processes. Some of the key pressure-sensitive processes described for *E. coli* have been listed in the table below.

Table 5.1 Pressure-sensitive processes in *Escherichia coli* [Bartlett, 2002]

Process	Pressure affecting/abolishing process (bar)	Reference
Motility	100	Meghnathan & Marquis, 1973
Substrate transport*	260	Landau, 1967
Cell division	200 - 500	Zobell, 1970; Zobell, 1963; Zobell, 1962
Growth	500	Yayanos & Pollard, 1969
DNA replication	500	Yayanos & Pollard, 1969
Translation	600	Yayanos & Pollard, 1969; Gross *et al.*, 1993
Transcription	770	Yayanos & Pollard, 1969
Viability	1000	Pagan & Mackey, 2000

Such extensive studies are lacking in case of fungi, with exception of yeasts like *Saccharomyces cerevisiae* and a few species of *Rhodotorula*.

5.2 Objectives

The main objectives targeted while studying the growth of fungi under elevated hydrostatic pressure were -

1. To enumerate the germination of spores under elevated hydrostatic pressure.
2. To compare the biomass production by the deep-sea and terrestrial fungi under elevated hydrostatic pressure and low temperature.
3. To try and adapt the cultures not showing growth under elevated hydrostatic pressure, to grow under these conditions.
4. To study the effect of temperature and pressure on the viability of spores.
5. To study the effect of nutrient concentrations on the germination of spores under high hydrostatic pressure.

5.3 Methodology

5.3.1 Growth and spore germination under elevated hydrostatic pressure

All the 181 deep sea fungal isolates obtained using different techniques and nutrient media were examined for spore germination and growth under elevated hydrostatic pressures.

5.3.1.1 Spore germination

The cultures were inoculated onto MEA plates and incubated at 1 bar pressure and 30 °C until sporulating fungal colonies appeared (within 3-5days). 8 mL of autoclaved seawater along with 2 mL 2 %-sterile Tween 80 (added since the spores being hydrophobic, do not mix homogeneously in the solution) was taken in sterile 15 mL centrifuge tubes. Spores were obtained by scraping the surface of the growing fungal colonies with a loop and were suspended in the seawater-tween 80 mixture in the centrifuge tubes. This was mixed vigorously by vortexing the tube for 15 to 20 min. This was used as master stock suspension for all further experiments. To this sterile sea water and tween 80 mixture, 1 mL of spore suspension prepared as described previously was added. The contents were mixed by vigorous vortexing. This is done serially till the appropriate spore count is attained, which is observed after each dilution directly under the microscope by direct examination using Neubauer counting chamber. A drop of conidial suspension was placed on the engraved grid and let to stand for 1-2 min to allow the spores to settle at the bottom. It was covered with a cover slip in such a way that no air bubbles were trapped. The number of spores in the four corners and the middle square were counted. The number of spores mL^{-1} of the suspension was calculated mathematically as follows:

$$Spores\ mL^{-1} = Average\ number\ of\ spores\ in\ one\ large\ square\ x\ 10^{4}\ cm^{-3}$$

The disadvantage of the above method was that sometimes an accurate spore count could not be done as germinating spores got trapped in the outer grids and hence could not be quantified giving false counts. As an alternative 25 µL of sample

was directly observed under the microscope on a plain slide and the total spores present on a whole slide were counted.

The spore concentration in the suspension was calculated as described above. From this suspension, 100 µL was inoculated in 5 mL of MEB. This was incubated for 20 h. At the end of this incubation period, 50 µL was spread-plated on MEA and the plates were incubated at 1 bar and 30 °C until fungal colonies appeared (within 8–10 days). The percentage of germinating conidia was calculated by counting in a Neubar chamber and CFU (colony forming units) on the MEA plates. The plates were observed to determine the dilution at which well-isolated colonies were observed. Based on these results, it was found that a spore concentration of not more than 1,00,000 and with an optimum of 50,000 to 70,000 spores mL^{-1} was ideal and this was used as the standard for all further experiments.

The spores were then inoculated in MEB to obtain a concentration of approximately 5 x 10^4 mL^{-1}, in pouches made of sterilized polypropylene and sealed without trapping any air bubbles. These pouches were suspended in deep-sea culture vessel that was filled with sterile water and pressurized to the desired hydrostatic pressure and incubated at 30° and 5°C. Three replicates were maintained for each treatment. After 7 days of incubation, the deep-sea culture vessels were decompressed gradually (at the rate of 50 bar 15 min^{-1}). The percentage of germinating conidia was counted as described above.

The spores of all the cultures including the deep-sea cultures failed to germinate at low temperature of 5°C; hence all the further experiments involving spore germination under different conditions were carried out at 30°C (5.3.7).

5.3.1.2 Biomass production under elevated hydrostatic pressure

The cultures were grown in malt extract broth for 2-3 days (before the onset of sporulation) and the mycelial biomass was homogenized using sterile glass beads. A known weight of the finely broken mycelial suspension were inoculated in malt extract broth (MEB) and incubated under 200 bar pressure at 30 and 5°C 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 in the initial and final biomass determined as mycelial dry weight.

Fungi grown under elevated hydrostatic pressure at 5° and 30°C were observed under microscope and their morphology under different culture conditions was recorded by photomicrography.

5.3.1.3 Effect of type of inoculum on growth under elevated hydrostatic pressure and low temperature

In case of a sporulating fungus, the spores germinate to give rise to vegetative mycelia, which in turn give rise to new spores. The requirements for the germination of the spores and the growth of mycelia are quite different. Due to this, spore and mycelia may react differently to the high hydrostatic pressure and low temperature. To investigate this, spores and mycelia (before sporulation sets in) of a deep-sea fungus, # A 4634 (*Aspergillus terreus*) were inoculated in sterile MEB in plastic pouches and incubated at following conditions:

1. 1 bar & 30°C
2. 1 bar & 5°C
3. 200 bar & 30°C
4. 200 bar & 5°C

After an incubation period of 20 days at the above conditions, the plastic bags were cut open and the results were recorded in terms of biomass produced.

5.3.2 Screening and acclimatization of fungal isolates for growth under elevated hydrostatic pressure

Many of the deep-sea fungal isolates failed to grow at elevated pressures of 300, 400 and 500 bar when incubated at these pressures directly. Therefore, an experiment was carried out to examine if these could be gradually acclimatized to grow 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 on MEA at 30°C / 1 bar pressure.

5.3.3 Comparative growth of deep-sea and terrestrial fungi under elevated hydrostatic pressure

A comparison was done between the deep-sea isolates, terrestrial isolates (obtained from IMTECH, Chandigarh) and some shallow water isolates (Lakshadweep lagoon) with respect to the biomass production and conidial germination under

elevated hydrostatic pressure at 30°Cand 5°C. The experimental set up was as described below.

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) and a few representative deep-sea fungi and shallow water fungi from Lakshadweep coral lagoons were used for this study. Two types of inocula were used. In one, conidia from the fungi were inoculated in malt extract broth containing 1 % glucose and incubated under elevated hydrostatic pressure of 200 bar and different temperatures (30 and 5°C) for comparing their germination ability. The number of conidia showing germination were determined microscopically after 5 days of incubation. In the other, fungal mycelia, before sporulation, were broken into fine pieces using sterilized glass beads and inoculated in malt extract broth with 1% glucose. The dry weight of the inoculum was recorded. The cultures were incubated under 200 bar at 30°C and 5°C for 20 days. The biomass as fungal dry weight was determined as described above.

5.3.4 Adaptation of cultures not showing growth under elevated hydrostatic pressure using chemical additive in the growth medium

The fungal cultures which failed to grow at an elevated pressure of 50 bar (lowest hydrostatic pressure used during the screening at which the cultures were able to grow and survive) were tried to adapt to make them grow at elevated pressures using a chemical additive in the form of DMSO (Dimethyl sulfoxide) in the growth medium.

Four deep-sea fungal cultures were used for these studies. The medium used for this was MEB with 1% glucose, 1 % DMSO and 1% TTC (2,3,5-Triphenyl Tetrazolium Chloride). DMSO is known to increase stress tolerance [Komatsu *et al.*, 1991] while TTC was used as a vital stain [Bäckor & Fahselt, 2005]. A control medium was used in the form of MEB with 1% glucose. The culture in the form of fresh mycelia, just before sporulation, were inoculated in the above medium (15 ml each) in plastic bags and incubated under an elevated pressure of 200 bar at 30°C and 5°C. The bags were opened after 20 days of incubation in the above conditions and the results recorded microscopically and in terms of biomass produced.

5.3.5 Effect of elevated pressure on viability of spores

The viability of spores incubated under elevated pressure was checked using five cultures viz. A 4634, NIOCC 20, MTCC 479, A 6137 and A617. The spore suspensions were prepared as described previously and inoculated in MEB to give approximate concentration of 6 x 10^4 spores mL^{-1} and incubated at 100, 200, 300 and 500 bar at 30°C. The viability of spores was checked after 10 days of incubation at the above mentioned hydrostatic pressures by spread plating the spore suspension on MEA plates and incubating at 1 bar / 30°C. The results were recorded in terms of CFU mL^{-1}.

5.3.6 Effect of low temperature on viability of spores

As the spores were not showing any germination at low temperature, the viability of spores at low temperature was determined. Four cultures used for this

study were A 4634, NIOCC 20, MTCC 479 and A617. The spore suspensions were prepared as described previously and inoculated in MEB to give approximate concentration of 6 x 10^4 spores mL^{-1} and incubated at 1 bar and 5°C. The viability of spores was checked randomly up to 16 days for all the cultures by spread plating the spore suspension on MEA plates and incubating at 1 bar/30°C. The results were recorded in terms of cfu mL^{-1}.

5.3.7 Effect of nutrient concentration on spore germination under elevated pressure

The effect of various nutrients and the concentration of the nutrients on the spore germination of the deep-sea and the terrestrial IMTECH cultures were studied. The nutrients were supplied in the form of MEB and sediment extract (described in detail in chapter 8) in different dilutions as 1:10, 1:20, 1:40, 1:60, 1: 80 and 1:100. The sediment extract was prepared from the deep-sea sediments obtained from Central Indian Basin. For preparation of sediment extract, 35 g of sediment was suspended in 100 mL of sterile seawater. To the sediment suspension, EDTA was added (final concentration of 1 %) [http://www.ar.wroc.pl/~weber/ekstrak2.htm] to facilitate the extraction of water-soluble nutrients into the seawater. The whole mixture was kept on a rotary shaker overnight. Next day, the suspension was filtered sequentially through sterile GF/F and 0.22 μ duraphilic filter papers (Millipore, USA). This filtrate termed sediment extract was used for the further experiment. During the extraction process, antibiotics (penicillin 40,000 U/ 100 mL and ampicillin 0.1 g / 100 mL) were added to the sediment suspension to avoid bacterial growth.

Five cultures used for this were A 4634, NIOCC 20, MTCC 479, A 617 and A 6133. The cultures were inoculated on MEA and allowed to grow and sporulate at 1 bar / 30°C. After the cultures were properly sporulating, the spores were scrapped and suspended in sterile seawater with 2 % tween 80. The mixture was vortexed vigorously to get a homogeneous spore suspension with minimum spore clumps. The spore concentration was estimated by taking the spore count on a haemocytometer. These spores were inoculated in different dilutions of MEB and sediment extract as stated above to give a spore concentration of approximately 5 x 10^4 spores mL^{-1}. These were incubated at 1 bar for 20 and 24 h and at 500 bar for 10 days. At the end of the incubation period, the spores were examined microscopically for germination and the results recorded in terms of total number of spores showing germination.

Effect of some additives like sugars on the spore germination of these cultures was also determined. The sugars checked were glucose and sucrose. The spores were inoculated in MEB with 10, 20 and 40 % glucose and sucrose and incubated under the same conditions as described above. At the end of the incubation period, the germinated spores were counted microscopically and the results recorded in terms of total number of spores showing germination.

5.4 Results

5.4.1 Biomass production under elevated hydrostatic pressure

A large number of the fungi (109 in total) did not show growth when directly incubated under elevated pressures of 500, 400 and 300 bar. Spore germination was also not seen amongst all the cultures under elevated pressure. All the cultures failed

Table 5.2 Biomass produced by a few deep-sea fungi under different pressure and temperature conditions

Isolate #	Fungi	Biomass produced (mg dry wt.)		
		200 bar and 30°C	200 bar and 5°C	1 bar pressure and 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**

to show germination when incubated at low temperature of 5°C at both 1 bar and elevated pressures. From the cultures showing growth at 200 bar, 27 isolates were used for biomass production. Amongst these, a few cultures (Table 5.2) were found to show better growth at 200 bar and 5°C than at 200 bar / 30°C.

5.4.2 Effect of type of inoculum on growth at elevated hydrostatic pressure

The deep sea *Aspergillus terreus* # A4634, showed a lot difference in the biomass produced when the inoculum was in the form of mycelia and spores (Table 5.3). The biomass produced by the culture was more or less similar at all the four incubation conditions when the inoculum was in the form of mycelia (inoculated just before sporulation sets in). When the spores were used as inoculi, there was a drastic reduction in the biomass produced at 5°C at both the pressures, 1 bar and 200 bar (Table 5.3).

Table 5.3 Biomass produced by deep sea *Aspergillus terreus* (# A 4634) at different hydrostatic pressures and temperatures when the inoculum used was in the form of mycelia and spores

	Biomass produced (mg)			
	Mycelial inoculum		Spore inoculum	
	1 bar	200 bar	1 bar	200 bar
30°C	17.6	13.4	15.7	11.6
5°C	12.4	8.9	0.41	0.2

5.4.3 Acclimatization of fungal isolates for growth at elevated hydrostatic pressure

Majority of the 109 fungal isolates that did not grow at 300 bar pressure showed growth at 50 bar pressure (Table 5.4). 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 # A 344 grew at 300 bar and only the latter grew at 400 bar pressure.

Table 5.4 Number of fungi surviving and showing active growth after exposure to sequential increase of hydrostatic pressure

Total fungi tested – 109		
Incubation pressure	Surviving	Actively Growing
50 bar	106 (97 %)	74 (68 %)
100 bar	81 (74 %)	42 (39 %)
200 bar	22 (20 %)	16 (15 %)
300 bar	11 (10 %)	3 (3 %)
400 bar	3 (3 %)	1 (0.9 %)

All the incubations were done at room temperature. The numbers within bracket represent percentage values.

Most of the fungi used for this experiment were fungi isolated at 1 bar pressure from sediments that were enriched with 1/5 diluted malt extract broth and incubated at 300 bar pressure at 5°C for 20 days. Fungi that showed active growth were included in the numbers that show survival.

5.4.4 Comparative growth of deep-sea and terrestrial fungi under elevated hydrostatic pressure

The terrestrial cultures obtained from IMTECH also showed growth at 200 bar pressure at both 30°C and 5°C, but the biomass produced by these was less than the similar deep sea isolate of *Aspergillus terreus* (Table 5.5).

Table 5.5 Comparison of biomass produced by the deep-sea isolates, the terrestrial species obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and some shallow water cultures obtained from Lakshadweep coral reef slope

Culture	Biomass produced by the fungi (mg)			
	1 bar / 30°C	200 bars / 30°C	1 bar / 5°C	200 bars / 5°C
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
Deep-sea isolates				
A. terreus (# A 4634)	11.2 (0.2)*	8.9 (0.8)	5 (0.3)	9.6 (1.1)
Aspergillus sp (#A 61 P4)	4.6 (1.0)	3.0 (0.3)	2.5 (0.6)	2.0 (0.2)
Unidentified (#A 3415)	10.5 (2.3)	8.0 (0.8)	6.2 (16.0)	5.0 (0.2)
Aspergillus sp (# A 6128)	10.0 (3.0)	3.3 (1.0)	1.9 (0.3)	1.6 (0.2)
Cladosporium sp (#A 6136)	3.7 (0.2)	0.9 (0.1)	1.4 (0.2)	1.5 (0.2)
Non-sporulating (# A 3428)	1.9 (0.3)	0.4 (0.3)	0.7 (0.2)	0.6 (0.2)
Orange yeast (#A 61P63)	1.9 (0.2)	1.3 (0.2)	1.5 (0.3)	1.3 (0.1)
Off-white yeast (#A 344)	6.6 (0.7)	1.0 (0.7)	3.3 (0.4)	1.7 (0.8)

Mycelia, before sporulation used as the inoculum; figures within brackets represent standard deviation

5.4.5 Germination of spores of deep sea and terrestrial cultures at elevated hydrostatic pressure and low temperature

The deep sea *Aspergillus terreus* (A 4634) showed germination at elevated pressure of 200 bar at 30°C (Table 5.6), but failed to germinate at 5°C (Fig. 5.1). Out of the remaining 3 terrestrial isolates, two cultures showed negligible (1 % and 0.5 %) germination and the third one failed to show germination. All 3 cultures failed to show germination at 5°C under 1 bar and 200 bar pressure.

Table 5.6 Germination of spores of the deep-sea fungus # A4634 and the terrestrial species from Microbial Type Culture Collection (MTCC), Chandigarh, India

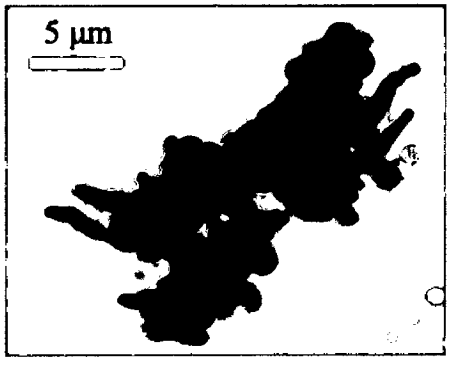
Culture	% germination			
	200 bar / 30°C	200 bar / 5°C	1 bar / 30°C	1 bar / 5°C
Aspergillus terreus MTCC 279	1	No germination	92	No Germination
Aspergillus terreus MTCC 479	0.5		94	
Aspergillus sydowii MTCC 635	0		91	
Aspergillus terreus - deep sea isolate A 4634	66		94	

5.4.6 Effect of additive in the growth medium on growth of deep sea cultures at elevated hydrostatic pressure

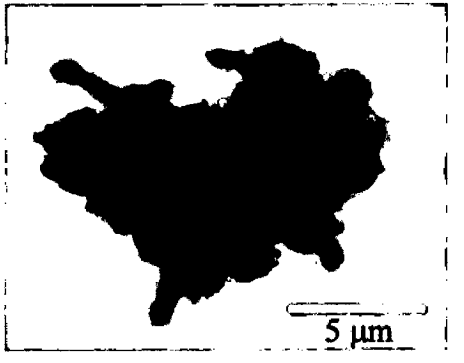
The addition of DMSO did not show significant difference in the biomass production, under different conditions of growth in all the isolates tested (Table 5.7). Generally, with increasing hydrostatic pressure, there was a decrease in biomass production in all the isolates. Only the different growth conditions influenced the biomass production significantly (Table 5.8).

Fig. 5.1 Germination of spores of deep sea *Aspergillus terreus* at different hydrostatic pressures and temperatures.

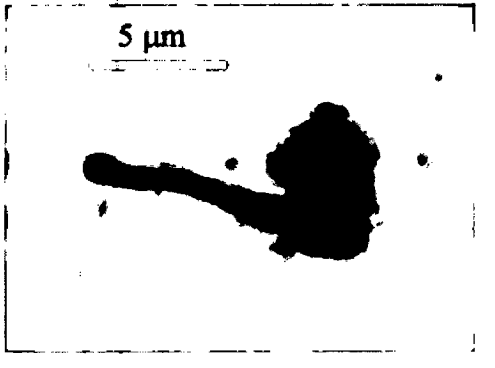
30°C and 1 bar



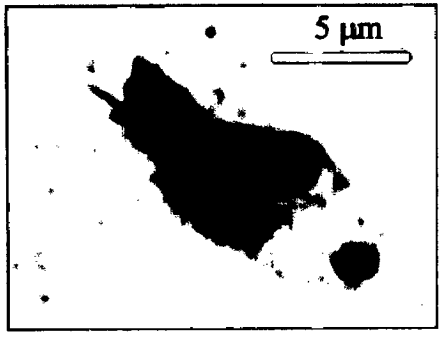
30°C and 50 bar



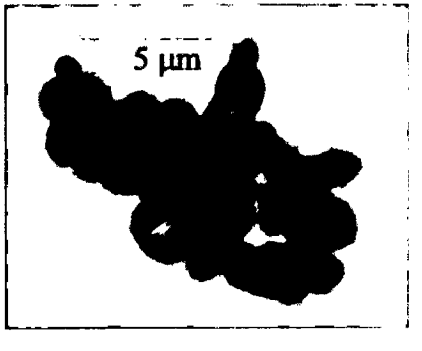
30°C and 100 bar



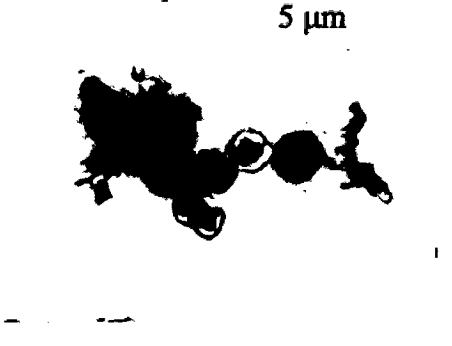
30°C and 200 bar



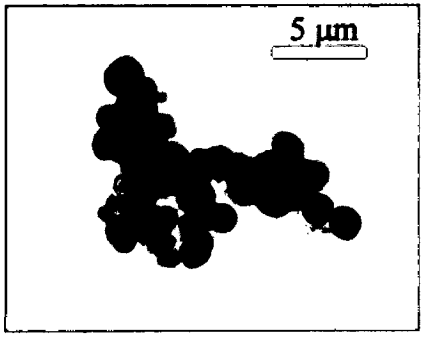
5°C and 1 bar



5°C and 50 bar



5°C and 100 bar



5°C and 200 bar

Table 5.7 Biomass (mg mL^{-1})produced by fungal isolates in the presence of DMSO

Incubation conditions	A 6136		A 6126		A 6127		A 6134	
	MEB	MEB + DMSO	MEB	MEB + DMSO	MEB	MEB + DMSO	MEB	MEB + DMSO
200 bar / 30°C	0.33	0.23	0.06	0.10	0.03	0.17	0.05	0.05
200 bar / 5°C	0.23	0.63	0.21	0.13	0.11	0.44	0.13	0.03
1 bar / 30°C	2.49	4.12	4.41	3.97	8.58	4.39	7.55	7.47
1 bar / 5°C	0.29	0.41	0.22	0.17	0.28	0.31	0.21	0.37

Table 5.8 Statistical interpretation of the effect of DMSO on biomass production by the deep-sea isolates under different incubation conditions of pressure and temperature

Culture		Df	F critical	F - value	P - value
A 6136	Growth conditions	3	9. 277	14.621	0.027
	Presence of DMSO	1	10.128	1.76	0.277
A 6126	Growth conditions	3	9.27663	366.271	0.00024
	Presence of DMSO	1	10.128	1.574	0.2985
A 6127	Growth conditions	3	9.2766	8.2427	0.0584
	Presence of DMSO	1	10.128	0.715	0.4599
A 6134	Growth conditions	3	9.2766	3892.303	0.000006
	Presence of DMSO	1	10.128	0.0072	0.9379

* F-value greater than F critical indicates statistical significance in the parameters

5.4.7 Effect of elevated hydrostatic pressure on germination of spores

The elevated pressure decreased the germination rate of all the deep-sea as well as terrestrial isolates, except in few cases The deep-sea isolate # NIOCC 20 showed highest germination rate at all the pressure used, closely followed by # A 617 (Table 5.9). Surprisingly, the terrestrial *A. terreus* (MTCC 479) showed better germination rate than the deep-sea *A. terreus* (# A 4634). In the same experiment, the

Table 5.9 Germination of different fungal isolates under elevated hydrostatic pressure

Incubation pressure (bar)	Germination of spores (%)				
	A 4634	NIOCC 20	A 6137	A 617	MTCC 479
100	10	28.5	15.7	22.1	21
200	9.2	25.1	4.2	20	19
300	4.6	16.4	12.8	11.5	4.5
500	3	7.8	4.2	5.2	6.4

* The germination results were recorded by taking haemocytometer counts after 10 days of incubation at the above-mentioned pressures at 30°C.

viability of spores decreased with increasing pressure. The deep-sea isolates showed a lot of variability amongst themselves. The spores of the deep-sea isolate # A 4634 showed highest viability at all the pressures tested (Table 5.10). The viability decreased gradually from 92.3 to 12.8 % at 100 and 500 bar respectively in case of # A 4634, while in case of other deep-sea and terrestrial isolates, the viability was not much affected by the incubation pressure, but was on the lower side.

Table 5.10 Viability of spores incubated under elevated hydrostatic pressure

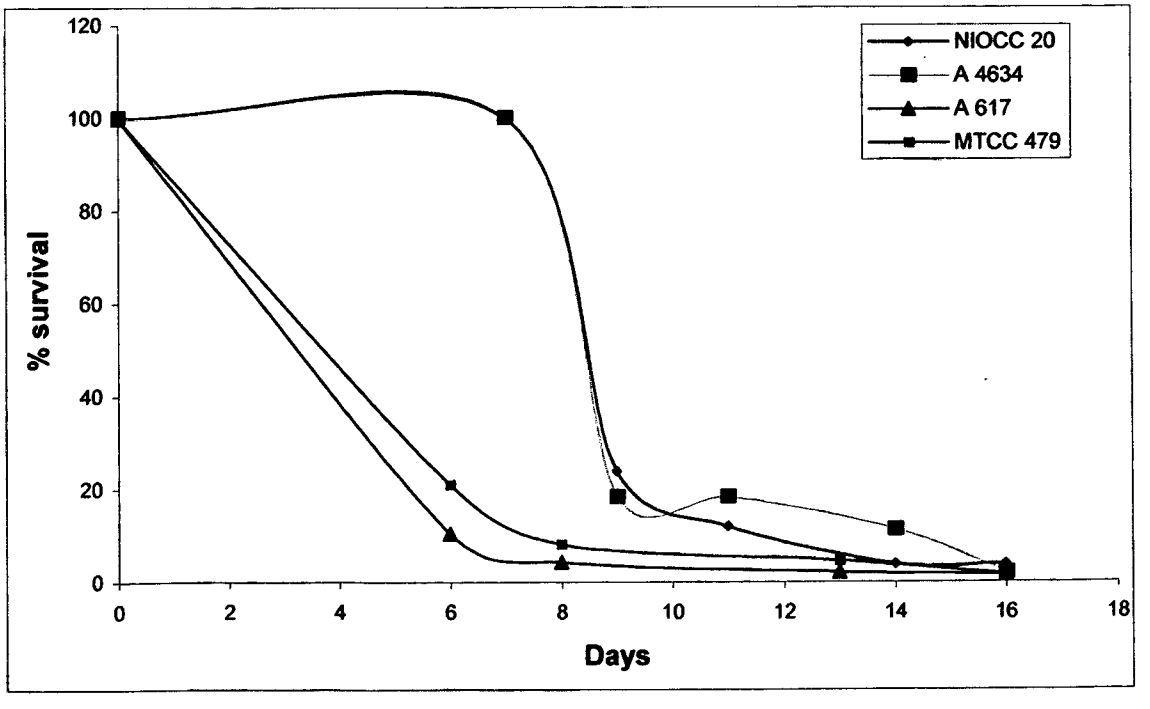
Incubation pressure (bar)	Viability of spores (%) based on CFU mL^{-1} on MEA				
	A 4634	NIOCC 20	A 6137	A 617	MTCC 479
100	92.3	14.2	9.6	13.2	14.3
200	86.9	14.2	5.4	16.8	25.2
300	62	12.1	7	14.7	21.8
500	12.8	7.1	11.5	7.4	24

* After the incubation period of 10 days at the above-mentioned hydrostatic pressures at 30°C, the aliquots were spread plated on MEA plates and the CFU counts recorded after an incubation of 2 to 3 days at 30°C.

5.4.8 Effect of low temperature on viability of spores

The viability of spores decreased over a period of time at 5°C. The deep-sea cultures A 4634 and NIOCC 20 showed 100 % viability up to 8 days while the terrestrial culture MTCC 479 and the other deep sea isolate A 617, showed a reduction

Fig. 5.2 Viability of spores incubated at low temperature over a temporal scale



in viability by more than 80 % on 6th day itself (Fig 5.2). There was a drastic drop in viability for NIOCC 20 and A 4634 by 7th to 9th day by 80 % after which the viability went on decreasing gradually, to 4 and 2 % respectively on the 16th day. The other two cultures showed more or less similar viability by the 16th day (1.7 and 1.8 %).

5.4.9 Effect of nutrient concentration on germination of spores

The spore germination was not affected by the nutrient concentrations in the sediment extract at 1 bar/ 30°C. There was a random increase or decrease in the germination percentage of the spores of both the deep sea as well as the terrestrial isolate (Table 5.11).

Table 5.11 Effect of nutrient concentration on spore germination – Sediment extract dilutions

Culture #	Dilutions					
	1:10	**1:20**	**1:40**	**1:60**	**1:80**	**1:100**
NIOCC 20	100	90	100	64	99	91
A 4634	80	71	69	67	82	59
A 617	63	73	50	73	84	77
MTCC 479	62.5	57	48	60	69	93

* The spores were incubated at 1 bar / 30°C for 24 h and the % germination of spores calculated (mentioned in the columns). There was no germination at 500 bar / 30°C after 10 days of incubation, but the spores were showing swelling, a sign of initiation of germination.

5.5 Discussion

Zobell & Johnson [1949] and Yamasato *et al.* [1974] have shown that yeasts isolated from the terrestrial and marine environments grow at elevated hydrostatic pressure, with an upper limit of approximately 500 bar. However, they grew the cultures in closed glass tubes, and only the oxygen dissolved in the medium was available for the growth of the cells. This was also true for other methods used for bacteria such as cultivation in thick-walled silicone tubing [Kosmoswosky, 1990; Groß *et al.*, 1994] and in plastic syringes [Yayanos *et al.*, 1981; Alongi, 1990]. Helmke [1980] demonstrated for actinomycetes that such cultivation techniques do not produce sufficient biomass for growth assessment. To overcome this limitation, Helmke cultured the actinomycete cells in polypropylene bags in a fluorocarbon-filled pressure vessel. Berger & Tam [1970] cultured aerobic bacteria in plastic bags but used water, which had less oxygen solubility, and due to no exchange of oxygen, the system becomes anaerobic very rapidly. In the present study also, polypropylene bags were used which resulted in a good yield of biomass, which was used for subsequent studies as reported in the later chapters.

Most of the isolates obtained were described to be terrestrial cultures based on their morphology. These could have blown from land by the winds as discussed in the previous chapter. These take some time to adapt to the conditions prevailing in the deep sea, and only a few sturdy forms are able to adapt and lead a normal life, the rest either die or remain in dormant but viable state. This could be the reason why the cultures did not grow when incubated directly under elevated pressure, but were showing growth when subjected to gradual increase in the hydrostatic pressure. This is similar to what might be occurring in the deep sea when the fungal hyphae or spores

are transported to the deeper parts, at a rate depending on the particle to which it is associated. Thus it can be said that slower the descent, more the chances for adaptation and thereby survival of the fungus. This is in concurrence with the hypothesis stated by Turner and Raghukumar *et al.* as discussed in the previous chapter.

The results indicated that the fungi should be able to grow up to 5°C at a depth of at least 3000 m (equivalent to pressure of 300 bar). But again as previously reported [Jannasch, 1987; Yayanos & Delong, 1987], there was no correlation found between isolation depths of fungi and the maximum or optimum growth pressure for them.

Low temperature is known to decrease the metabolic activities and all the cellular processes. Reduced growth and low metabolic rates under deep-sea conditions are well studied in bacteria [Wirsen & Jannasch, 1975]. The spores were unable to germinate at low temperature although they were able to germinate at elevated hydrostatic pressures, thus showing that it is temperature, which is the limiting factor and not the high hydrostatic pressure. The viability of spores and thus in effect culture was also found to be affected by the pressure as well as by the low temperature explaining the low recovery of fungi from the deep - sea sediments as compared to Lakshadweep coralline sediments.

Although DMSO has been shown to increase the chances of growth under high hydrostatic pressure in case of *S. cerevisiae* [Komatsu *et al.*, 1991], that was not the case here. DMSO did not seem to affect the biomass produced under elevated pressure. Even the spore germination did not show much effect in the presence of DMSO. This showed that DMSO may be effective osmoprotectant for yeast cells as reported previously, but not in the present studies. Komatsu *et al.* [1991] have shown

that addition of DMSO helps in structurization of water molecules which prevents yeast cells from hydrostatic pressure injury.

The spore germination doesn't seem to be affected by the concentration of the nutrients as recorded in the experiments in these studies. Thus, in the deep-sea sediments, sudden increase or decrease of nutrients (Feast & Famine) in the vicinity may not affect the germination of spores. This could be also due to the fact that the metabolic activities of the spores are much lower than those of mature hyphae, hence **less requirement of nutrients [Hawker & Madelin, 1976].**

To answer the questions about how cells respond to changes in hydrostatic pressure, a new research field called baro-(piezo-) physiology was developed. In this field, intermolecular interactions among various biological molecules, aspects of cellular metabolism and chemical reactions with large volume changes are the different topics of interest. In the next chapter we will deal with two of these aspects viz. stress proteins and an osmoprotectant, trehalose.

Chapter 6

Adaptation of Fungi to Deep-sea Conditions

6.1 Introduction

The deep-sea microbial community is very complex as described in the previous chapters. Fast sinking particles from surface water contain surface bacteria, cyanobacteria, phytodetritus, etc. These particles can reach a depth of 4500 m in only 4 to 6 weeks [Turley, 1995]. Thus, there must be numerous '1-bar-adapted' organisms as well as high-pressure adapted organisms at the bottom of the sea. Additionally, large number of non-peizophiles, the growth rate of which are reduced by elevated hydrostatic pressure, are present in the deep-sea sediments. Therefore to understand the complete diversity of microbial life under deep-sea conditions, we must consider the survival strategies not only of peizophilic organisms but also of 1 - bar - adapted organisms.

Hydrostatic pressure in the range of 300-500 bar usually inhibits the growth of various organisms. The basis of all pressure effects arises from the single fact, that is, the change in the system volume that accompanies a biological reaction. When a reaction is accompanied by a volume increase, it is inhibited by increasing pressure. When a reaction is accompanied by a volume decrease, increasing pressure enhances it. For example, dissociation of ribosome subunits is significantly enhanced by increasing pressure because solvation of charged groups is accompanied by a volume decrease [Abe & Horikoshi, 2001].

DNA synthesis is one of the most pressure-sensitive cellular processes, with the initiation of DNA replication rather than polymerization itself being particularly sensitive. By contrast, RNA synthesis is relatively pressure resistant and occurs at pressures up to 680 bar [Yayanos & Pollard, 1969]. Protein synthesis, however, is highly sensitive to elevated pressure. Ribosomes associated with both mRNA and

tRNA show perfect stability at elevated pressures up to 1000 bar. However, uncharged ribosomes (ribosomes without mRNA or tRNA) become dissociated at pressures greater than 600 bar [Gross, 1993]. Hence, the destabilization of uncharged ribosomes is one of the factors limiting cell growth under conditions of high hydrostatic pressure. In this sense, peizophiles which can grow at pressures > 600 bar, are likely to have undergone critical changes in their ribosomal proteins.

In *Escherichia coli*, a pressure of 546 bar induces a unique stress response that results in higher levels of heat-shock proteins (Hsps) or cold-shock proteins (Csps), as well as many proteins that are produced only in response to high pressure [Welch *et al.*, 1993]. DnaK and GroEL, which are molecular chaperones [Craig & Gross, 1991; Hartl, 1996], are induced at 546 bar. In the yeast *Saccharomyces cerevisiae*, an extreme pressure of 1500 bar causes severe damage to membrane integrity and protein structure, resulting in cell death [Kobori *et al.*, 1995]. It has been found that a short period of heat shock treatment allows the cells to survive at this lethal level of pressure [Iwahashi *et al.*, 1991]. A molecular chaperone, Hsp 104, and intracellular trehalose are known to play a role in cell survival at extremely high pressures [Iwahashi *et al.*, 1997].

Studies aimed at ascertaining what happens intracellularly as hydrostatic pressure is applied to living cells can provide an insight into the dynamics of metabolic events that occur in response to pressure. In one such study, the intracellular pH in *S. cerevisiae* was measured under high hydrostatic pressure using pH sensitive fluorescent probes [Abe, 1995 & 1997]. Hydrostatic pressure promotes the acidification of vacuoles in a manner dependent on the magnitude of the pressure

applied, up to 600 bar. Pressure-induced vacuole acidification is caused by the production of carbon dioxide [Abe, 1997].

Because the effect of pressure on membrane structure is considerable, the function of membrane proteins must also be affected by increasing pressure. *Photobacterium fundum* SS9 has been investigated genetically in great detail, particularly the synthesis of the outer membrane proteins OmpH and OmpL. OmpH is most abundant when SS9 is grown at its pressure optimum of 280 bar, whereas OmpL is produced in the greatest quantity at 1 bar [Bartlett *et al.*, 1989; Welch & Bartlett, 1996]. OmpH is thought to function as a nutrient transporter in nutrient-limited environments such as the deep sea.

The significance of nutrient availability was genetically documented in *S. cerevisiae*, the growth of which is inhibited by high pressure. Under high-pressure conditions, the uptake of tryptophan via the high-affinity tryptophan permease TAT2 is impaired and the expression of TAT2 is down regulated, leading to growth arrest [Abe & Horikoshi, 2000]. These results are consistent with those of transport studies in marine bacteria, which indicate that the rate of uptake of many substrates, including glutamate and acetate is low at a pressure up to 180 bar. Interestingly, the addition of excess tryptophan or over expression of TAT2 protein enables *S. cerevisiae* cells to grow at 250 bar [Abe & Horikoshi, 2000]. These results suggest that the uptake of tryptophan is one of the most pressure-sensitive processes in living yeast cells.

There is evidence that the composition and structure of the lipid bilayer of the cell membrane, respiratory proteins and a membrane-localized signaling system contribute significantly to baro- (peizo-) adaptation. However the main difficulties in

understanding pressure regulated metabolic processes in living cells are that --- 1) the effects of pressure on living organisms are usually analyzed after decompression and; 2) it is still difficult to predict the orientation of a given metabolic pathway at elevated pressure. Therefore direct measurements under high-pressure conditions are required to investigate the association and/or dissociation of proteins, metabolic flux and electron transfer through respiratory system [Abe *et al.*, 1999].

The fungal stress response often involves the production of various protective molecules [Jennings, 1993] and these form complexes with essential enzymes keeping them functional.

Due to all these facts, there is a great interest in elucidating the mechanisms of high-pressure adaptation in peizophiles. However, even non-peizophiles respond to changes in hydrostatic pressure. These organisms may well be able to place themselves in a state of suspended animation without significant loss of viability until the pressure is reduced and growth conditions become more favourable.

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

Stress proteins were first described in cells from *Drosophila melanogaster* in response to exposures to high temperatures [Ritossa, 1962], and so the term 'Heat Shock Proteins' (Hsp) was coined. Since then a range of environmental stresses have been shown to induce heat shock proteins, and the term 'Stress Proteins' has subsequently been used to describe these proteins. The environmental stresses, which induce these proteins, include trace metals [Sanders *et al.*, 1991], changes in osmolarity [Kultz, 1996], hypoxia/ anoxia [Mestril *et al.*, 1994; Myrmel *et al.*, 1994] and exposure to UV radiation [Nepple& Bachofen, 1997]. These proteins are among the most highly conserved proteins in existence [Lindquist & Craig, 1988]. Either the stress proteins themselves or their close relatives present in all organisms at normal growth conditions play vital roles in normal cell function also.

In all organisms, the induction of Hsps is remarkably rapid and intense showing that it is an emergency response [Daniels *et al.*, 1984; Kimpel & Key, 1985; Li & Laszlo, 1985] and there is a striking relationship between the induction condition and the organism's environment. For example, there are many dimorphic pathogens that cycle between relatively cool temperatures in one phase of their life cycle and the warmer temperature of their mammalian hosts in another phase. This change in temperature is accompanied by the strong induction of Hsps in both prokaryotic and eukaryotic pathogens.

In recent years, major focus on the role of the stress proteins has been mostly in -

1. Cell biology in regulatory concerns of protein folding-unfolding and transport.
2. Immunobiology of the involvement in the immune response during tissue or cell damage and infectious diseases

3. Pathophysiology and medicine on the effect of stress response in human disease.

At present, the stress proteins are classified into six different classes as –

1. **Hsp 110 kDa and above** – Most of the eukaryotes produce proteins of greater than 100 kDa in response to high temperatures. This is a constitutively expressed protein in eukaryote. Its synthesis increases about 5-fold after stress conditioning. The synthesis of the protective heat shock produced in response to high temperature is regulated by the 110 kDa heat shock transcription factor (hstf).
2. **Hsp 90** – This group is one of the most abundant cytoplasmic proteins in most of cells even under normal conditions. This is found in association with several cellular proteins. Association with Hsp 90 either enhances or inhibits the normal function of these proteins. The normal abundance of Hsp 90 may limit the extent to which its synthesis may be induced on exposure to stress.
3. **Hsp 70** – The gene HSP 70 encodes the abundant heat inducible 70 kDa Hsp. HSP 70 of most eucaryotes is a member of a multigene family whose genes are expressed under a variety of physiological conditions. The Hsp 70 family accounts for a majority of the translational activities in cells in response to the environmental changes.
4. **Hsp 60** (or chaperonin) – The major bacterial hsps belong to this class. It is also found in the mitochondria and chloroplasts of the eukaryotic cells. These may be associated transiently with the newly synthesized unfolded peptides. This class of proteins is also required in bacteriophage assembly and host DNA

replication at normal conditions. This is also called as Gro-EL in *E. coli* and in other related bacteria.

5. **Small Hsps** – The small Hsps are a very diverse group. These are 15 – 30 kDa, low molecular weight (LMW), stress proteins. These proteins are more species specific than the larger stress proteins and less conserved, with significant variation occurring within the same class of organism [Mansfield & Key, 1987]. The small Hsps share the property of being induced at specific stages in development at normal temperatures. These proteins are not synthesized under normal conditions. Their synthesis is regulated during development and differentiation is modified by environment [Ayme & Tissiers, 1985; Ingolia, 1982].
6. **Ubiquitin** – This is highly conserved 76-residue protein, which is found in all eukaryotic cells, and is induced by heat. Its synthesis is increased with increase in temperature. It has a complementary role to Hsp70 in resolubilizing and stabilizing proteins by targeting denatured proteins for degradation and removal.

Cold shock proteins (Csps) – These are relatively new types of stress proteins, first reported in *Escherichia coli*, viz. Csp A. Both eukaryotes and prokaryotes exhibit a cold shock response upon an abrupt temperature downshift [Thieringer *et al.*, 1998]. Membrane fluidity decreases with decreased temperature, affecting the membrane-associated cellular functions. Cold shock also causes stabilization of secondary structures in RNA and DNA resulting in reduced efficiency of translation, transcription and DNA replication [Phadtare *et al.*, 1999]. These deleterious effects are overcome by induction of cold shock proteins. Cold-shock-inducible proteins are the

key factors needed for cellular adaptation to lower temperature. Identification of cellular thermosensors and elucidation of the functions of the cold-shock proteins is essential for understanding cold-shock response and adaptation.

Stress proteins are studied by a variety of molecular and cell biology techniques like-

1. **Polyacrylamide gel electrophoresis** - The classical way to visualize the total protein expressed by a cell is two-dimensional IEF/SDS-PAGE as developed by O'Farrell [O'Farrell, 1975]. This method can, under favourable conditions, resolve more than 1000 proteins in one gel and needs only minimal amounts of cell extract. Proteins are separated according to their isoelectric point by isoelectric focussing in the first dimension and in the second dimension, it separates out according to molecular weight of the proteins. The technique has been successfully applied to investigate the pressure response of bacteria like *Escherichia coli* and *Thermotoga maritima*, filamentous fungi like *Asteromyces cruciatus* and *Dendryphiella salina*, and marine yeasts *Debaromyces hansenii*, *Rhodosporidium sphaerocarpum* and *Rhodotorula rubra* [Groß *et al.*, 1994].
2. **Northern blotting** – In this, the total RNA is extracted from the tissue/cells. This is subjected to agarose-formaldehyde or glyoxylic gel electrophoresis, and the RNA separated on nylon or nitrocellulose. After fixation, membrane is hybridized with desired labelled cDNA probes. Visualization can be done by radiography.

3. **PCR** – Since stress proteins are gene-induced products, the study of the structure of individual stress genes in living organisms is very important. Molecular cloning allows such study.
4. **Western blotting** – Western blotting describes the transfer of proteins from an electrophoresis gel to membrane (usually nitrocellulose), and immunoblotting (or immunoassay), the subsequent probing (detection) of these proteins using immunological techniques (i.e. the use of antibodies). Western blotting may be performed with chromophores or radio-labelled samples, either to compare the protein detected by immunoblotting with those labeled with radio-isotope, or because a particular sample is only available as a radiolabelled protein.

Trehalose as a stress protectant

One of the protective compounds known to be accumulated during stress is the sugar, trehalose (α-D-glucopyranosyl (1-1) - α-D-glucopyranoside) [d'Enfert & Fontaine, 1997]. Trehalose is a disaccharide composed of two glucose molecules bound by an alpha, alpha-1, 1 linkage. Since the reducing end of a glucosyl residue is connected with the other, trehalose has no reducing power. It is widely distributed in nature. It is known to be one of the sources of energy in most living organisms, including bacteria, fungi, insects, plants, and invertebrates. It is known to protect organisms against various stresses such as dryness, freezing, pressure, etc. Trehalose plays a key role in stabilizing membranes and other macromolecular assemblies under extreme environmental conditions. Apart from trehalose, other stress protectants reported in microorganisms are osmolytes like glycine betaine, ectoine, sucrose, mannitol and glycerol [DasSarma & Arora, 2001]. Organic osmolytes occur at high

levels in marine invertebrates. These are mostly free amino acids such as taurine, which are compatible with cell macromolecules and methylamines such as trimethylamine oxide (TMAO), which may have nonosmotic role as protein stabilizer, and present in higher amounts in many deep sea animals [Yin *et al.*, 2000]

Trehalose has been detected in a variety of organisms and is known to serve numerous functions [Crowe *et al.*, 1992]. In fungi, the role of trehalose has been subject to great debate but large amounts of evidence have established the disaccharide as a protectant of cellular structures during stress [Thevelian, 1996]. In yeast, the investigations of arguments for trehalose as a stress protectant are numerous. The survival of *S. cerevisiae* grown in 3M NaCl correlated significantly with intracellular levels of trehalose, while mutants unable to produce trehalose were more sensitive to the severe salt stress treatment [Hounsa *et al.*, 1998]. The accumulation of trehalose has also been seen in cells of *S. cerevisiae* grown media lacking in carbon, nitrogen, phosphorus or sulphur and in cells subjected to desiccation or freezing [Thevelian, 1996].

Whether trehalose is accumulated in all fungal species during stress and whether this accumulation is universal or relates to specific stress factors remains to be established. The regulated trehalose hydrolyzing enzymes have mainly been investigated in zygomycetes and yeasts even though higher fungi may be capable of regulating the level of cytosolic trehalose.

6.2 Objectives

The main objectives kept in mind while studying the adaptive response to pressure and temperature stress by the deep-sea fungi were –

1. To compare the difference in whole cell protein profiles of the cultures when grown at different temperatures and hydrostatic pressures.
2. To investigate the response of the cultures to hydrostatic pressure when exposed for different time durations
3. To estimate accumulation of trehalose by the cultures in response to exposure to different temperatures.

6.3 Methodology

6.3.1 Response of one bar grown cultures to elevated hydrostatic pressure and low temperature

The cultures used for these studies were those of 2 filamentous fungi (one deep sea *Aspergillus terreus* and one *Aspergillus terreus* from IMTECH) and one deep-sea yeast. The response of these cultures with respect to proteins produced under the stress of pressure and temperature was studied.

The studies were carried out in 2 different parts --------

1. Effect of elevated pressure when exposed for different time periods
2. Effect of different pressures for a fixed time period.

6.3.1.1 Effect of pressure for different time periods

The cultures were grown in malt extract broth (MEB) prepared in seawater for 2 to 3 days. Just before sporulation, the mycelia were homogenized and inoculated in MEB with 1% glucose in plastic bags and incubated under hydrostatic pressure of 300 bar at 30°C and 5°C, and at atmospheric pressure at 30°C and 5°C. After 6, 12, 24 and 48 hours of incubation at the above conditions, one bag of each was harvested and lyophilized for protein extraction. The second set of bags at the 300 bar was further incubated at atmospheric pressure at the respective temperatures, while the bags at

atmospheric pressure were continued to be incubated at the same conditions. After 6 days of incubation, the bags were opened, cultures harvested and lyophilized for protein extraction later.

6.3.1.2 Effect of different pressures for a fixed period (48 hours)

The cultures were grown in malt extract broth (MEB) prepared in seawater for 2 to 3 days. Just before sporulation, the mycelia were homogenized and inoculated in MEB with 1% glucose in plastic bags and incubated at 50, 100, 200, 300, 400 and 500 bar for 48 hours. After 48 hours of incubation at the above conditions, one bag of each was harvested and lyophilized for further protein extraction. The second set of bags at the 50 bar was further incubated at atmospheric pressure at the respective temperatures, while the bags at atmospheric pressure were continued to be incubated at the same conditions. After 6 days of incubation, the bags were opened, cultures harvested and lyophilized for further protein extraction.

From the biomass harvested in the above experiments, the total protein was extracted using ProteoPrep^{TM} Sample Extraction Kit as described below. The total protein concentration in the extract was determined using Folin Phenol method and electrophoresed using SDS-PAGE and 2-D PAGE.

6.3.2 Protein extraction methods

6.3.2.1 Whole cell protein extraction

The protein was extracted from the lyophilized biomass using ProteoPrep Sample Extraction Kit (PROT-TOT, Sigma) (Appendix A – III). The protocol described by the supplier was used which is described herein. 100 mg of lyophilized,

powdered biomass was suspended in 2 mL of Reagent 4 (Cellular and Organelle Membrane Solubilizing reagent) and sonicated. During sonication care was taken that the temperature of the mixture did not rise above 30°C. The temperature should also not fall below 15°C since the urea and thiourea will precipitate out of the solution. The sonicated suspension was then centrifuged at 15,000 g for 30 minutes at 15°C. The supernatant was transferred into a clean tube and the cell debris was discarded. The supernatant was then reduced by adding tributyl phosphine to a final concentration of 5 mM and incubated at room temperature for 1 hour. After reduction, the sample was alkylated by adding iodoacetamide to a final concentration of 15 mM and incubating for 1.5 hours at room temperature. The final reduced and alkylated sample was centrifuged at 20,000 g for 5 minutes to pellet any insoluble material. This was the final protein extract. The total protein concentration of the samples was measured by reading absorbance at 280 nm.

6.3.2.2 Urea buffer extraction method

Urea sample buffer extraction method was followed. The lyophilized and powdered biomass (approx. 100 mg) was mixed with urea buffer (Appendix A - IV). The mixture was mixed vigorously and boiled for 2 minutes. Then the mixture was vortexed and again boiled for 1 minute. The mixture was cooled to room temperature, centrifuged and the supernatant collected. Total protein estimation in the supernatants was carried out using Folin phenol method [Lowry *et al.*, 1951] (Appendix A - V). Ten μg protein of each extract was loaded on to 12 % resolving gel for electrophoresis [Laemmeli, 1970] (Appendix A - VI). The electrophoresis was carried out at a constant 60V. After electrophoresis, the gels were stained as per silver staining

method [Heukeshovan & Dernick, 1985]. The gels were first fixed in a fixative solution for a minimum period of 60 minutes. The fixed gels were then dehydrated in methanol. The gels were rehydrated in 0.02 % $Na_2S_2O_3$. After the gels were rehydrated, a rinse with distilled water was carried out. The gels were then transferred to silver stain solution. After an incubation period of 30 minutes, the gels were rinsed thoroughly with distilled water to remove the residual unreacted silver stain. The developer was added to the gels and gently mixed till the protein bands appeared. After the intensity of the bands was sufficient enough for recording, the developing reaction was stopped by discarding the developer and adding the fixative solution once again (Appendix A - VII). The stained gels were photographed using Gel documentation system.

6.3.3 Intracellular protein profiles of cultures grown in different conditions of hydrostatic pressure and temperature

Two filamentous fungi, *Aspergillus terreus* from the deep-sea sediment (#A 4634) and from Microbial Type Culture Collection (MTCC 479), Chandigarh, India and one yeast isolate (# A344) obtained from the deep-sea sediments were used for this experiment. The cultures were inoculated in MEB and incubated under 1 bar / 30°C for 48 hours; in case of the yeast, they were incubated in a shaker water bath. After sufficient growth in MEB, these cultures were homogenized with glass beads and inoculated in MEB with 1 % glucose in sterile plastic pouches and incubated under following conditions -

1. 1 bar / 30°C
2. 1 bar / 5°C
4. 100 bar / 5°C
5. 500 bar / 30°C

3. 100 bar / 30°C 6. 500 bar / 5°C

The inoculum was harvested, lyophilized and kept as control.

After 20 days of incubation under the above conditions, the pressure vessels were depressurized as described previously and the biomass harvested, washed with sterile distilled water, lyophilized and used for preparing the total cell protein extracts. The proteins were extracted using the urea buffer extraction method. The total protein content in the extracts was measured using Folin phenol method. Ten µg protein of each extract was loaded on to 12 % resolving gel for electrophoresis [Laemmeli, 1970] (Appendix A - VI). The electrophoresis was carried out at a constant 60V. After electrophoresis, the gels were stained by silver staining method described above. The stained gels were photographed using Gel documentation system (AlphaImager 2200, U.S.A).

6.3.4 Western blotting

The whole cell proteins extracted using the Sigma total protein extraction kit were used to detect the type of proteins produced in response to the stress of pressure and temperature by the fungal cultures. After electrophoresis, the proteins on the polacrylamide gels were electroblotted onto nitrocellulose membranes using the electroblotting apparatus (Bio-Rad, USA). The blotting was carried out at 500V for 3 h in the transfer buffer (Appendix A - VIII). After transferring the proteins on to the membrane, the membrane was stained with Ponceau R in order to stain the marker and check for the presence and location of the proteins in the samples on the gel. After marking the positions with a lead pencil, the membrane was washed with TBST buffer (Appendix A - VIII) to remove the Ponceau stain. Then the proteins on the membrane

were blocked with blocking agent (5% milk) for 60 min. Again the membrane was washed with TBST buffer (10 min x 3 times). Primary antibody (anti Hsp 25 and anti Hsp 70) was then added on to the membrane and allowed to react for 60 minutes. The excess antibody washed away using TBST buffer (5 min x 3 times). Then the secondary antibody (goat anti-rabbit IgG – ALP, Sigma Chemicals, Cat. A 3687) was added on to the membrane and allowed a reaction time of 60 minutes. The excess secondary antibody was washed using TBST buffer (5 min x 3 times). The substrate for alkaline phosphatase, conjugated to secondary antibody, BCIP/NBT was added and the membrane gently shaken till purple bands appear. The excess substrate was immediately washed with distilled water and membrane allowed to dry, and the results recorded. The two anti – hsps used (anti-Hsp 25 & anti-Hsp 70) were from Stressgen, Canada (Cat. SPA – 810C & SPA 811 respectively).

6.3.5 Trehalose accumulation by yeast cells in response to elevated hydrostatic pressure and low temperature

Three yeast cultures were used for these studies. One of them was a filamentous yeast (A 344) and the other two were the orange yeasts (A61P35 & BC7E1) isolated from the deep-sea sediments.

To raise the inoculum, the cultures were inoculated in 20 mL sterile MEB and incubated at 30°C on a shaker for 48 hours. From these flasks, the cultures were inoculated to 100 mL MEB and incubated for a further 48 hours at 30°C on a shaker. These growing cultures were then added to sterile plastic pouches to be incubated under following conditions –

1. 100 bar / 30°C
2. 200 bar / 30°C
3. 300 bar / 30°C
4. 500 bar / 30°C
5. 1 bar / 30°C
6. 1 bar / 15°C
7. 1 bar / 5°C

One part of the culture, used for adding to the pouches was harvested by centrifugation, washed with distilled water and lyophilized for trehalose extraction.

The cultures were incubated at the above-mentioned conditions for a period of 18 hours, at the end of which, the cultures were harvested by centrifugation, washed with distilled water and lyophilized for trehalose extraction and estimation.

6.3.5.1 Trehalose extraction

Trehalose Assay Kit, K-TREH, manufactured by Megazyme International Limited (Ireland) was used for the estimations. The assay is specific for trehalose, being linear over a range of 4 to 80 μg of trehalose per reaction. Before the estimation, it is essential to extract trehalose and remove the free glucose. Accurately, 100 mg of lyophilized, powdered biomass was weighed and suspended in 1 mL of hot water (~ 80°C). The contents were placed in a water bath at 80°C with intermittent mixing with vortexing, for 15 minutes, till the sample was completely dispersed. The final volume was made up to 1 mL again, wherever there was reduction in the volume. The contents were allowed to cool to room temperature. The samples were then centrifuged and the supernatant stored at 4°C till assayed.

Being a total cell extract, it may be having reducing sugars, which need to be removed before the trehalose content is estimated. For this, 0.2 mL aliquot of the solution was dispensed in a glass test tube and 0.2 mL of alkaline borohydride solution

was added to it. This was mixed vigorously and incubated at 40°C for 30 minutes to effect complete removal of reducing sugars to sugar alcohols. At the end of the incubation period, 0.5 mL of 200 mM acetic acid was added to the tube with vigorous stirring on a vortex mixer to remove the excess borohydride solution and adjust the pH to approx. 4.5. After 5 minutes, 0.2 mL of 2M imidazole buffer pH 7 was added to the mixture to adjust the pH to approximately 7. This was the final sample solution, which was used for the trehalose estimations.

6.3.5.2 Trehalose estimations

The samples treated as above were used for the trehalose estimations. Trehalose is hydrolyzed to D-glucose by trehalase, and the D-glucose released is phosphorylated by the enzyme hexokinase (HK) and ATP to glucose-6-diphosphate (G-6-P) with the simultaneous formation of ADP

Trehalose + H_2O ---------- 2 x D-glucose

D - glucose + ATP ---------- G-6-P

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by $NADP^+$ to gluconate-6-phosphate with the formation of reduced NADPH.

G-6-P + $NADP^+$ ---------- Gluconate-6-phosphate + NADPH + H^+

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose and thus twice the amount of trehalose. It is the amount of NADPH that is measured by the increase in absorbance at 340 nm. For the estimations, following steps were followed,

Pipette into cuvettes	**Blank**	**Sample**
Distilled water (at ~ 25°C)	2.2 mL	2.0 mL
Sample solution	-	0.20 mL
Imidazole buffer	0.20 mL	0.20 mL
$NADP^+$/ATP	0.10 mL	0.10 mL
HK/G-6-PDH	0.02 mL	0.02 mL
Mix, read the absorbances of the solutions (A_1) after approximately 5 minutes and start the reactions by addition of:		
Trehalase suspension	0.02 mL	0.02 mL
Mix, read the absorbances of the solutions (A_2) at the end of the reaction (approximately 5 minutes). If the reaction has not stopped after 5 minutes, continue to read the absorbances at 2 minutes intervals until the absorbances remain the same over 2 minutes		

The absorbance difference (A2 – A1) for both blank and sample were calculated. The absorbance difference of blank was subtracted from the absorbance difference of the sample, which is $\Delta A_{trehalose}$. The concentration of trehalose was calculated as:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 2} \times \frac{1.1}{0.2} \times \Delta A_{trehalose}$$

where, V = final volume (mL)

MW = molecular weight of trehalose (g/mol)

ε = extinction coefficient of NADPH at 340 nm

= 6300 [l x mol^{-1} x cm^{-1}]

d = light path [cm]

v = sample volume [mL]

2 = 2 molecules of D-glucose released from each molecule of trehalose hydrolyzed

1.1 / 0.2 = 0.2 mL of the sample extract is treated with borohydride and the final volume after treatment and neutralization is 1.1 mL

For trehalose, this can be solved as,

$$C = \frac{2.54 \times 342.3}{6300 \times 1 \times 0.2 \times 2} \times \frac{1.1}{0.2} \times \Delta A_{trehalose}$$

$$= 1.8976 \times \Delta A_{trehalose}$$

From this, the content of trehalose in terms of g / 100 g of biomass was calculated based on the amount of biomass used for the extraction.

6.4 Results

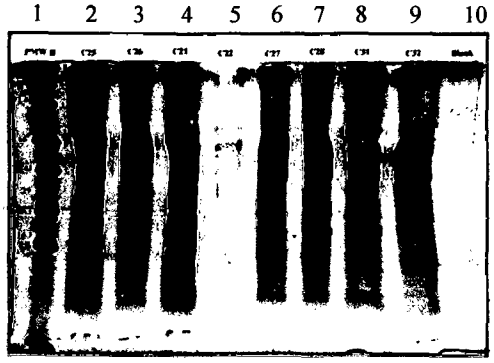
6.4.1 Protein profiles of normal and stressed deep-sea culture

The protein profile for the deep-sea yeast culture # A 344, grown under elevated pressure for 48 h, and given a pressure shock of 48 h and again grown at 1 bar pressure for 96 h, was obtained after electrophoresing the protein extracts. There were subtle differences visible to naked eye in the protein profiles (Fig. 6.1) obtained from cultures grown under different conditions; hence the profiles were reconstructed to get a diagrammatic representation. This was done using the AlphaImager FC 2200 software and the Band Scoring analysis method therein (Fig. 6.2a - 6.5b). The protein profiles of the deep-sea yeasts are given here.

50 bar: The number of proteins detected was more under the elevated pressure of 50 bar at both 30°C and 5°C, and at 1 bar/5°C than at 1 bar/30°C (Fig. 6.2a). Quite a large number of low molecular weight proteins were detected in the 48 hours old culture grown at 50 bar/30°C (lane 4; positions 2, 4, 6, 7, 8 & 11), 50 bar/5°C (lane 3;

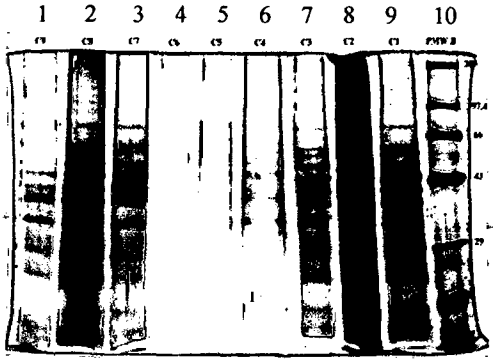
Fig. 6.1 Protein profiles (SDS PAGE) of deep sea yeast # A 344 grown at different hydrost pressure and given a pressure shock during growth

Gel I



Corresponding to Fig. 6.2a & 6.2b

Gel II

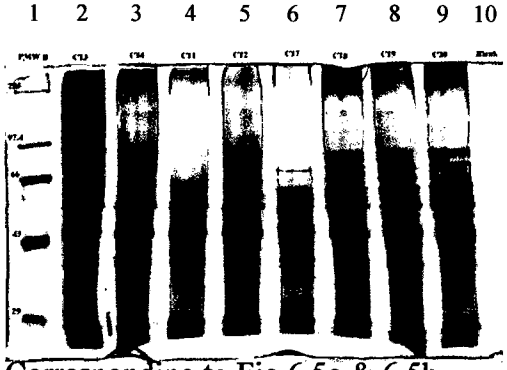


Corresponding to Fig.6.3a & 6.3b

Gel III

Corresponding to Fig.6.4a & 6.4b

Gel IV



Corresponding to Fig.6.5a & 6.5b

Lane No.	Gel I	Lane No.	Gel II	Lane No.	Gel III	Lane No.	Gel IV
1	3 to 205 kDa	1	200 bar / 30°C – 48 h	1	3 to 205 kDa	1	3 to 205 kDa
2	1 bar / 30°C – 48 h	2	100 bar / 5°C – 144 h	2	1 bar / 30°C – 48 h	2	1 bar / 30°C – 48 h
3	1 bar / 5°C – 48 h	3	100 bar / 30°C – 144 h	3	1 bar / 5°C – 48 h	3	1 bar / 5°C – 48 h
4	50 bar / 30°C – 48 h	4	100 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	4	200 bar / 30°C – 48 h	4	300 bar / 30°C – 48 h
5	50 bar / 5°C – 48 h	5	100 bar / 5°C – 48 h & 1 bar / 5°C - 96 h	5	200 bar / 5°C – 48 h	5	300 bar / 5°C – 48 h
6	50 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	6	1 bar / 5°C – 48 h	6	200 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	6	300 bar / 30°C – 48 h & 1 bar / 30°C - 96 h
7	50 bar / 5°C – 48 h & 1 bar / 5°C - 96 h	7	1 bar / 30°C – 48 h	7	200 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	7	300 bar / 30°C – 48 h & 1 bar / 30°C - 96 h
8	1 bar / 30°C – 144 h	8	100 bar / 5°C – 48 h	8	200 bar / 30°C – 144 h	8	300 bar / 30°C – 144 h
9	1 bar / 5°C – 144 h	9	100 bar / 30°C – 48 h	9	200 bar / 5°C – 144 h	9	300 bar / 5°C – 144 h
10.	Extraction Reagent Blank	10	3 to 205 kDa	10.	Extraction Reagent Blank	10.	Extraction Reagent Blank

* In all the above gels, the differences in the protein profile were not easily visible to the naked eye, hence to understand the pictur better, the profiles were reconstructed to get diagrammatic representations using Alpha Imager FC 2200 software and the Ban Scoring analysis method therein. In the Fig. 6.2a to 6.8b, the protein bands are labeled serially in each lane from top to down an these serial numbers do not correspond to the numbers in the different lanes. The molecular weight is denoted by the colours of th protein band.

positions 1, 3, 6, 7, 8, 9) and 1 bar/5°C (lane 1; positions 3, 4, 6, 7, 8 & 10) as compared to 1 bar/30°C (lane 2; Fig. 6.2a). The culture when grown at 50 bar for 48 h at 30°C and again allowed to grow at 1 bar/30°C for 96 h showed newly expressed proteins (with reference to 1 bar/30°C) at positions 4, 6, 8, 9 & 11 (lane 4; Fig. 6.2b) as compared to the culture grown at 1 bar/30°C for 144 h (lane 2), the total duration of the incubation. Similar pressure shock given at 5°C and then grown at 1 bar/5°C, showed new proteins at positions 3, 5 & 6 (lane 3; Fig. 6.2b) as compared to the culture grown at 1 bar/5°C for 144 h (lane 1). The proteins at positions 4, 6, 7, 8, 9 & 11 of the 1 bar/ 30°C (144 h, lane 2) grown culture were not seen in the culture grown at 1 bar/5°C for 144 h (lane 1; Fig. 6.2b).

100 bar: The culture grown at 100 bar / 30°C for 48 h showed large number of new proteins expressed at positions 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13 & 14 (lane 4; Fig.6.3a). Almost same situation was seen in the 1 bar grown culture, with the culture grown at 30°C (lane 2) showing more number of proteins than the one grown at 5°C (lane 1; Fig. 6.3a). The culture when given a shock of 100 bar at 30°C for 48 h and allowed to grow at 1 bar/30°C for 96 h, showed new proteins at positions 2, 3, 4 & 8 (lane 4) as compared to the culture grown at 1 bar/30°C for 144 h (lane 2; Fig. 6.3b). The culture when given a shock of 100 bar at 5°C and allowed to grow at 1 bar/ 5°C for 96 h (lane 3; Fig. 6.3b) showed a completely new protein of high molecular weight at position 1 as compared to the culture grown at 1 bar/5°C for 144 h (lane 1; Fig. 6.3b), while the rest of the proteins were detected in more or less same region with probable shifts; while the proteins seen at positions 4, 7 & 9 at 1 bar/5°C (lane 1) were not seen in the 100 bar shocked culture (lane 3; Fig. 6.3b).

200 bar: The 48 h old culture at 1 bar/ 30°C (lane 2; Fig. 6.4a) showed maximum number of detectable proteins as compared to the 1 bar/ 5°C (lane 1) and the pressurized cultures at 200 bar (lane 3). The proteins at positions 1, 3, 6, 8, 10 & and 13 at 1 bar/30°C (lane 2; Fig. 6.4a) were not seen at 200 bar/ 30°C, while the proteins at positions 2, 3, 6, 8, 9 & 10 at 200 bar/5°C (lane 3) were newly expressed as compared to protein profile of culture at 1 bar/ 5°C (lane 1) after 48 h growth.

A lot of variability in the protein profile was seen in cultures given pressure shock of 200 bar for 48 h at 30°C and 5°C (lanes 3 & 4; Fig. 6.4b). Although the proteins expressed were appearing as new, there were only minor difference in the molecular weights. The proteins at positions 2, 7, 11, & 13 (Fig. 6.4b) at 1 bar/30°C (lane 2) for 144 hours were not seen in the 200 bar shocked culture (lane 4), while the proteins at positions 6 & 7 of pressure shocked culture were newly expressed as compared to culture grown at 1 bar/30°C for 144 h. The culture when given shock of 200 bar at 5°C expressed many new proteins (lane 3, positions 1, 2, 3, 4, 11 & 14; Fig.6.4b) as compared to the culture grown at 1 bar/ 5°C for 144 h (lane 1).

300 bar: Many of the proteins detected in the cultures grown at 1 bar and 300 bar for 48 h were found to be similar (Fig. 4a). The proteins at positions 1, 2, 7 & 9 (lane 4; Fig. 6.5a) at 300 bar were newly expressed as compared to the culture grown at 1 bar (lane 2), while the protein at position 3 at 1 bar was not detected at 300 bar (lane 1 & 3 respectively). In case of 5°C also, there was similarity in the protein profile of cultures grown at 1 bar and 300 bar. Most of the proteins were nearly similar, with minor shifts in the molecular weight.

Fig. 6.4 a & b Protein profile of deep sea yeast # A344 grown at 200 bar pressure

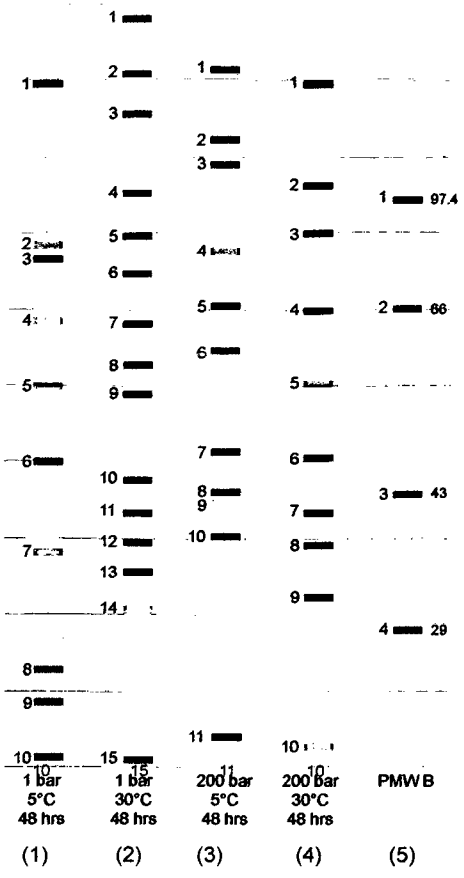


Fig. 6.4a

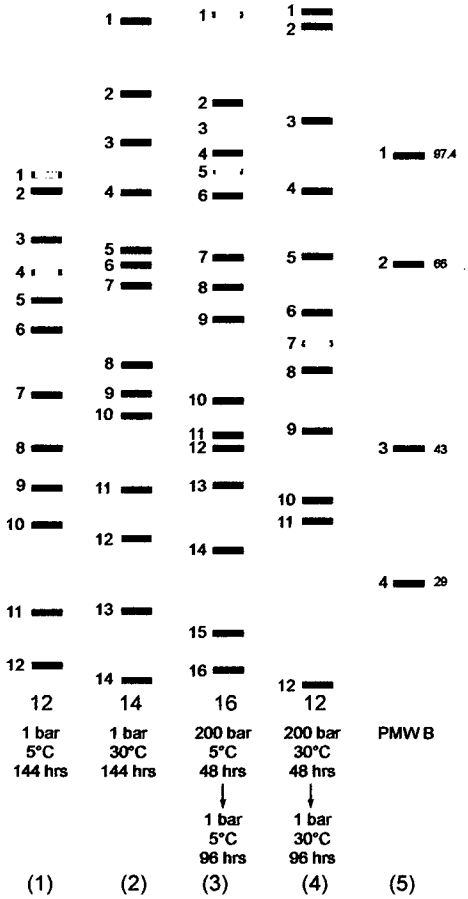


Fig. 6.4b

Fig. 6.5 a & b Protein profile of deep sea yeast # A344 grown at 300 bar pressure

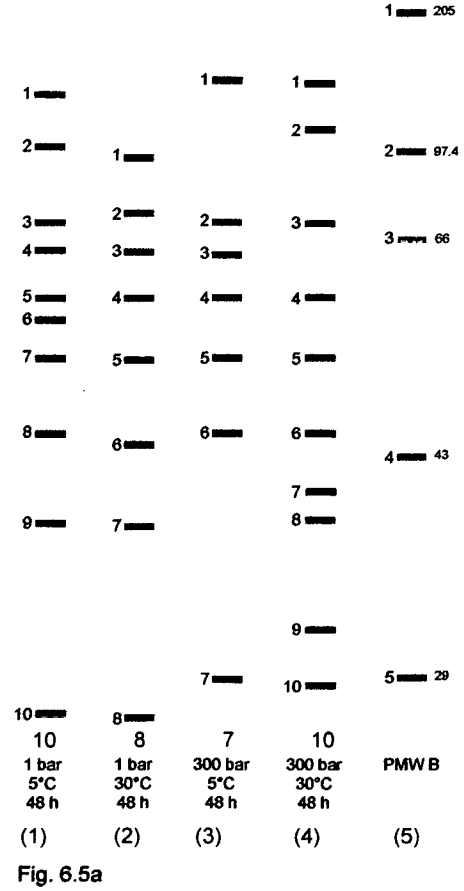
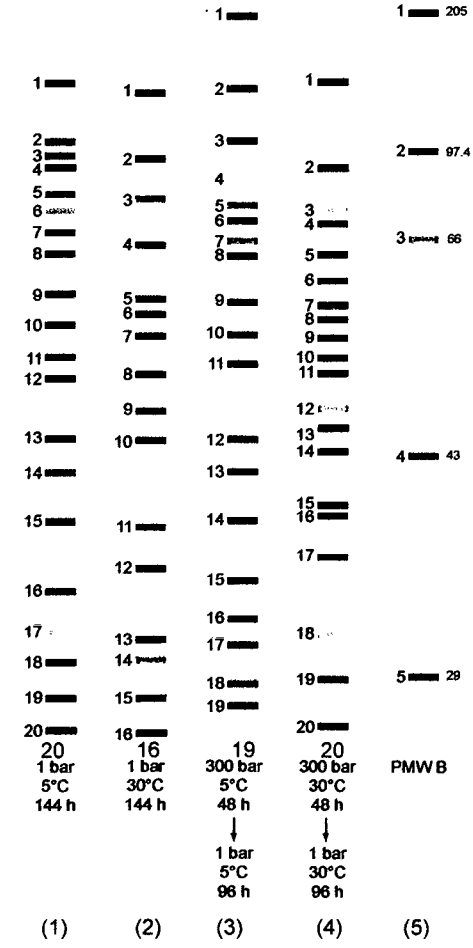


Fig. 6.5a



The 300 bar shocked cultures showed maximum number of detectable proteins in all the experiments conducted. Although the number of proteins detected was quite high, there were not many newly expressed proteins, but only minor shifts with respect to molecular weight. The proteins at positions 4, 6, 14, 15 & 16 (Fig. 6.5b) in 300 bar shocked cultures (lane 4) were newly expressed as compared to 1 bar/30°C grown culture (lane 2). In case of 300 bar shocked culture at 5°C (lane 3), the proteins at positions 6, 7, 9 & 16 were not detected in culture grown at 1 bar/5°C for 144 h (lane 1), while the proteins at positions 3, 4, 12 & 18 (lane 1; Fig. 6.5b) in culture grown at 1 bar/5°C were not detected in the pressure shocked culture.

6.4.2 Western blotting

The proteins from the cultures grown under 1 bar pressure and then given a shock for different time intervals did not show any positive signals with both the anti - Hsps, anti Hsp 25 & anti Hsp 70, used.

6.4.3 Protein profiles of deep-sea and terrestrial cultures grown under elevated pressure

The proteins extracted by the urea buffer extraction method were electrophoresed to obtain the protein profiles of the cultures grown under different conditions of pressure and temperature. The SDS-PAGE profile of the protein extracts of the deep-sea and terrestrial isolates grown under pressure followed the similar pattern that the proteins being not expressed under elevated pressure as compared to 1 bar grown cultures.

At all the conditions, the terrestrial culture of *Aspergillus terreus* (MTCC 479) showed more number of proteins than the deep sea isolate (# A 4634). When the detectable proteins expressed under 1, 100, 500 bar at 30°C by the deep-sea isolate #A4634 were compared, maximum number of proteins were detected at 1 bar/ 30°C (lane 2; Fig. 6.6). The proteins at positions 2, 4, 5 & 7 (Fig. 6.6) at 1 bar/30°C were not seen at 100 & 500 bar (lanes 3 & 4, respectively). At 100 bar/30°C (lane 3), new proteins appeared at positions 2, 3 & 4 (Fig. 6.6), while at 500 bar/30°C (lane 4) new proteins appeared at positions 2 & 4 (Fig. 6.6). The number of proteins detected at 5°C was more than at 30°C for all the 3 conditions of pressure (lane 5, 6 & 7). Even here, maximum numbers of proteins were detected at 1 bar (lane 5) as compared to 100 and 500 bar pressure (lanes 6 & 7 respectively). The proteins at positions 4 & 11 at 1 bar/5°C (lane 5; Fig. 6.6) were not detected at 100 & 500 bar. Quite a few proteins were eliminated in the higher molecular weight range, i.e. more than 40 kDa at 500 bar. The high molecular weight proteins at positions 3, 4, 5 at 100 bar (lane 6; Fig. 6.6) disappeared at 500 bar (lane 7), whereas the low molecular weight proteins at positions 5, 6 & 7 (Fig. 6.6) at 500 bar (lane 7) were not present at 100 bar (lane 6).

In case of the terrestrial isolate MTCC 479, the number of proteins detected at 30°C was more than those detected at 5°C (Fig. 6.7), unlike the deep-sea isolate (Fig. 6.6). The number of proteins in the lower molecular weight was much higher at 1 bar/30°C (lane 1) as compared to 100 & 500 bar (lane 3 & 4). The proteins at positions 4, 6, 7 & 8 detected at 1 bar/ 30°C (lane 1; Fig. 6.7) were not detected at 100 bar (lane 3), while the proteins at positions 4, 5, 6, 7, 8 & 9 (Fig. 6.7) were not detected at 500 bar (lane 4). At 100 bar/5°C (lane 6), proteins at positions 2 & 3 were newly expressed

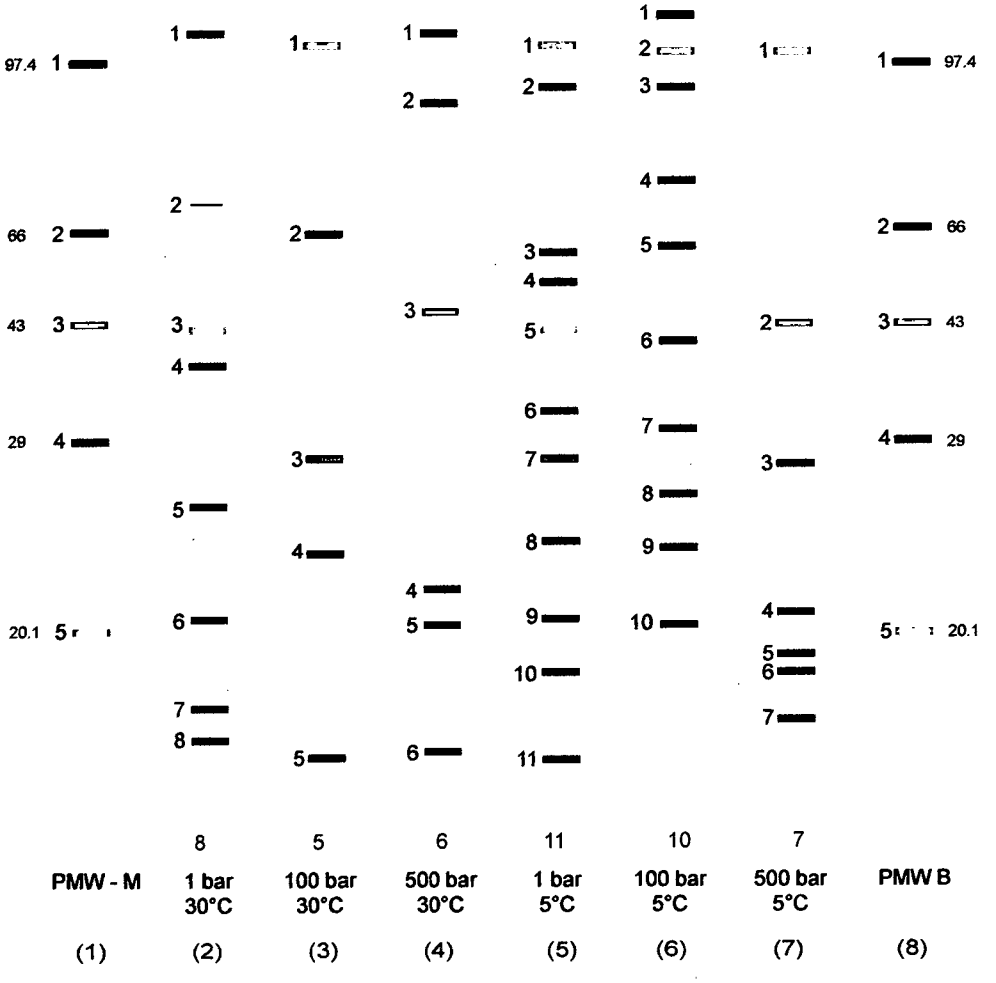


Fig. 6.6 Protein profile of deep sea *Aspergillus terreus* # A4634 grown at different hydrostatic pressures and temperatures

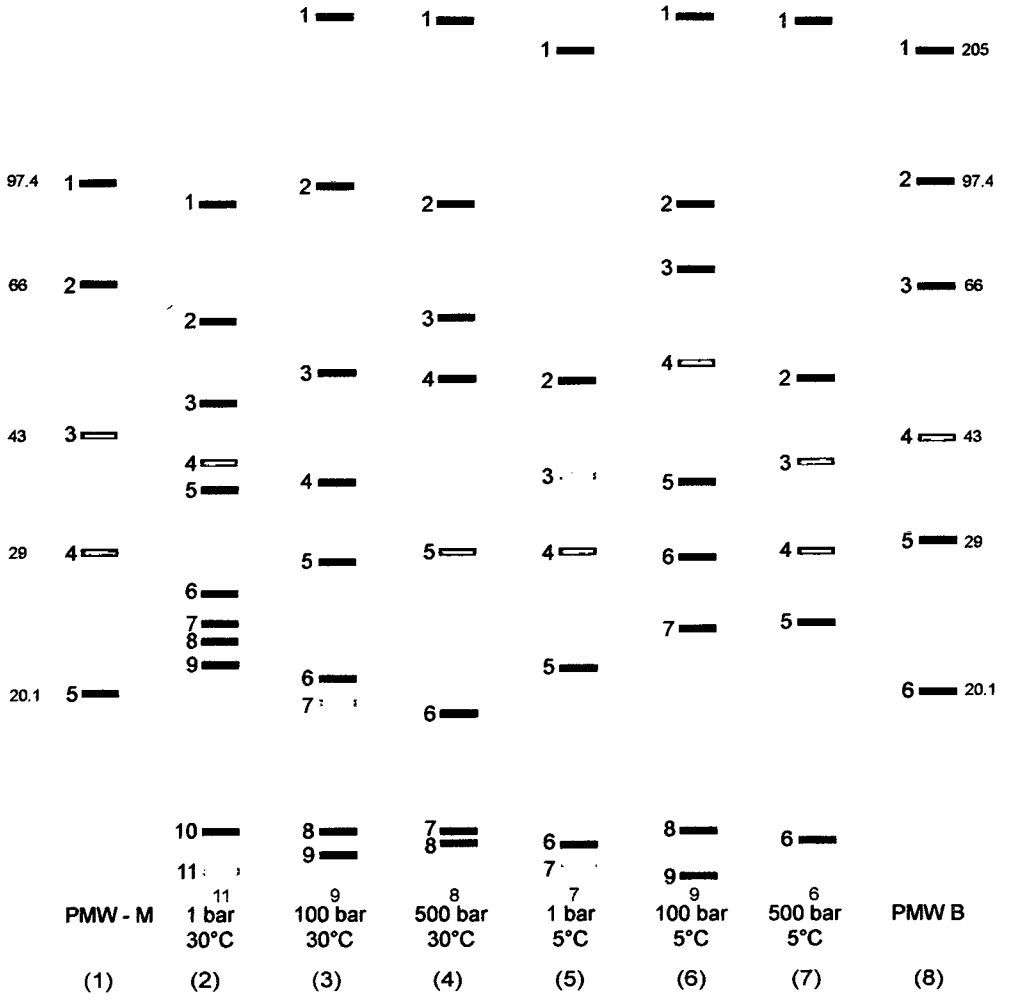


Fig. 6.7 Protein profile of deep sea *Aspergillus terreus* # MTCC 479 grown at different hydrostatic pressures and temperatures

as compared to 1 bar (lane 5) and 500 bar (lane 7), while the rest of the proteins were more or less similar with slight shift in their positions.

When the deep sea yeast # A 344 was grown at 1 bar/30°C, 300 bar/30°C and 300 bar/5°C, there was a huge dissimilarity in protein expression as detected by SDS electrophoresis (Figs. 6.8 a - c). At each interval of time, new proteins were expressed, which is a common pattern observed in any growing culture. The culture grown at 1 bar / 30°C for 6 h (Fig. 6.8a, lane 1) showed totally different proteins, except at position 5 which was also seen in the 24 h old culture (lane 3). The 48 h old culture showed maximum number of proteins (lane 4) as compared to the 6 h, 12 h and 24 h old cultures (Fig. 6.8a, lanes 1, 2 & 3 respectively). When the culture was grown under 300 bar at 30°C (Fig. 6.8b), similar trend was seen with new set of proteins being expressed at different time intervals. But here, more number of proteins was seen in the 6 h old culture (lane 1, Fig. 6.8b) than the 12 h, 24 h and 48 h old cultures (Fig. 6.8b, lanes 2, 3 & 4 respectively). When the culture was grown at 300 bar/ 5°C (Fig. 6.8c), the number of proteins detected at all the time intervals was almost the same, indicating that the culture is probably taking more time than what was used in our experiment to show appearance of new proteins as compared to the 300 bar/ 30°C grown (Fig. 6.8b) or 1 bar / 30°C culture (Fig. 6.8a). This is again in accordance with earlier observations that it is the temperature which is really retarding the growth of the fungus more than the elevated hydrostatic pressure. In case of 300 bar/30°C (Fig. 6.8b), the change in protein profile is seen immediately by 12 h, which is changed considerably by 24 and 48 h.

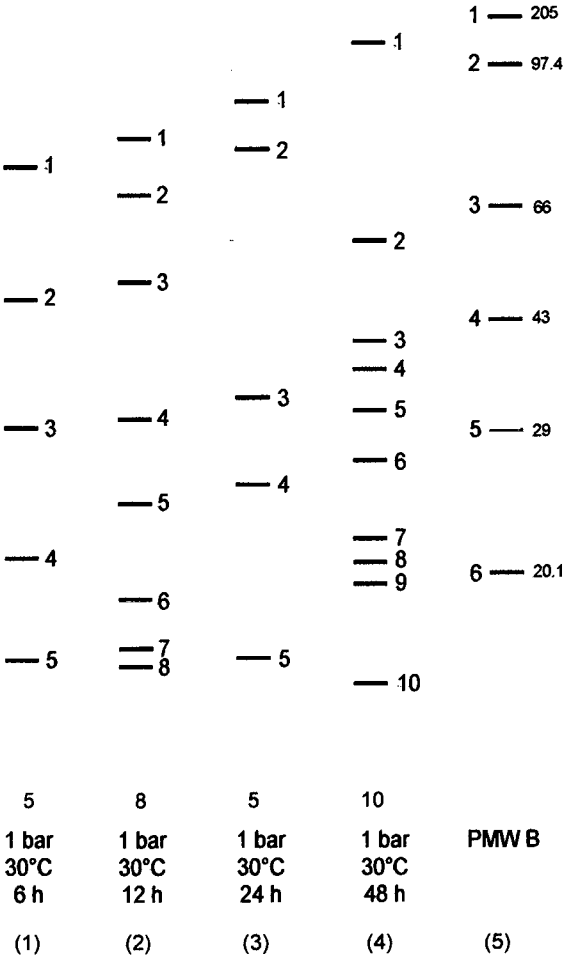


Fig. 6.8a Protein profile at 1 bar / 30°C at different time intervals by the deep sea yeast # A344

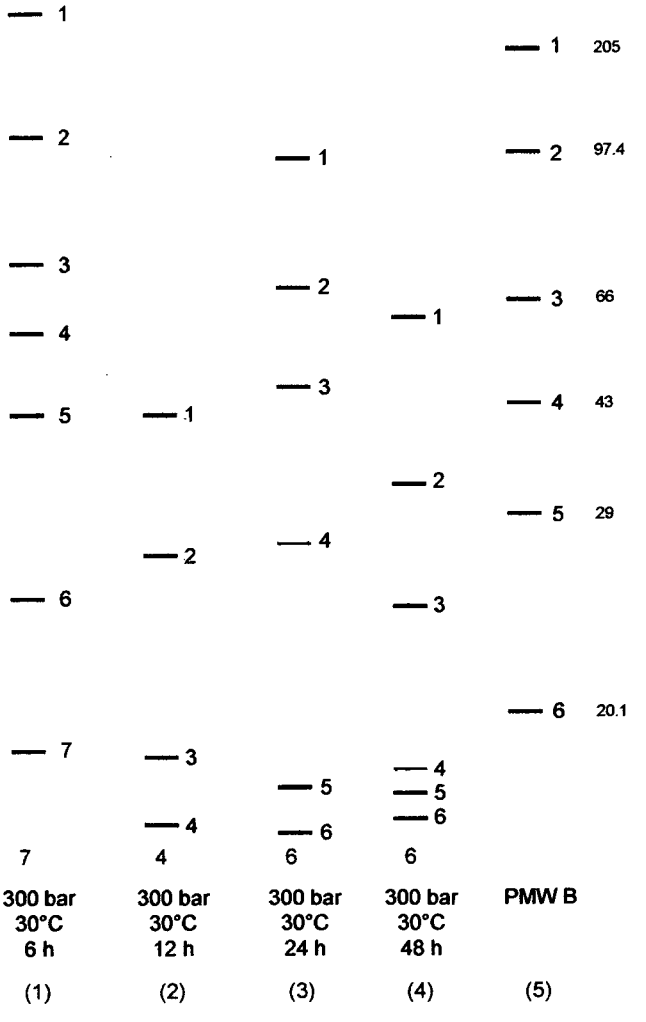


Fig. 6.8b Protein profile at different time intervals at 300 bar / 30°Cby the deep sea yeast # A344

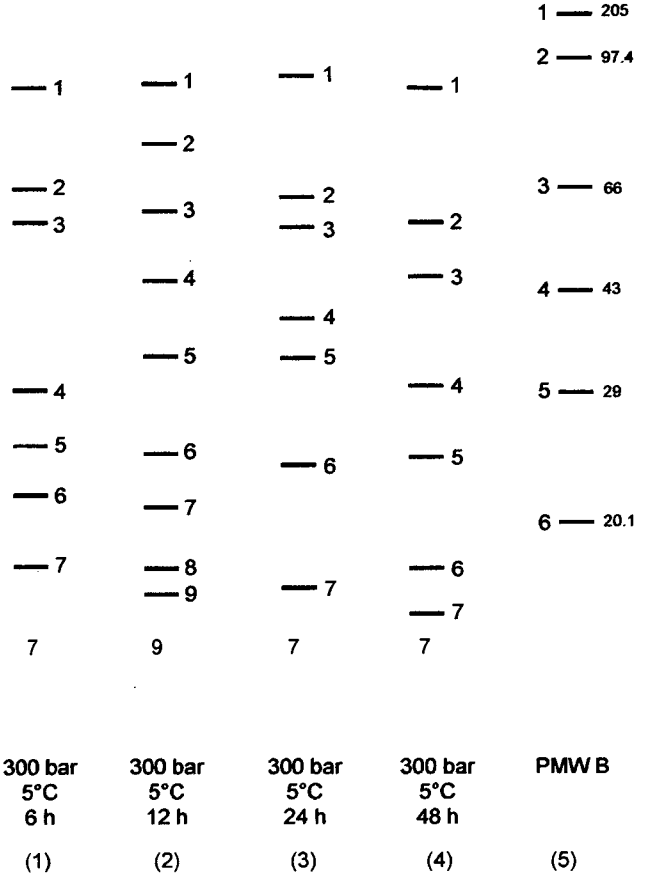


Fig. 6.8c Protein profile at different time intervals at 300 bar / 5°Cby the deep sea yeast # A344

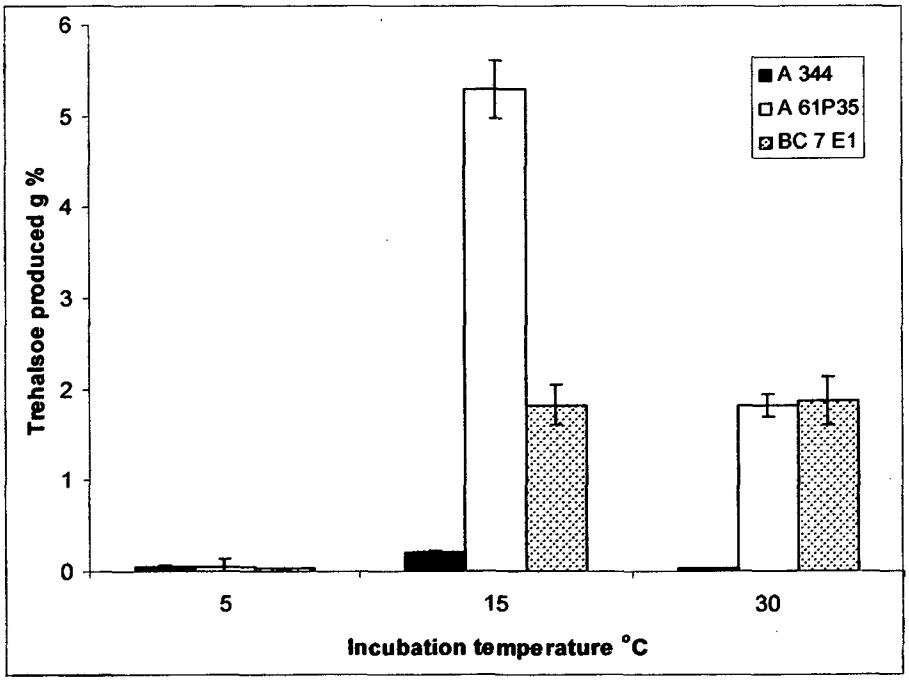
6.4.4 Trehalose accumulation

All the deep sea yeast cultures checked were showing trehalose production. The culture A 61P35 showed maximum trehalose accumulation at 15°C (Table 6.1 & Fig. 6.9). All the cultures showed least trehalose production at 5°C.

Table 6.1 & Fig. 6.9 Trehalose production by the deep-sea yeasts at different temperatures

Culture	Growth condition (°C)	Trehalose produced (g %)
A 344	5	0.06 ± 0.01
	15	0.22 ± 0.09
	30	2.92 ± 0.01
A 61P35	5	0.03 ± 0.01
	15	5.30 ± 0.32
	30	1.35 ± 0.22
BC7E1	5	0.03 ± 0.01
	15	1.82 ± 0.13
	30	1.87 ± 0.27

Fig. 6.9

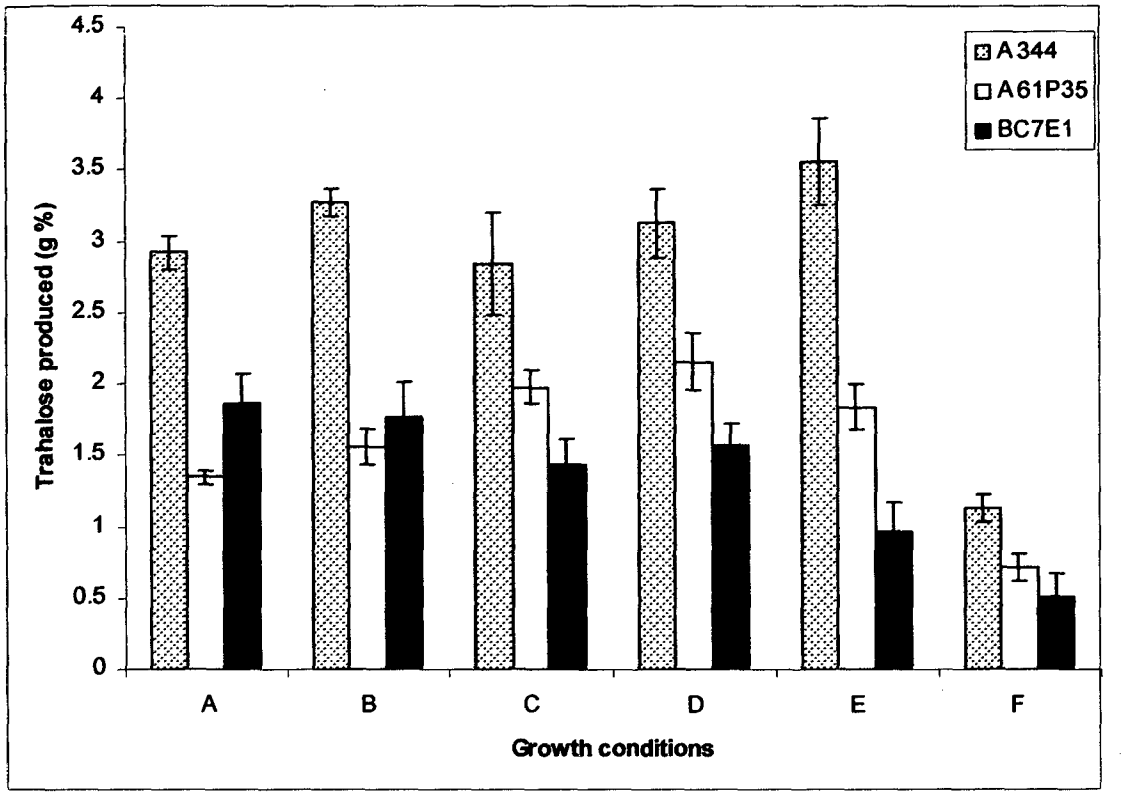


The deep sea yeasts (A 344, A 61P35 & BC7E1) when incubated under elevated pressure did not show any drastic increase or decrease in trehalose accumulation (Table 6.2 & Fig. 6.10). The off-white, filamentous yeast, A 344, showed highest trehalose accumulation at 500 bar / 30°C, while the orange yeast BC7E1 showed least at the same condition. In case of A 61P35, there was slight increase in trehalose accumulation from 1 bar to 300 bar, but again there was decrease at 500 bar, while in case of the other two yeasts used for the experiment, there was random increase or decrease in the trehalose levels without any direct correlation to the growth conditions.

Table 6.2 & Fig. 6.10 Trehalose production by the deep-sea yeasts at different hydrostatic pressures

Growth conditions (18 h)	Trehalose accumulation (g %)		
	A 344	A 61P35	BC7E1
1 bar / 30°C	2.92 ± 0.12	1.35 ± 0.05	1.87 ± 0.2
100 bar / 30°C	3.27 ± 0.1	1.56 ± 0.13	1.76 ± 0.25
200 bar / 30°C	2.84 ± 0.36	1.98 ± 0.12	1.43 ± 0.18
300 bar / 30°C	3.13 ± 0.24	2.16 ± 0.2	1.57 ± 0.15
500 bar / 30°C	3.56 ± 0.3	1.84 ± 0.16	0.96 ± 0.22
Control (Inoculum) 1 bar / 30°C (48 h)	1.13 ± 0.1	0.72 ± 0.1	0.51 ± 0.16

Fig. 6.10



A	1 bar/30°C
B	100 bar/30°C
C	200 bar/30°C
D	300 bar/30°C
E	500 bar/30°C
F	Control (Inoculum) 1 bar / 30°C (48 h)

6.5 Discussion

In the present study, the growth of the cultures at high pressure and low temperature was not inhibited indicating the versatility and tolerance of cultures to extreme growth conditions. However, more number of proteins were seen in the cultures grown at 1 bar rather than at the elevated pressures. This could be pointer to the fact that all these cultures were isolated from the deep sea and the elevated

pressure conditions being the native conditions existing, the atmospheric pressure may be acting as a stress, thereby expressing more number of proteins. But this was not supported by other growth experiments to satisfaction. This needs to be studied to the genetic level as in case of bacteria [Bartlett, 2002; Horikoshi, 1998]. This might also be indicating that the metabolic rates are very low at the elevated pressure and low temperature, correspondingly giving less number of proteins or this could be due to biochemical adaptation of cells by transition to a specific surviving state, which is characterized by deceleration of vital activity (DVA), a process related to decreased metabolism [Feofilova, 2003].

The first attempt to characterize the pressure response of an unicellular organism at the protein level targeted the methanogenic archaeon *Methanococcus thermolithotrophicus* [Jaenicke *et al.*, 1988], partly because the negative reaction volume of the methane-forming reaction suggested that this organism might thrive at elevated pressures. In spite of the positive results of this study, further characterization of the detected pressure-induced proteins was hampered by the extremely difficult handling of this organism. Four Pressure-induced-proteins (PIPs) were observed in case of *Rhodotorula rubra* at 450 bar. The profiles obtained at 20, 40 and 45 MPa, revealed no change in protein expression as compared to samples grown at atmospheric pressure. However, at 400 to 500 bar, approaching threshold of complete growth inhibition, significant alterations occur [Groβ *et al.*, 1994]. Cultivation of cultures at temperatures from 5 to 30°C showed no inhibition of growth. The pressure shock induces heat and cold shock proteins [Welch *et al.*, 1993], and ribosomes appear to play a role as sensors for these two stress conditions [VanBogelen, 1990], strongly suggesting a similar role for ribosomes in response to hydrostatic pressure.

It has been reported previously [Julseth & Innis, 1990] that stress protein production can occur both during the absence and presence of cell growth. Further, time needed for induction of proteins subjected to shock at low temperature is more than the time needed for induction at higher temperature because of the decreased rate of overall protein synthesis at lower temperature. We also had similar observations (Fig. 6.8 a - c), with more number proteins observed at 30°C grown cells than 5°C grown cells under high pressure.

The filamentous fungi did not give very good protein profiles. The main problem for the filamentous fungi was the amount of biomass produced. The biomass produced was too little to carry out the liquid nitrogen crushing to obtain detectable amount of proteins. It was observed during the standardization of protein extraction methods using, 1 bar-grown culture, that maximum amount of protein was obtained using the liquid nitrogen crushing.

Both the anti hsp antibodies checked did not give positive signals with the proteins present in the sample indicating their novel nature. To get a positive confirmation, these proteins need to be gel purified, and polyclonal or monoclonal antibodies will have to be raised against them. Other approach to study these proteins would be to purify the proteins and do the amino acid sequencing to find out if the proteins are really new.

It has been shown in previous studies with bacteria [Niven *et al.*, 1999] that effect on ribosomal structure observed after cold shock was similar to the effect observed after high pressure treatment, suggesting that there is overlap in the responses to these two types of stress. This was also reported in case of *Listeria monocytogenes* LO28 [Wemekemp-Kamphuis *et al.*, 2002]. In the present studies also

the cultures showed similar response to pressure and low temperature (5°C), with many proteins being induced (Fig. 6.1 to 6.8) in both deep sea and terrestrial isolates. The maximum trehalose accumulation was observed at 15°C or 30°C. Trehalose, being a stress protectant is known to be produced under the stress of low temperature, but the results obtained here were on similar lines of the growth and the protein profiles. More trehalose accumulation at mesophilic temperatures is a further proof of these yeasts being deep-sea inhabitants. Similarly, high hydrostatic pressure also doesn't seem to induce or repress trehalose production (Table 6.2 & Fig. 6.10), except in case of one of the cultures, A 61P35. But even this is also not very high to be called as a response against the increasing pressure, and thereby playing a role in the defense of the cell. A possible role of trehalose in the defense of the yeast *Saccharomyces cerevisiae* against osmotic stress was suggested by Piper [1993]. In case of a thermophilic fungus *Chaetomium thermophilum* var. *copriphilum* [Jepsen & Jensen, 2004], it was shown that cytosolic trehalose accumulation was observed only in case of temperature stress and not osmotic stress caused due to increased concentration of sodium chloride. In the present studies also there was no increase in trehalose accumulation when the cells were exposed to the stress of decreasing temperature or increasing hydrostatic pressure, suggesting that trehalose alone may not be playing a role as stress protectant. Other compatible solutes that accumulate in cells are amino acids and polyols, e.g. glycine betaine, ectoine, sucrose, mannitol and glycerol [DasSarma & Arora, 2001]. Halotolerant yeasts and green algae accumulate polyols, while many halophilic and halotolerant bacteria accumulate glycine betaine and ectoine. Compatible solute accumulation may occur by biosynthesis, *de novo* or from storage material, or by uptake from the medium. A major exception is for the

halobacteria and some other extreme halophiles, which accumulate KCl equal to the external concentration of NaCl [DasSarma & Arora, 2001]. The role of glycine betaine is very well documented in case of prokaryotes, *Listeria monocytogenes* [Mendum & Smith, 2002]. TMAO (Trimethylene N-oxide) is another such protectant found in deep-sea animals [Yancey *et al*, 2002] which is a universal protein stabilizer. Further, TMAO works as a protein stabilizer for organisms that cannot produce it, when provided externally.

Chapter 7

Proteases of Deep-sea Fungi

7.1 Introduction

The deep-sea environment is a source of unique microorganism with great potential for biotechnological exploitation. Very few studies concerning the isolation and characterization of deep-sea microorganisms have been carried out, and investigations in this field may lead to many new discoveries. Microorganisms living in the deep sea have special features that allow them to live in this extreme environment, and it seems likely that further studies of these organisms will provide important insights into the origin of life and its evolution.

Fungi from marine habitats have received much attention in recent years for the production of useful secondary metabolites [Liberra & Lindequist, 1995; Yu *et al.*, 2003; Jensen & Fenical, 2002]. Research on marine fungi and biotechnologically useful enzymes produced by them, however, has been restricted to those isolated from coastal habitats and to lignocellulose degrading enzymes for application in bioremediation and paper industries [Raghukumar, 2000; 2002]. Fungi and their enzymes from the deep-sea environment have received scant attention. The deep-sea inhabitants being present in extreme conditions produce extracellular enzymes, which are active under such conditions. Amongst the extracellular enzymes produced, proteases are important class of enzymes, which occupy a pivotal position with respect to their physiological role. Proteolytic enzymes play an important role in remineralisation processes in the sea, mainly because proteins and peptides constitute a substantial portion of the organic nutrients present in the deep-sea sediments as well as suspended particulate matter [Banse, 1990; Smith *et al.*, 1992]. Raghukumar &

Raghukumar [1998], reported production of protease enzyme under simulated deep-sea conditions by two filamentous fungi isolated from deep-sea calcareous sediments.

Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. They are ubiquitous in nature. Proteases differ in their ability to hydrolyze various peptide bonds. They are highly complex group of enzymes, which vary enormously in their physicochemical and catalytic properties. Each type of protease acts on a specific kind of peptide bond. They are produced intra- and extracellularly. Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. Proteases play a critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, and transport of secretory proteins across membranes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism [Kalisz, 1988; Rao *et al.*, 1998].

Previously, proteases were classified by Hartley (1960) into four classes as,

1. Serine proteases,
2. Aspartic proteases,
3. Cysteine proteases and
4. Metalloproteases

According to the Nomenclature Committee of the International Union of Biochemistry, proteases are classified in subgroup 4 of group 3 (Hydrolases) [Dixon & Webb, 1979,]. This classification was further recommended by Nomenclature Committee of 1992 (International Union of Biochemistry, 1992). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure.

Currently, proteases are classified on the basis of three major criteria as,

1. Type of reaction catalyzed,
2. Chemical nature of the catalytic site and
3. Evolutionary relationship with reference to structure [Barret, 1994]

Even though a lot of work has been carried out to explore the possibility of harnessing extremophiles as potential protease producers, fungi from extreme conditions of deep sea have not yet been tapped for this purpose. Barophilic enzymes having characteristic substrate specificity would be very useful for industrial applications such as proteases and glucanases for detergents and DNA polymerases for PCR amplification. Michels & Clark [1997] purified and characterized a protease from *Methanococcus jannaschii*. This enzyme is the first protease to be isolated from an organism adapted to a high pressure and high temperature environment. The partially purified enzyme has a molecular mass of 29 kDa and narrow substrate specificity. Enzyme activity increased as the temperature increased up to 116°C and enzyme activity was measurable up to 130°C, one of the highest temperatures reported for the function of any enzyme. These results suggest that enzymes produced by these high-pressure adapted bacteria should be more functional under high-pressure conditions

than at atmospheric pressure [Horikoshi, 1998]. Thus, such enzymes may be very useful in high-pressure bioreactor systems.

Proteases are a unique class of enzymes, since they are of immense physiological as well as commercial importance. Since proteases are physiologically necessary, they occur ubiquitously in animals, plants and microbes. However, microbes are a gold mine of proteases and represent the preferred source of enzymes in view of their rapid growth, limited space required for cultivation, and ready accessibility to genetic manipulation. The proteases isolated from extremophilic organisms are likely to mimic some of the unnatural properties of the enzymes that are desirable for their commercial applications. Hence deep-sea fungi could be one of the candidate organisms for harnessing the proteases produced by them.

Apart from their ecological significance, proteases are one of the most valuable commercial enzymes. They are being used in food industry, dairy, detergents, pharmaceuticals, leather industry, research laboratories, bioremediation, effluent treatments, etc. More than 25% of the worldwide sale of enzymes is contributed by proteases alone, where mainly alkaline proteases are used.

7.2 Objectives

The objectives for the present studies were –

1. To screen the deep-sea fungi isolated by different method for the production of alkaline and low temperature active protease.
2. To characterize protease produced by these cultures.

7.3 Methodology

7.3.1 Screening for protease producing fungi

All the isolates obtained from the deep-sea sediments, deep-sea organisms as well as from the lagoon sediments were screened for the production of protease as per method described by Molitoris [2000]. The assay was a qualitative one indicating whether the culture is capable of producing protease. Briefly, mycelia of a growing culture were spot inoculated on a Czapek Dox (CD) agar plate supplemented with 1 % of skimmed milk. The plates were incubated till good growth was observed (4 to 5 days). The protease producers show a zone of clearance around them. If the zone was not clearly visible, the plates were flooded with coomassie blue solution for 10 minutes. The area around the protease producers is seen as a clear colourless zone as against blue coloured area where the milk protein is not degraded.

7.3.2 Protease production

The protease positive cultures were used for further quantification of the enzyme. For this, the cultures were first grown in MEB at 30°C. After 4 to 5 days, when sufficient growth was achieved, the biomass was homogenized mechanically using sterile glass beads and this was used as inoculum (the same procedure was followed for all the experiments involving protease production). This was inoculated in CD broth (Czapek Dox broth without agar) with 0.3% skimmed milk powder (Appendix A - IX). Protease production was compared at 5° and 30°C by growing the cultures at these temperatures. The cell free culture filtrates were obtained by filtering

through GF/F and 0.22 μm nitrocellulose filter papers, successively. These filtrates were used to assay the protease enzyme activity.

The cultures were also tested for their ability to grow and produce protease at elevated hydrostatic pressures. Sterile plastic pouches containing 45 ml of Czapek Dox broth with 0.3% skimmed milk solution were inoculated with 5 ml of culture suspension (finely broken mycelia and spores in seawater). These were suspended in deep-sea culture vessels (Tsurumi & Seiki Co., Japan) filled with sterile distilled water. These vessels were pressurized at 50 bar and 100 bar (10 bar = 1 MPa) and incubated at 30°C and 5°C for 20 days. Three replicates were maintained for each treatment. After 20 days, the biomass was separated from culture broth by centrifugation and further filtered through sterile 0.22 μm hydrophilic durapore membrane filters (Millipore, USA). Protease activity of the filtrate was estimated using azocasein as the substrate. The assay was carried out at the optimal conditions of the enzyme (pH = 9; 45°C and 1 bar pressure). The activity was also assayed under 50 and 100 bar pressure at pH 9 and 30°C.

The extracellular protease activity of the culture grown under atmospheric pressure (1 bar pressure) and 30°C and 50 bar/30°C was compared with activity under elevated hydrostatic pressure of 50, 100, 200 and 300 bar at pH 9 and 30°C. The K_m constants of the protease activity from cultures grown at 30°C and 1 bar pressure were measured at 1 bar/5°C and 50, 100, 200 and 300 bar/45°C using the substrate azocasein at pH 9.

7.3.3 Protease assay

The protease activity was assayed with 150 µL of crude culture filtrate and 250 µL of the substrate azocasein (Sigma Chemicals, USA) at 2 % concentration prepared in 2 buffers, 0.1 M boric acid-borax buffer pH 9 and 0.1 M phosphate buffer pH 7. Protease activity was measured by incubating the reaction mixture at 30°C and 5°C for 30 min. The reaction was stopped by addition of 1.2 mL of 10% trichloroacetic acid solution. The contents were centrifuged at 10000 rpm for 10 min. To the supernatant, 1.4 mL of 1N NaOH was added and the absorbance read immediately at 440 nm against appropriate blanks in a spectrophotometer (Shimadzu, Model 1210, Japan). One ACU (Azocasein Digestion Unit) is defined as the increase in absorbance by 0.001 per minute under the assay conditions [Hamamato *et al.*, 1995].

Protease activity under elevated hydrostatic pressure was assayed in microcentrifuge tubes of 0.5 mL capacity [Raghukumar & Raghukumar, 1998]. The lids of the tubes were snapped off and sealed with parafilm after adding the reaction mixture, which contained the substrate azocasein, buffer at pH 9 and appropriately diluted enzyme. Care was taken to avoid trapping of air bubbles while sealing. The tubes were suspended in deep-sea culture vessel pressurized to the desired hydrostatic pressure and incubated under desired temperature for 30 min. At the end of the incubation period, the vessels were depressurized and without any delay the enzyme activity was arrested by adding TCA. Protease activity was measured as described above. Control was kept keeping in view the delay in pressurizing the vessels and that was considered as the starting point for the calculation of enzyme activity.

7.3.4 Characterization of protease produced by one of the deep sea fungus

One of the cultures NIOCC # 20, identified as *Aspergillus ustus* (Bain.) Thom and Church (deposited at the Institute for Microbial Technology, Chandigarh, India under the accession number MTCC 5102) showed the highest protease activity at pH 9, both at 5° and 30°C temperatures among all the deep-sea fungal cultures and therefore was selected for further studies. The conditions for obtaining maximum protease production were partially optimized with reference to the time of harvesting and different inducers.

7.3.4.1 Temporal production of biomass and protease

The culture, NIOCC # 20, was inoculated in Czapek Dox broth with 0.3 % skimmed milk. Every day, culture filtrate was collected by filtering first through glass fibre (GF/F) filters (Whatman, USA) and then by durapore 0.22µm (GVWP04700, Millipore, USA). The biomass was dried to constant weight and recorded. The culture filtrate was assayed for protease activity at pH 9 and 45°C, the optimum pH and temperature for activity.

7.3.4.2 Substrate inducers

The culture was inoculated in Czapek Dox broth supplemented with different inducer substrates like molasses, corn steep liquor, skimmed milk, tween 80 and also in plain CD broth and MEB. The culture filtrate was collected after 7 days of incubation at 30°C and 1 bar as described above and assayed for protease activity at pH 9 and 45°C.

7.3.4.3 Harvesting of enzyme

It was observed that the culture is producing a lot of exopolysaccaharides, which might be binding the extracellular protease and thereby resulting in the loss of the amount of protease being reported. To circumvent this problem, just before harvesting the culture filtrate, EDTA was added to it, mixed properly and then the contents filtered as described previously.

7.3.4.4 Purification and characterization of purified enzyme

The culture was inoculated in CD broth with 0.3 % skimmed milk and incubated at 30°C. After 7 days of incubation, the culture filtrate was collected as described previously and used for further studies. The cell free culture filtrate was concentrated in a vacuum concentrator (Biotron, Korea) to $1/10^{th}$ volume. The concentrate was passed through an anion exchange column 'Resource Q' (Amersham Biosciences, Upssala, Sweden). It was eluted using a gradient of NaCl (0 – 0.25 M) prepared in 10 mM phosphate buffer at pH 7. The flow rate was adjusted to 0.1 mL min^{-1} and eluted fractions of 1 mL each were collected. Fractions showing protease activities were pooled, concentrated and further subjected to size exclusion column chromatography using Superdex 200 column (Amersham Biosciences, Upssala, Sweden). The enzyme was eluted with 0.2 M NaCl prepared in 100 mM acetate buffer pH 4.5. The flow rate was adjusted to 0.5 mL min^{-1} and the eluted fractions of 2 mL each were collected. Fractions showing protease activity were pooled and used for characterization of the enzyme.

At each step of purification, the enzyme activity and protein content [Lowry *et al.*, 1951] (Appendix A-V) were assayed to calculate the efficiency of the purification process.

The homogeneity of the fractionated enzyme was confirmed by running a native PAGE with 10% acrylamide at 60 V. The gel was blotted on 1.5% agarose containing 1% casein for 1 h. The agarose blot was stained with 0.15% amido black (Sigma Chemicals, USA) and 0.3% coomassie blue in methanol: acetic acid: water at a ratio of 4:1:5 [Oh *et al.*, 1999]. The molecular weight of the purified enzyme was determined using SDS-PAGE [Laemmeli, 1970] (Appendix A-VI). The electrophoresis was carried out on 12% resolving gel at a constant voltage of 60V. The protein was detected using silver staining method [Heukeshovan & Dernick, 1985] (Appendix A-VII). The pI of the purified enzyme was estimated by running an isoelectric focusing tube gel. Broad range ampholytes of pH 3-10 were used. The pH gradient was formed by running the gel at 250 V for 30 min following which the sample was loaded onto the gel. The isoelectric focusing was carried out at 500 V for 4 hours.

7.3.4.5 Optimum pH for enzyme activity

The optimum pH for the activity of the purified enzyme activity was determined using eight different buffers (100 mM): sodium acetate (pH 5), citrate phosphate (pH 5-7) phosphate (pH 6-8), tris HCl (pH 8 & 9), borax-boric acid (pH 8 & 9), glycine-NaOH (pH 9 & 10) borax-NaOH (pH 10) and carbonate-bicarbonate

(pH 10 & 11) at 45°C. The substrate was prepared in the above buffers and the assay was carried out at 45°C as described previously.

7.3.4.6 Optimum temperature for enzyme activity

The optimum temperature for the protease activity was determined by assaying at 2, 10, 15, 30, 45, 50, 60, 70, 80 and 90°C at pH 9, the optimum pH for the enzyme activity.

7.3.4.7 K_m constant and V_{max} for the protease

K_m constant and V_{max} for the enzyme were determined from Lineweaver Burke plot at pH 9 and 45°C using the substrate azocasein. The enzyme assay was carried out with varying substrate concentrations of 1 to 10 mg mL^{-1}.

7.3.4.8 Thermostability of purified enzyme

Thermostability of purified enzyme at different temperatures was assayed by incubating the enzyme samples at 40, 50, 60, and 70°C for 10 minutes and then the residual activity was measured at it's optimum pH and temperature i.e. pH 9 and 45°C. Thermostability of purified enzyme at its optimum temperature of activity (45°C) was estimated by incubating the enzyme at 45°C and the residual activity was assayed at an interval of 10 minutes. The assay was carried out at pH 9 and 45°C as described previously.

7.3.4.9 pH stability of the purified enzyme

The stability of purified enzyme at different pHs was carried out by incubating 25 µL of enzyme with 75 µL of different buffers as given above for one hour and then the residual protease activity was measured at 45°C and pH 9. Different additives were used to increase the thermostability of the enzyme at 60°C.

7.3.4.10 Effect of inhibitors and stabilizers

The effect of different protease inhibitors like PMSF, EDTA, chlorides of heavy metals like Ni, Zn, Cu, Hg, Fe, reducing agents like DTT, glutathione, β-mercaptoethanol, sodium thioglycolate, on the enzyme activity was determined by assaying in presence of these substances. The effect of varying ionic strength on the protease activity was also determined by assaying the enzyme in presence of varying sodium chloride concentration up to 1M. Keeping in mind one of the possibilities of the enzyme being a potential candidate for use in detergents, the activity was determined in presence of various commercially available detergents and bleaching agents at pH 9 and 45°C.

7.3.5 Protease production by terrestrial *Aspergillus ustus* (MTCC 2200)

A comparison was carried out with respect to protease production between the deep-sea isolate # NIOCC 20 (*A. ustus*) and the terrestrial isolate MTCC 2200 (*A. ustus*). The protease of the two cultures was compared with respect to the temporal production, different salinites, pH and temperature for activity. The biomass produced by the two in CD broth was also compared.

7.4 Results

7.4.1 Screening of deep sea fungi for protease production

A total of 221 and 22 fungi from deep-sea and shallow depth sediments respectively were obtained by employing different techniques of isolations.

Table 7.1 Growth and alkaline (pH 9) protease production by deep-sea and shallow water isolates of fungi

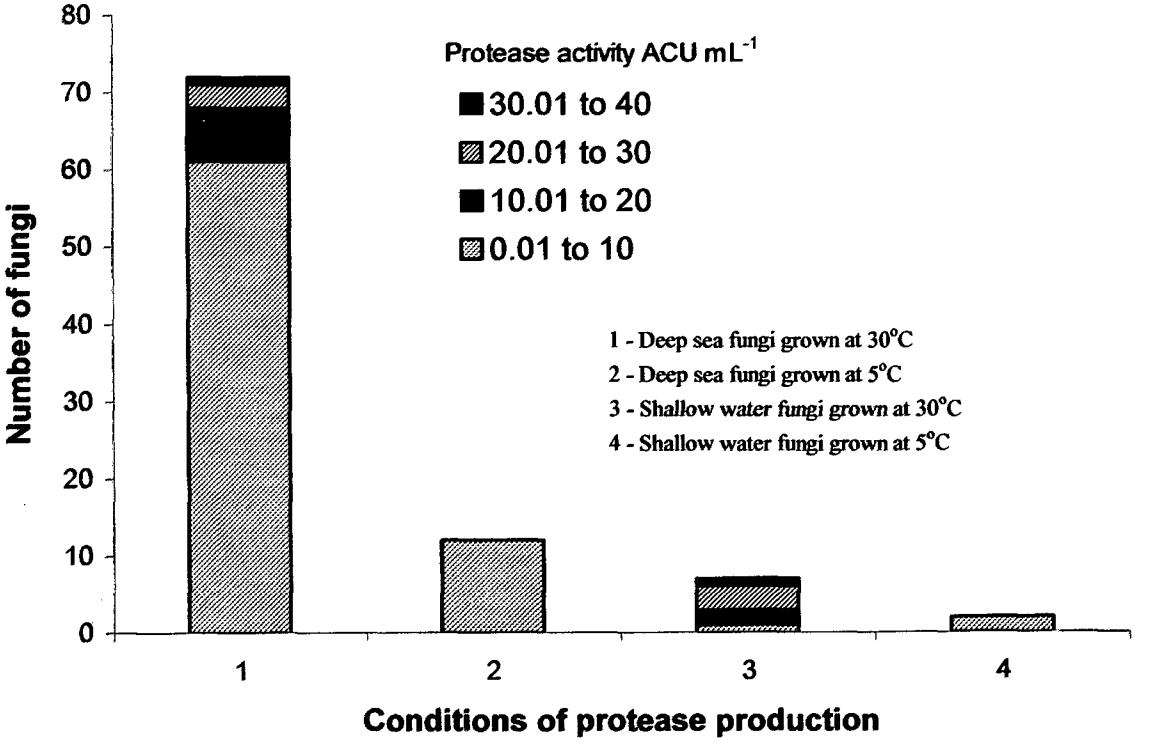
Source of Fungi	Total Number isolated	Culture condition	Number and (%) isolates showing growth	Cultures with protease activity at 30°C*	Cultures with protease activity at 5°C*
Deep sea	221	1 bar and 30°C	221 (100 %)	105 (48 %)	73 (33 %)
		1 bar and 5°C	113 (51 %)	15 (13 %)	12 (11 %)
Shallow water	22	1 bar and 30°C	22 (100 %)	10 (45 %)	3 (14 %)
		1 bar and 5°C	7 (32%)	2 (29 %)	2 (29 %)

* percentage of cultures with protease activity was calculated from the total number of cultures showing growth (numbers in the 4th column)

Among these, 33 % of the deep-sea and 14 % of shallow water fungal isolates when grown at 30°C and 1 bar pressure-produced protease, which was active at alkaline pH and low temperature of 5°C. Up to 51% of the deep-sea isolates and only

32 % of the shallow water isolates showed ability to grow at low temperature of 5°C. Among the cultures showing growth at low temperature, 11 % of the deep-sea isolates and 29 % of the shallow water isolates produced protease that was active at alkaline pH (9.0) and 5°C (Table 7.1). The number of isolates showing protease activity at 30°C was much higher than those showing activity at 5°C. A large number of isolates showed alkaline protease activity within a range of 1 – 10 ACU mL^{-1} (Fig. 7.1).

Fig. 7.1 Number of fungi isolated from deep-sea and shallow–water sediments showing protease activity in the range of 1 to 40 ACU mL^{-1} when grown at 5° and 30°C. The NIOCC #20 belonged to the group showing 30-40 ACU mL^{-1}



Measurement of protease activity using azocasein was found to be very sensitive because as low as 1 ACU could be detected

7.4.2 Protease production by NIOCC 20 under different conditions

A culture of *Aspergillus ustus*, (NIOCC # 20) isolated from the deep-sea sediments, during the cruise AAS 34, from core # BC 3, subsection 15-20 cm, from a depth of 5294 m by particle plating method showed maximum protease production initially in the range of 30-40 ACU mL^{-1} when assayed at 30°C and 1 bar pressure. It showed growth at elevated hydrostatic pressure and 5°C and was selected for further characterization of the enzyme. The culture showed growth under elevated hydrostatic pressures at 30 and 5°C (Table 7.2).

Table 7.2 Characteristics of the deep-sea isolate NIOCC # 20 when grown for 20 days in CD broth with 0.3 % skimmed milk powder under various pressure and temperature conditions

Growth temperature	1 bar	50 bar	100 bar	Parameters
30°C	208.2	69.4	44.1	Biomass produced (mg dry weight.)
5°C	121.4	93.7	85.8	
30°C	36.02	3.67	0.78	Protease activity (ACU mL^{-1}) assayed at 30°C and 1 bar
5°C	4.45	0.45	0.22	
30°C	100	10.2	2.17	% activity against the highest value
5°C	12.35	1.25	0.61	
30°C	0.173	0.15	0.014	Protease activity /mg dry biomass
5°C	0.037	0.013	0.007	

Protease production by this fungus was assayed both at 30 and 5°C. The protease produced at 1 bar pressure at both 30°C and 5°C was active up to 300 bar. The activity was fairly stable but the protease production seemed to be repressed considerably at 5°C (Table 7.3).

Table 7.3 Activity of the protease of the deep-sea fungus NIOCC #20 assayed under elevated hydrostatic pressures and 30°C

Pressure (bar)	Protease activity (ACU mL^{-1})	
	A	B
1	427.33 ± 4.51	ND
50	356 ± 2.12	3.67 ± 0
100	363.33 ± 4.72	3.33 ± 0
200	417.33 ± 10.24	4.33 ± 0.24
300	428.33 ± 4.95	4.67 ± 0

A – Protease produced when grown at 1 bar pressure /30°C

B – Protease produced when grown at 50 bar pressure / 30°C

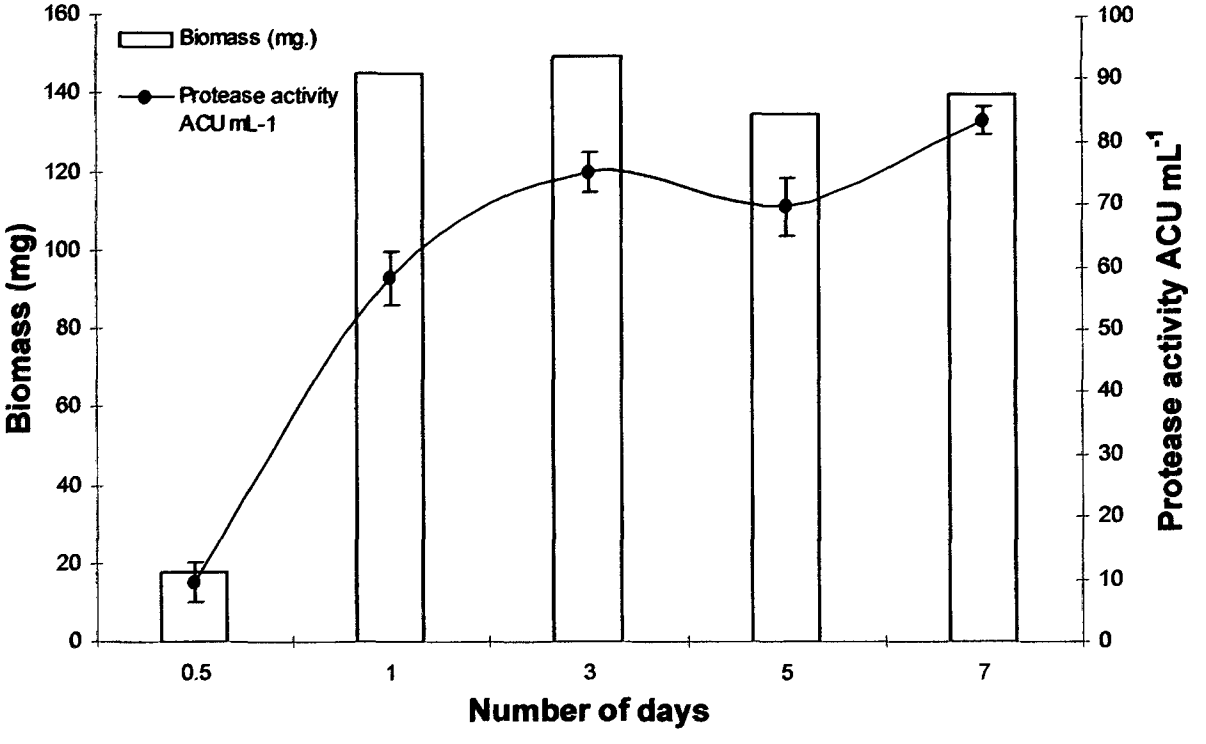
ND – not detectable

The enzyme produced at elevated hydrostatic pressure and low temperature revealed negligible or non-detectable levels of protease in all the assays at 45°C. Therefore further comparisons of protease production under different growth conditions of elevated hydrostatic pressures were made by assaying at 30°C.

7.4.3 Optimization of culture conditions for protease production by NIOCC 20

The culture was showing highest amount of protease production after 7 days of incubation at 30°C (Fig. 7.2). The biomass production was very high by day 1 with the

Figure 7.2 Temporal production of biomass and protease by NIOCC #20 grown in CD broth with 0.3 % skimmed milk powder

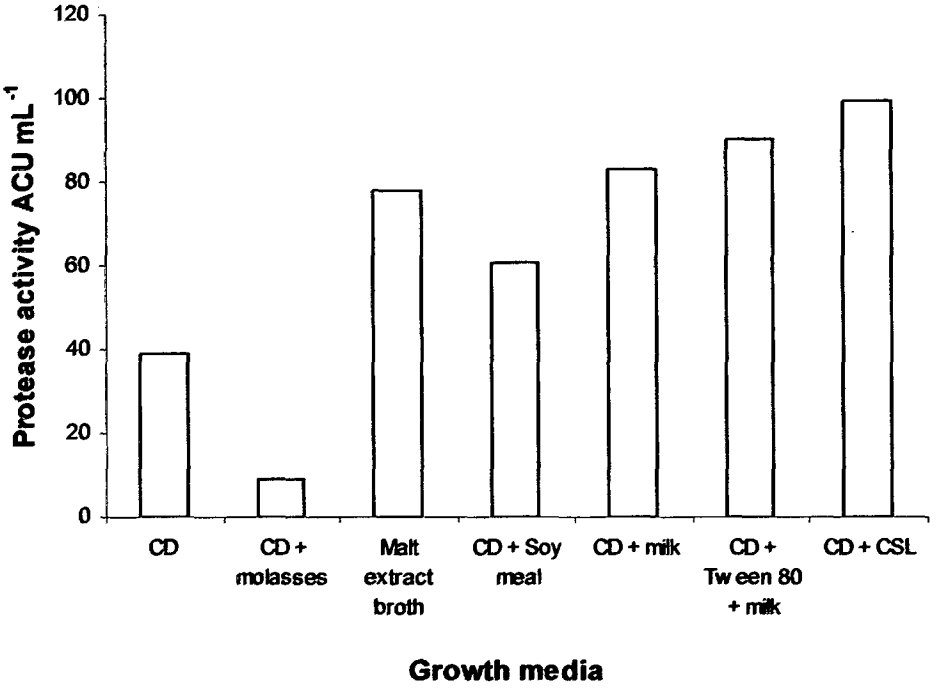


maximum being observed by day 2. The protease production attained maxima by day 7, which was more or less similar from day 3.

There was increase in the amount of protease production in the presence of inducers (Fig.7.3) **Protease production was also observed constitutively in CD broth** with only glucose as an organic source. The maximum protease production was observed in the presence of corn steep liquor (CSL), with the production decreasing in

CD + tween 80 + skimmed milk, CD + skimmed milk, malt extract, CD + soy meal and plain CD. The protease production was repressed in the presence of molasses.

Figure 7.3 Protease production by NIOCC #20 when grown at 30°C in different media



Addition of EDTA to the culture before harvesting was found to increase the protease yield by 1.5 to 2 times. When the enzyme was harvested after addition of EDTA, the yield of the enzyme was around 2400 ACU mL^{-1} as against that of 1200 ACU mL^{-1} without addition of EDTA.

Amongst the different methods of concentrations used, vacuum concentration was found to be the most efficient as compared to dialysis and ultra filtration (Table 7.4).

Table 7.4 Efficiency of different concentration techniques

Concentration method	Total volume (mL)		Protease activity (ACU mL^{-1})	
	Before concentration	After concentration	Before concentration	After concentration
PEG concentration using dialysis	100	10	1639	1890
Ultrafiltration using 3 kDa cut off	100	20	1639	1485
Vacuum concentration	100	15	1639	2365

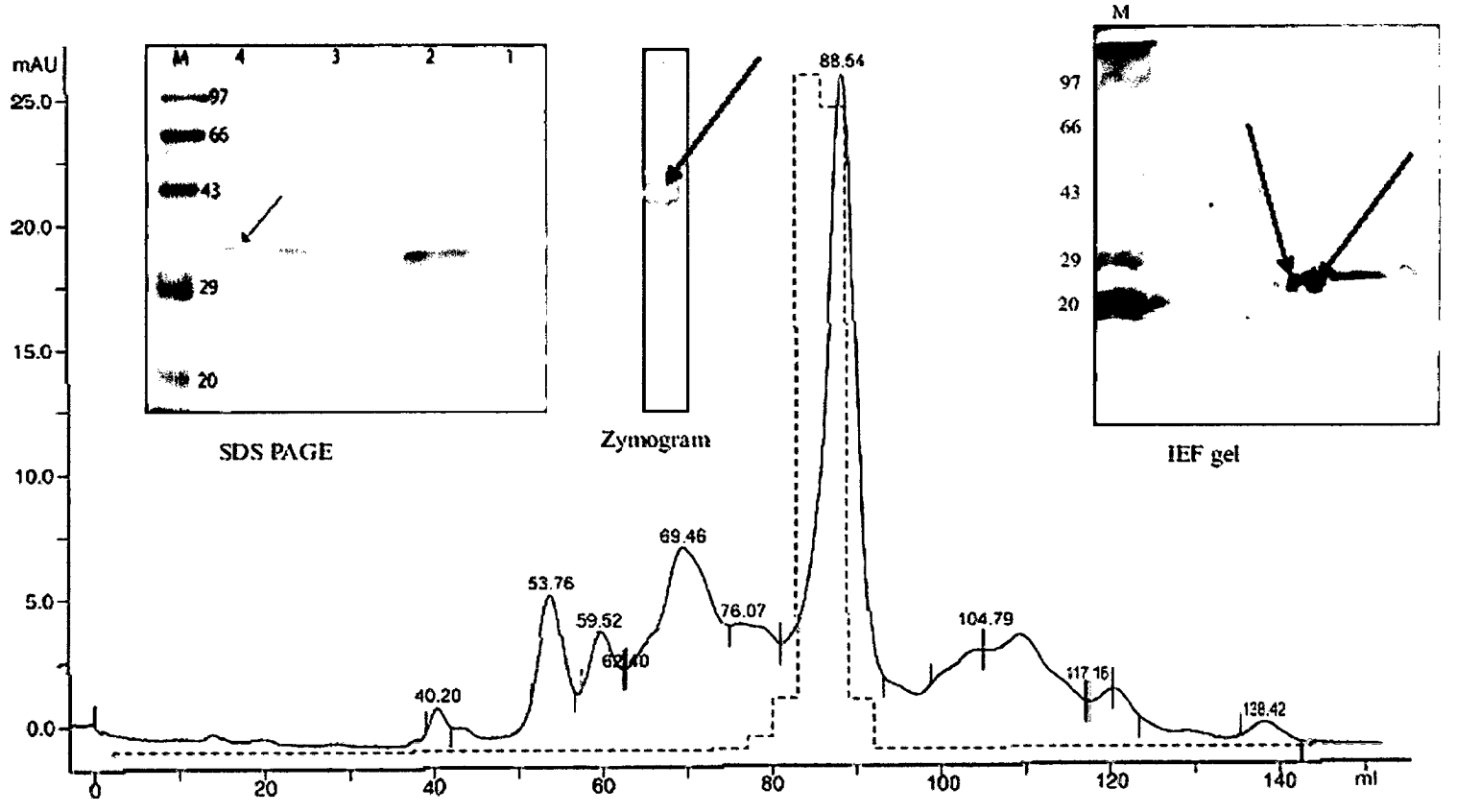
7.4.4 Purification and characterization of protease produced by NIOCC 20

The concentrate when passed through Resource Q column, protease enzyme was eluted as an unbound fraction. There was no protease activity recorded in any of the bound fractions. These fractions when pooled, concentrated and subjected to size exclusion chromatography, resolved in different proteins. The protease enzyme was

Table 7.5 Purification of protease of the deep-sea fungus NIOCC #20 grown at pH 9.0 and 30°C

	Total enzyme units ACU	Total Protein mg	Sp. Activity (ACU mg^{-1} protein)	Purification (fold)	% Recovery
Crude filtrate	981,000	131.08	7,484	1	100
Vacuum concentrated	205,800	93.06	2,212	0.3	21
Resource Q	166,786	82.61	2,019	0.27	17
Superdex-200	206,800	6.82	30,323	4.05	21

Figure 7.4 FPLC profile with Zymogram, SDS and pI profile as insets



FPLC profile after passing through Superdex 200, showing active enzyme fraction (dotted line, solid line indicates protein)

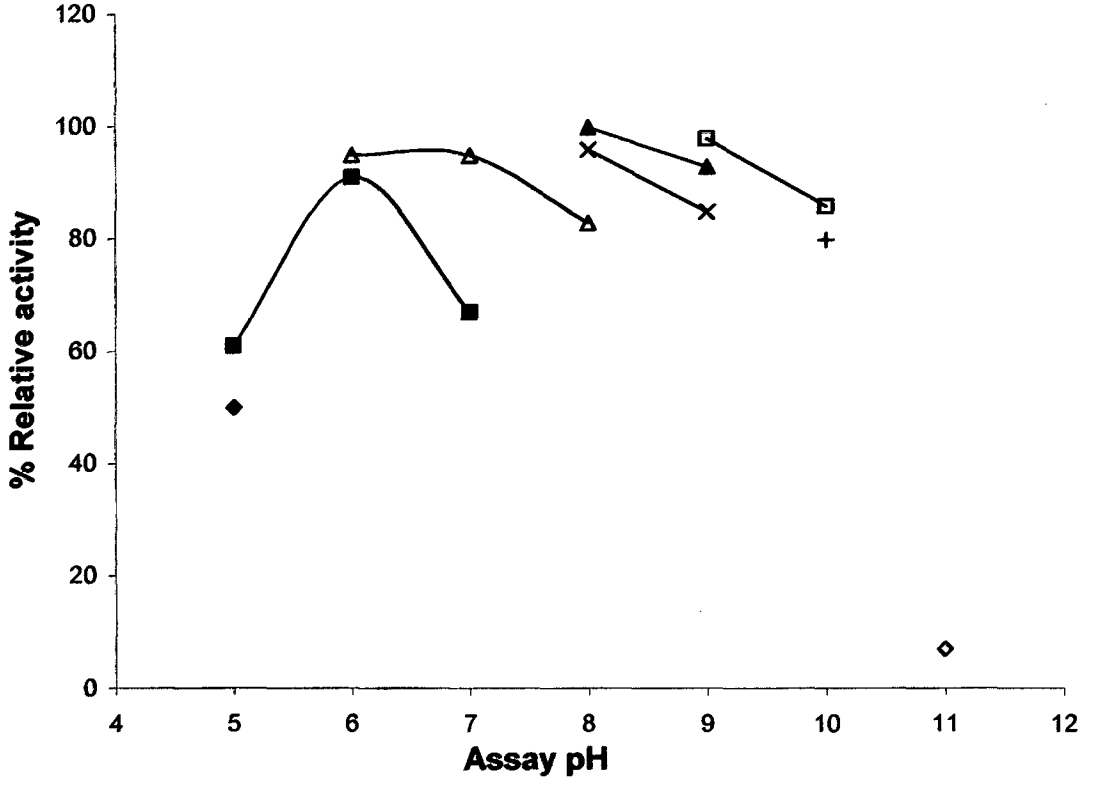
Zymogram of the active fraction on native page showed activity staining for protease. The clear zone in casein as a result of protease activity appears as a band (arrow);

The SDS-PAGE of the protease; Lane 1= crude culture filtrate, Lane 2=vacuum-concentrated culture filtrate, Lane 3= Resource Q-pooled fraction, Lane 4= pooled active fraction obtained by gel filtration using Superdex 200 showing single band of 32 kDa (arrow);

IEF gel showing isozymes with pI values of 6.6 and 6.9 (arrows).

obtained in pure form distributed over 10 mL (Fig. 7.4). This part was concentrated under vacuum and when checked for homogeneity showed a single activity band on a zymogram, with casein as substrate. The protease enzyme showed a single band of 33 kDa on a SDS PAGE, indicating that it is monomer (Fig. 7.4). On the isoelectric gel, the enzyme was found to give two bands corresponding to pI values of 6.6 and 6.9 (Fig. 7.4). The enzyme was purified 4-fold with the specific activity of the pure enzyme being more than 30000 ACU mL^{-1} and a total yield of 21 % (Table 7.5).

Figure 7.5 Relative activity of protease of NIOCC #20 at different pHs and 45°C



◆ acetate buffer; ■ citrate phosphate buffer; △ phosphate buffer; × Tris-HCl buffer; ▲ boric-borax buffer; □ Glycine-NaOH buffer; + borax-NaOH; ◇ carbonate-bicarbonate buffer.

The enzyme exhibited pH optima of 9 with >80% of activity between pH 6-10 at 45°C (Fig. 7.5). The maximum enzyme activity was recorded at 45°C and about 45% of the maximum activity was detected at 20°C and about 10 % at 2°C (Fig.7.6). The Q_{10} values at 2 and 15°C were above 1 while those at 30, 45 and 60°C were less than 1 (Table 7.6).

Figure 7.6 Relative activity of protease at different temperatures measured at pH 9

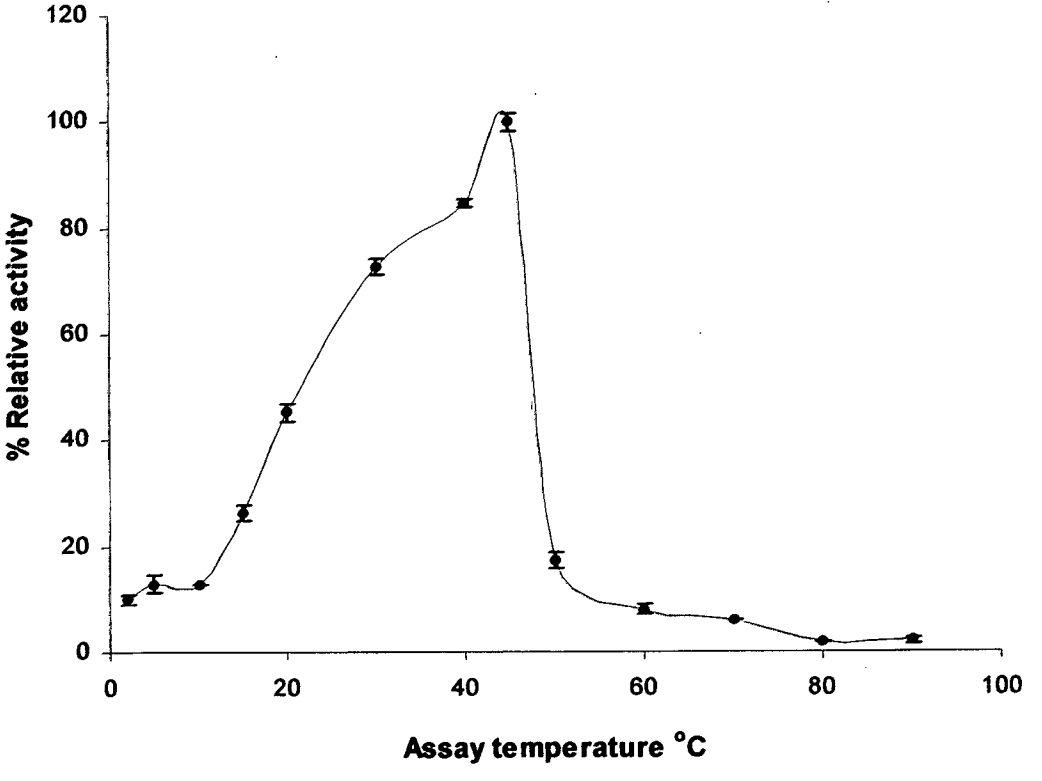
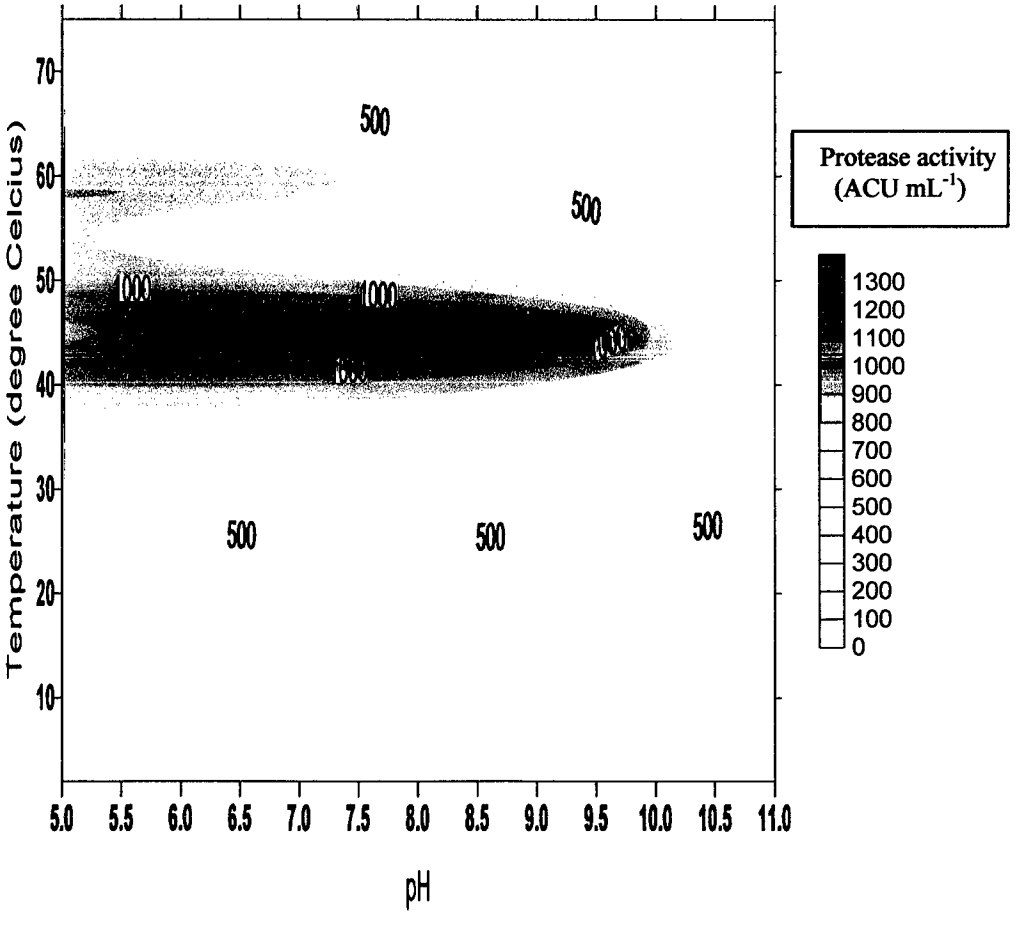


Table 7.6 Q_{10} values for the purified protease enzyme produced by NIOCC # 20

T_1	T_2	Q_{10} value	T_1	T_2	Q_{10} value
2	15	1.53	30	45	1.74
2	30	1.78	30	60	0.89
2	45	1.77	30	75	0.73
2	60	1.22	30	45	1.74
2	75	1	30	60	0.89
15	30	1.82	30	75	0.73
15	45	1.78	45	60	0.46
15	60	1.13	45	75	0.47
15	75	0.92	60	75	0.49

Figure 7.7 Activity of purified protease at different pH and temperature



When the enzyme activity was assayed at all the pH values at all the temperatures mentioned above, it showed a fairly constant activity in the pH range 6 to 8 and a temperature range of 30 to 50°C (Fig 7.7).

The protease of the culture grown at 1 bar pressure showed a K_m constant of 2 mg mL^{-1} when measured at pH 9 and 45°C and this increased marginally at elevated hydrostatic pressures of 50 and 100 bar (Table 7.7). There was a marked increase in K_m constant at 5°C/1 bar, 45°C/ 200 and 300 bar pressure. The V_{max} values were not much affected by the elevated hydrostatic pressures except a substantial drop at 50 bar pressure from that at 1 bar pressure (Table7.7).

Table 7.7 K_m constant and V_{max} values of the purified protease of the deep-sea fungus NIOCC # 20 measured under different conditions

Enzyme activity assayed at	K_m constant (mg mL^{-1} azocasein)	V_{max} (ACU mL^{-1})
1 bar and 45°C	2.0	26.3
50 bar and 45°C	2.2	19.2
100 bar and 45°C	2.4	24.4
200 bar and 45°C	3.3	25.0
300 bar and 45°C	5.0	25.0

Thermostability studies showed that the enzyme retained 80 and 55% of its activity at 50 and 60°C respectively for 10 min at pH 9 (Fig. 7.8). At its optimum temperature of activity (45°C), the enzyme retained 100 % of its activity for 30 min after which it steadily decreased (Fig. 7.9). The enzyme was most stable at pH 4.5 in the absence of the substrate (Fig. 7.10), with the stability decreasing with alkalinity.

Figure 7.8 Residual activity of protease measured after incubating the enzyme sample at pH 9 for 10 min at different temperatures

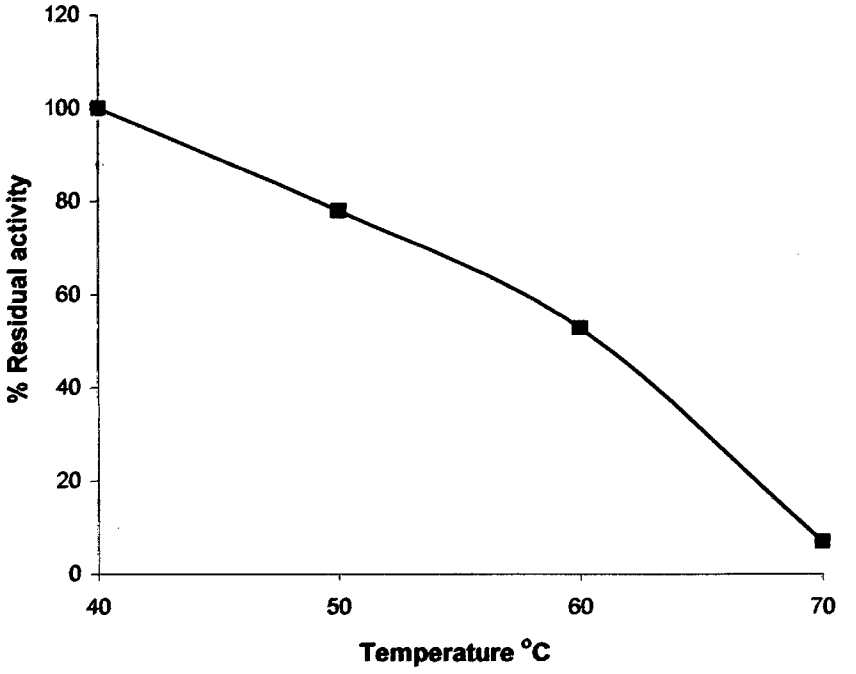


Fig. 7.9 Stability of purified protease of NIOCC # 20 at its optimum temperature of activity (45°C)

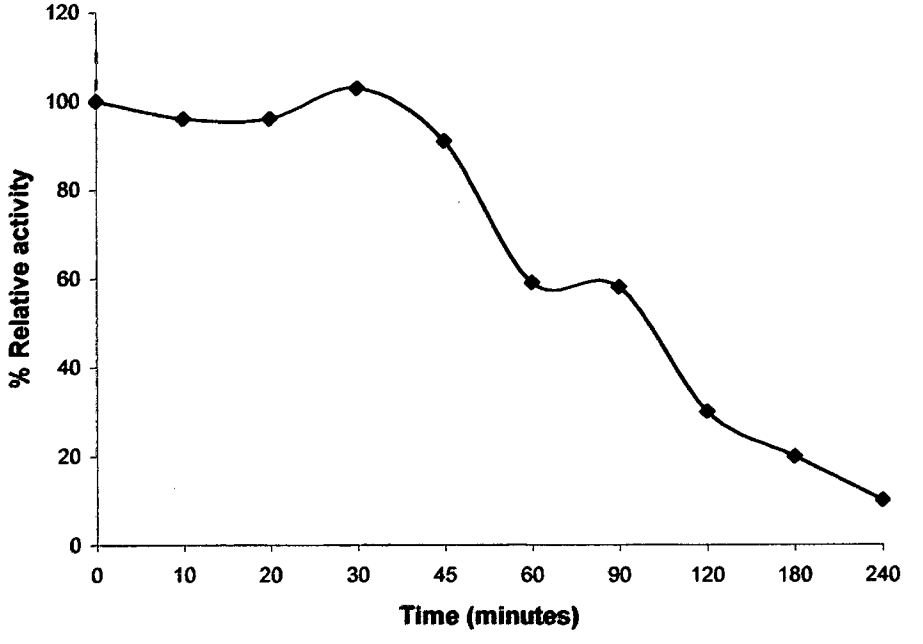
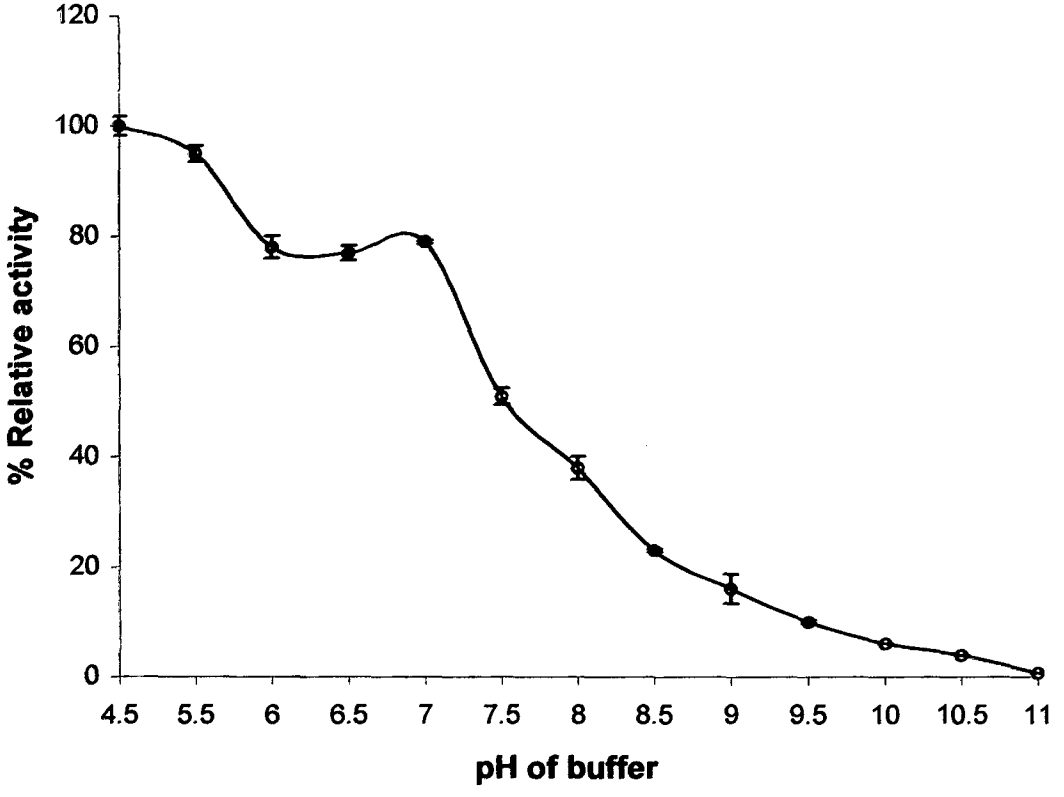


Fig. 7.10 Stability of protease at different pHs checked by incubating the enzyme at specified pHs without the substrate for 60 min. Activity was measured at its optimum temperature (45°C) and expressed as percent residual activity.



The protease activity was totally inhibited by 2 mM PMSF suggesting it to be a serine protease. EDTA at 5 and 100 mM concentration hardly inhibited the protease activity (Table 7.8) indicating that it was not a metalloprotease. The protease of NIOCC #20 is thiol-independent because reducing agents as DTT and mercaptoethanol did not affect the activity considerably (Table 7.8) At 1 mM concentration of Hg only 45 % inhibition of the enzyme was observed. Addition Fe, Ni and Zn at 1 mM hardly inhibited the enzyme activity. On the other hand, Cu at 1 mM concentration inhibited the protease activity totally (Table 7.8). About 70% of the activity was retained in the presence of sodium chloride of 0.5 M concentration, which equals to 29 ppt salinity of seawater (Table 7.8).

Table 7.8. Effect of various inhibitors, heavy metals, varying ionic strengths (sodium chloride) and reducing agents on protease activity of the deep-sea fungus NIOCC #20.

Inhibitors	Residual activity (%)
Protease Inhibitors (mM)	
None (control)	100
PMSF (2)	0
EDTA (5)	99
EDTA (100)	93
Heavy metals (mM)	
$NiCl_2$ (1)	72
$ZnCl_2$ (1)	81
$HgCl_2$ (1)	55
$CuCl_2$ (1)	0
$FeCl_3$ (1)	74
Sodium chloride (M)	
0	100
0.1	88.6
0.25	80
0.50	70
0.75	61.4
1.0	40
Reducing Agents (%)	
None	100
DTT Dithiothereitol (0.1)	91
DTT (0.5)	73
β-mercaptoethanol (0.1)	77
β-mercaptoethanol (0.5)	75
Sodium thioglycolate (0.1)	45
Sodium thioglycolate (0.5)	27
Sodium thioglycolate (1.0)	27
Glutathione (0.1)	0

Chemical surfactants like Triton X-100 and Tween 80 (both at 1.0%) and commercial detergents at 2 g L^{-1} concentrations did not reduce the protease activity, although SDS (0.1%) totally inhibited its activity (Table 7.9). About 50% of the enzyme activity was lost after treatment at 60°C for 10 min. However, thermostability of the enzyme at 60°C increased on addition of glycerol, $CaCl_2$, PEG 6000, sucrose, mannitol, sorbitol and starch during enzyme assay (Table 7.10).

Table 7.9 Effect of detergents and bleaching agents on protease activity of the deep-sea fungus NIOCC #20

Compound (Concentration in %)	Residual specific activity (%)
None	100
Tween 80 (1.0)	142
Triton X-100 (1.0)	139
SDS (0.1)	0
Ala (Commercial fabric bleach) (2.0)	20
Sodium hypochlorite (1.0)	2
H_2O_2 (2.0)*	40
Commercial Detergent Wheel (2 g L^{-1})	91
Mr. White	85

7.4.5 Comparison of protease production between deep sea (NIOCC 20) and terrestrial isolate (MTCC 2200) of *Aspergillus ustus*

Both the terrestrial (MTCC 2200) and the deep-sea culture (NIOCC 20) produced almost similar biomass in Czapek Dox broth (Fig. 7.11). By third day, the

cultures reached stationary phase, after which the biomass was almost constant. But the protease production showed a lot of difference, with the deep-sea isolate producing

Table 7.10 Effect of additives on thermostability of the crude enzyme of the deep-sea fungus NIOCC #20 with reference to 10 minutes of heat treatment at 60°C without any additive.

Additive present during the heat treatment	% Residual activity
Control (untreated)	100
No additives	51
Glycerol (1%)	101.5
$CaCl_2$ (1 mM)	108.5
$CaCl_2$ (5 mM)	110.6
$CaCl_2$ (10 mM)	102.4
PEG 6000 (1%)	111.1
Sucrose (1%)	84.8
Mannitol (1%)	79.2
Sorbitol (1%)	85.6
Starch (1%)	98.8

a maximum of 1600 ACU mL^{-1}, while the terrestrial culture showed only 102 ACU mL^{-1} (Fig. 7.12). The pH for the activity of the protease produced by both the

terrestrial and the deep-sea isolate were found to be similar with the activity increasing with the increasing pH, more in the alkaline range (Table 7.11).

NIOCC 20 showed about 50-fold increased protease production in the presence of sea water at different salinities as compared to MTCC 2200. In case of NIOCC 20, the protease production increased gradually up to 20 ppt, above which salinity did not affect it. Whereas in case of MTCC 2200, no uniform effect was observed (Table 7.12).

Fig. 7.11 Temporal production of protease by NIOCC 20 and MTCC 2200

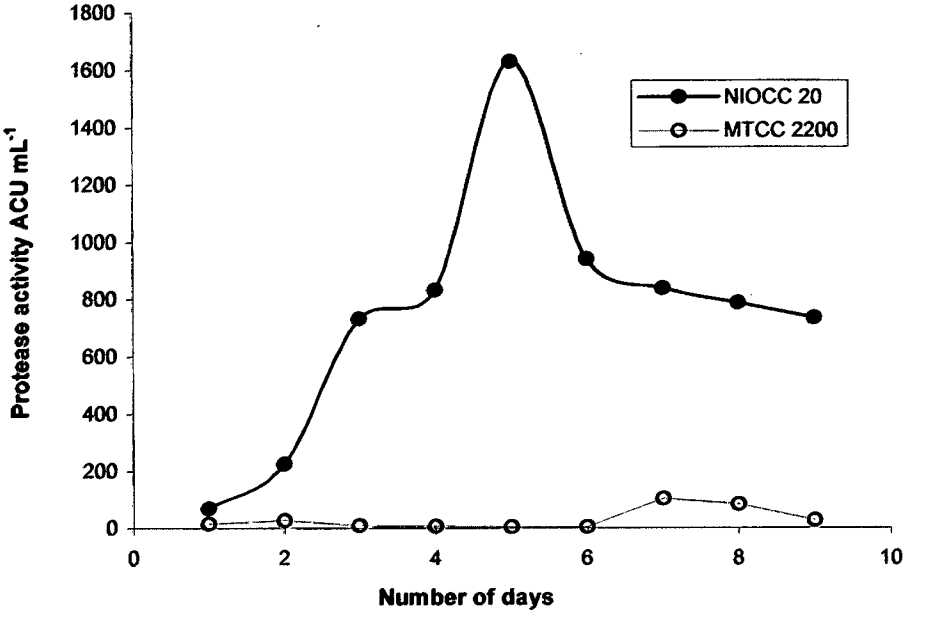


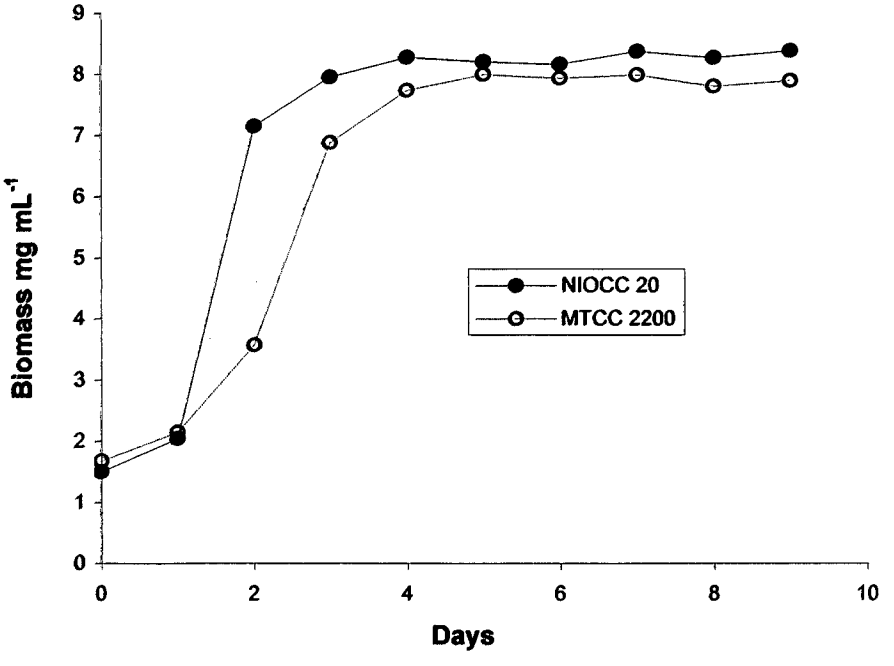
Table 7.11 Comparative activity of protease produced by the terrestrial isolate MTCC 2200 and the deep-sea isolate NIOCC 20 at different pH values

Assay pH	Protease activity ACU mL^{-1}	
	NIOCC 20	MTCC 2200
5	522	5.3
7	817	26
9.5	861	55.1

Table 7.12 Protease produced by the deep-sea isolate # NIOCC 20 and terrestrial isolate MTCC 2200 in Czapek Dox broth prepared in seawater with varying salinites after 8 days of incubation

Salinity (parts per thousand) ppt	Protease activity	
	NIOCC 20	MTCC 2200
0	729	16
5	828	20
10	926	28
15	1119	35
20	1140	17
25	1149	24
30	1151	28
35	1154	25

Fig. 7.12 Biomass produced by NIOCC 20 and MTCC 2200



7.5 Discussion

Protease enzyme was produced by majority of the fungal isolates tested suggesting that it is one of the major extracellular enzymes being produced by the fungi playing a major role in their nutritional requirements. But again, not all the fungal cultures were showing protease production, which was active in the alkaline pH range and at a low temperature of 5°C, suggesting that the protease being produced by one culture is enough to degrade the proteinaceous matter present into easily assimilable products and supply the required nutrients for growth to all the non-protease producers also. Further, although all the isolates were isolated from a cold habitat of deep sea, only 51 % of these showed protease production, which was active at low temperature. The number of fungi showing protease activity at 30°C was much higher than those showing activity at 5°C, with large number of isolates showing activity in the range of 1 - 10 ACU mL^{-1}. This is keeping in view with the theory put forth in previous chapter that these peizotolerant isolates are terrestrial forms that have managed to reach the ocean bottom and have got adapted to the conditions there.

The measurement of protease activity using azocasein was found to be very sensitive because as low as 1 ACU could be detected and also the assay was replicable with low standard deviation values.

Aspergillus ustus isolated from deep-sea calcareous substrates has been reported to produce protease [Raghukumar & Raghukumar, 1998], which was active under elevated hydrostatic pressure and low temperature. The culture, *Aspergillus ustus*, NIOCC # 20, isolated in the present study, showed highest amount of protease production active at alkaline pH and low temperature. It showed substantial growth at

5°C, under 50 and 100 bar pressure (corresponding to 1000 m). Under the above two conditions, it attained a biomass equivalent to 58 and 41 % respectively of that produced at 30°C/1 bar pressure (Table 7.2). However, when the fungus was grown at 5°C under 1, 50 or 100 bar and the enzyme activity assayed at 5 or 30°C under different hydrostatic pressures, little or negligible amount of cold-tolerant protease was detected. In contrast to the above, the enzyme produced by the fungus at 30°C and 1 bar was much more versatile and retained about 10 % of activity at 2°C at 1 bar compared to its activity at optimum temperature. The enzyme produced at 30°C under 1 or 50 bar also retained its activity when assayed under elevated hydrostatic pressures up to 300 bar (Table 7.3). However, the increasing K_m constants of protease at elevated hydrostatic pressures of 200 and 300 bar (Table 7.7) indicates low enzyme-substrate affinity under these extreme conditions. The total enzyme activity *per se* is not affected by elevated pressures (Table 7.3), which is also indicated by the almost constant V_{max} values (Table 7.7).

Although growth was fairly unaffected by elevated pressure and low temperature, protease production under similar conditions was reduced 10 to 100 folds. This appears to be a general trend with all the deep-sea isolates that were isolated (Table 7.1). Thus, a higher percentage of deep-sea isolates (51 %) were capable of growing at 5°C, when compared to those isolated from shallow waters (32 %). Besides, a high number of deep-sea isolates produced low-temperature active protease when grown at 1 bar and 30°C, but very few of these did so when grown at 1 bar and 5°C (Table 7.1). Compared to the deep-sea isolates, fewer isolates from shallow water produced cold-

tolerant protease when grown at 1 bar pressure and 30°C and their growth was also poor at 5°C.

A culture of *Aspergillus ustus* obtained from the culture collection IMTECH, showed negligible amounts of protease production, the activity of which was very low at both the pH and temperatures checked indicating that NIOCC # 20 is a totally different strain isolated from the deep sea sediments. NIOCC # 20 appears to be a strain adapted to deep-sea conditions, since it grew better at 5°C / 100 bar than at 30° C / 100 bar, although its production of protease under the former conditions was low (Table 7.3). This is in contrast to the report on a deep-sea bacterial strain DB6705, which grew better at higher rather than low temperatures when cultured at 500 bar hydrostatic pressure [Kato *et al.*, 1996]. Growth and metabolic activities under extreme conditions are known to be low [Wirsen & Jannasch, 1975]. Our results with NIOCC #20 also show similar trends.

During the standardization of the medium components, it was found that the presence of corn steep liquor induced maximum protease production followed closely by skimmed milk (Fig. 7.3). But for all the standardization and characterization of the enzyme, skimmed milk was used as the inducer due to its easy availability and more defined nature. Apart from this CSL, being dark coloured might also interfere in the enzyme assay.

The enzyme yield was found to be better when EDTA was added to the culture broth just before harvesting of the enzyme. This could have happened due to release of the enzyme that was trapped in the exopolysaccharides produced by the culture that came in solution form due to chelating of the EPS by EDTA [Comte *et al.*, 2006]. The

protease produced by NIOCC # 20 showed several important features. Although the optimum temperature for activity of the enzyme is 45°C, it is described as a cold tolerant protease. Enzymes from cold-adapted organisms have been classified into 3 groups [Ohgiya *et al*, 1999]. Group I enzymes are heat-sensitive, but the other enzymatic characteristics are similar to mesophilic enzymes. Group II enzymes are heat-sensitive and relatively more active than mesophilic enzymes at a low temperature. Group III enzymes have same thermostability as mesophilic enzymes but are more active than mesophilic enzymes at a low temperature. Accordingly, the protease of this isolate falls into the group II. The protease of mesophilic *Penicillium* sp. did not show any activity below 20°C [Germano *et al.*, 2003]. The alkaline protease from another mesophilic species of *Penicillium* did not show any activity below 35°C [Agrawal *et al.*, 2004]. On the other hand, NIOCC # 20 showed about 45, 26 and 12% of the maximum activity at 20, 15 and 5°C. It looses about 50% of its activity at 60°C (Fig. 7.8). Moreover, at its optimum temperature of activity (45°C), it is stable only for 30 min after which it rapidly looses activity (Fig. 7.9).

The enzyme is stable for more than 24 h at 5°C and pH 4.5. Its half-life under these conditions is about 196 hours. These results suggest its placement in the group II. The effect of temperature on the enzyme activity is determined by the temperature coefficient Q_{10} [*http://www.csupomona.edu/~seskandari/Q10.html*]. It is the factor by which the rate increases when the temperature is raised by ten degrees. If the rate of the reaction is completely temperature-independent, the resulting Q_{10} will be 1.0. If the reaction rate increases with increasing temperature, Q_{10} will be greater than 1. Thus, the more temperature-dependent a process is, the higher will be its Q_{10} value. The

calculated Q_{10} value of 1 between 2° to 10°C in the present work (Table 7.6) indicates temperature-independent enzyme activity at lower temperatures. On the other hand, the increased Q_{10} values (above 1) at 15° to 45°C indicate direct relation to increasing temperatures. Above 45°C, the Q_{10} value is less than one suggesting negative effect of temperature on the enzyme activity.

The protease of NIOCC #20 is a serine protease because it was totally inhibited by PMSF, which is a known inhibitor of serine protease. EDTA did not inhibit it and therefore it is not a metalloprotease. It was not substantially inhibited by the heavy metals Ni and Zn, which are the inhibitors of cysteine protease. Its activity did not increase in the presence of thiol compounds, indicating that it was thiol-independent serine protease. Protease produced by this isolate showed several special features. It was active in the presence of several commercial detergents when assayed under its optimum conditions. The enzyme was active in the presence of 0.5 M NaCl equivalent to 29 ppt of seawater salinity. Its stability at 60°C increased in the presence of various stabilizing agents. Such features in proteases are often much sought after for use as additives in detergents for low temperature wash [Ohgiya *et al.*, 1999]. Most alkaline proteases have been reported to be significantly stabilized in the presence of additives such as those used in this study [Beg & Gupta, 2003].

Protease of *Aspergillus ustus* (NIOCC #20) with an optimum pH of 9 and showing about 45% and 25 % of its activity at 20°C and 15°C respectively is an alkaline protease. Its performance in terms of activity can be further improved by optimization of growth and other parameters. Terrestrial isolate of *Aspergillus ustus* (isolate # MTCC 2200) obtained from IMTECH culture collection showed extremely

low protease production (23 ACU mL^{-1}) under similar assay conditions. This indicates that deep-sea fungi are useful candidates in the search for alkaline proteases and that the enzyme can have multiple uses right from detergents to food to biotechnological field.

Chapter 8

Role of fungi in Deep - sea Sediments

8.1 Introduction

Roughly 62 % of the globe's surface, the ocean floor, is covered by seawater. This huge volume and area, however, has to be viewed as a desert of life where life is strictly limited by the source of organic carbon and biochemically useful energy [Jannasch, 1994]. The sea surface overlaying the deep-sea provides only about 21 % of the total global photosynthetic useful energy. About 95 % of this amount is recycled in the upper 100 - 300 m of surface water, while the remaining 5 % sink down in the water column in particulate form by sedimentation. Small portions of this material are large chunks of organic matter such as fish carcasses, decapods and large shrimp. Their relatively high sinking rates of 50 – 500 m $hour^{-1}$ [Vinogradov & Tseitlin, 1983] prevent a substantial en route decomposition. Another portion of organic matter is represented by faecal pellets of jellyfish and zooplankton with sinking rates of 2700 and 50 - 200 m/day, respectively [Bruland & Silver, 1981; Komer *et al.*, 1981; Madin, 1982]. The potentially much lower sinking rates of phytoplankton debris often aggregate to "marine snow" particles [Alldredge & Gotschalk, 1988] and much of it becomes part of the food for zooplankton, largely containing compounds undigestable for zooplankton. The faecal pellets overgrown with bacteria are eaten repeatedly on their way to the deep sea. These faecal pellets act as a matrix for bacterial growth, a system that can be termed as an extraorganismic enteric symbiosis [Jannasch, 1994].

Most of this material is decomposed during this descent providing food for the pelagic and deep populations of animal and microorganisms in the water column. The fraction of local plankton production reaching the sediments is mainly a function of water depth [Suess, 1980; Jørgensen, 1983]. The transport from the surface to the

deep-sea can be affected by various factors [Michaels *et al.*, 1990]. Where it is rapid and not diverted by lateral transport, seasonal signals of surface water productivity can be observed at the deep-sea floor [Asper *et al.*, 1992]. Generally, less than 1 % of the photosynthetically produced organic matter reaches the deep-sea sediment at a depth of about 4000 - 5000 m and even less in deeper parts of the oceans.

The major function of microorganisms in the deep sea is commonly seen in the conversion of dissolved organic matter into the particulate food for zooplankton often termed as the "microbial loop". The total concentration of organic carbon is of less importance for it's potential microbial decomposition than the metabolic availability of its constituents. The concentration of those compounds that are susceptible to microbial attack will decrease faster with depth than the concentration of total organic carbon, and the relative quantity of recalcitrant compounds will increase.

Because of their omnipresence and highly diverse metabolic capabilities, microorganisms are playing the major role as biocatalysts for the control of inorganic and organic carbon in the biosphere. Their ability to metabolize organic substrates at extremely low levels and to survive long periods of starvation, as well as adapt to life at low temperature and high pressure, helps them to use almost any biochemically exploitable form of organic carbon available at even low concentration [Jannasch, 1994].

The global carbon cycle is greatly influenced by the activities of both bacteria and fungi in terrestrial soils, which govern the rates of mineralization, as well as carbon sequestration [Bronick & Lal, 2005]. Individually, though, fungi and bacteria play different roles in soil biogeochemistry, bacteria have greater rates of

remineralization but lower C assimilation efficiencies than fungi, the latter thus being more significant in terms of carbon sequestration [Adu & Oades, 1978]. Therefore, it is important to consider both groups for a holistic understanding of microbial dynamics and their role in the carbon budget [Bailey *et al.*, 2002]. Particulate organic carbon deposition in deep-sea sediments is one of the mechanisms for removal of atmospheric carbon dioxide in the context of the global C cycle and marine sediments are the largest source of organic carbon on earth [Seiter *et al.*, 2004; Ittekot, 1996].

As in terrestrial soils, microorganisms are major players in transformation of organic matter in deep-sea sediments. In contrast to land, however, most studies on deep-sea sediments have focused exclusively on bacteria and have demonstrated their intense metabolic activities therein [Danovaro *et al.*, 1995; Turley & Dixon, 2002]. The fungi and their role in the deep-sea sediments have remained neglected mainly due to the fact that they are not easily seen / observed. This is because most of the times, fungi remain embedded in the sediments and hence go unnoticed. The fungi are known to stabilize the soil by the way of aggregation in the terrestrial ecosystems [Lynch & Bragg, 1985]. Soil aggregation is defined as the naturally occurring cluster or group of soil particles in which the forces holding the particles together are much stronger than the forces between the adjacent aggregates [Martin, 1955]. A number of studies have attempted to elucidate the role of soil microflora in the formation and stabilization of soil aggregates [Harris *et al.*, 1964; Molope *et al.*, 1987]. Although fungi are generally believed to be more effective at aggregate stabilization than other soil microflora, several studies have concluded that their primary contribution to aggregation is through hyphal entanglement of soil particles [Molope & Page, 1986].

Reducing sugars and amino acids, formed, as by-products of microbial metabolism in terrestrial sediments are known to undergo nonenzymatic polymerization to form a series of compounds like 3-carbon aldehydes, ketones and reductones and hydroxymethyl furfurals. These highly reactive compounds readily polymerize in the presence of amino compounds to form brown-coloured products, constituting humus [Tisdall & Oades, 1982]. The humic material combines with soil particles to form microaggregates. Fungal hyphae further act as binding agents themselves to form macroaggregates by trapping fine particles into the microaggregates [Kandeler *et al.*, 1999]. Fungi or bacteria can thus remain protected in certain particle size classes [Suberkropp & Meyers, 1996]. Cations such as Si^{4+}, Fe^{3+}, Al^{3+} and Ca^{2+} form bridges between particles in terrestrial microaggregates [Bronick & Lal, 2005].

Several enzymes are known to be involved in the cycling of nutrients and can be used as potential indicators of nutrient cycling processes. In aquatic ecosystems like that of deep sea where P-limitation exists, alkaline phosphatase activity (APA) plays an important role in the regeneration of inorganic phosphate through its catalysis of the breakdown of organic esters to inorganic P [Chrost, 1991]. Alkaline phosphatase activity is often repressed by highly dissolved reactive P (DRP) concentrations by feed back inhibition. The general name 'phosphatases' has been used to describe a broad group of enzymes that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid. Amongst these, the acid phosphatase has studied more as compared to the alkaline phosphatase.

Sediment microorganisms, which depend upon the activities of their extracellular enzymes to supply them with nutrients, are confronted with several

problems. In the first instance, the sediment environment is inhospitable for extracellular enzymes meaning, non-biological denaturation, adsorption and inactivation, and degradation by proteolytic microorganisms all conspire to take their toll on enzymes once they have left the protection of the cell [Burns, 1978]. However, it is obvious that if an enzyme is to be of value to the cell that secreted it, that enzyme must evade any destructive forces as long as it takes to locate and react with an exogenous substrate. There is also a strong possibility that at any particular moment, there is no suitable substrate in the vicinity of the enzyme-producing microorganism or that any substrate, which is present, is either too low a concentration to elicit a response or in form which is unavailable for enzymatic attack. So an enzyme has to survive long enough to locate and bind its substrate and even then, the physical conditions for catalysis may be inappropriate. Even if it is assumed that the enzyme overcomes all these obstacles then the next stage in the process is for the product of the catalysis to be relocated in the vicinity of the cell, which has produced the extracellular enzyme in the first place. Only when this stage is reached can adsorption of the product of extracellular catalysis take place and such responses occur as enzyme induction, chemotaxis and ultimately growth.

This is where fungi play a very important role, possibly in deep-sea sediments also. Fungi are known to produce a lot of exopolysaccharides [Selbmann *et al.*, 2003; Tang & Zhong, 2002], which in turn help the aggregation of these. This is particularly helpful feature in natural environment where it also helps other microorganisms like bacteria to stay together as consortia. This prevents the extracellular enzymes from diffusing away from the cells secreting it. Thus, these humic-enzyme complexes have

an important role to play in overall nutrient dynamics of the sediments [Burns, 1977, 1978].

8.2 Objectives

The main aim was to study the role of fungi in the deep-sea sediments with the following objectives:

1. To estimate the contribution of fungi to the carbon in the deep-sea sediments
2. To illustrate the microaggregation caused by fungi under laboratory conditions
3. To estimate the contribution of fungi to alkaline phosphatase in the deep-sea sediments.

8.3 Methodology

8.3.1 Fungal biomass (biovolume) in the deep-sea sediments

The fungal biomass in the sediments was estimated by measuring the total biovolume under a microscope. For this, about 0.5 g of sediment sample was suspended in 3 mL sterile seawater in a sterile vial and fixed with 1 mL formalin solution (final concentration of ~ 10 %). To 600 µL of this fixed sediment sample, 100 µL of 0.1 % solution of filter sterilized Calcofluor, an optical brightener, was added and mixed thoroughly. The excess stain was washed by centrifuging twice with sterile seawater. Microscopic mount of the sediment was then examined under ultraviolet light filter of an epifluorescence microscope to check for brightly fluorescing fungal hyphae. The same staining procedure was followed for the formalin fixed sediments mixed with 300 µL of sterile 10 % EDTA solution.

The biovolume of all the fluorescing fungal hyphae was calculated using an ocular micrometer and converted to μg of C g^{-1} of dry sediment [Van veen & Paul, 1979]. The approximate length and breadth (width) of the fungal hyphae detected under the microscope was measured. Using this, the volume of the filament was calculated assuming it to be a cylinder. The volume of the hyphae was then converted to the equivalent biomass in terms of C using the formula, 1 μm^3 of fungal biomass is equivalent to 1 pg C [Van veen & Paul, 1979]. This was then converted to μg of C g^{-1} of dry sediment to get the contribution of fungal biomass in terms of C in the deep-sea sediments in the Central Indian Basin (CIB).

8.3.2 Microaggregation by fungal cultures under simulated laboratory conditions

The ability of cultures for microaggregation was detected by growing them in sediment extract as sole nutrient source. The sediment extract was prepared as described in Chapter 5.

The deep-sea isolate # A 4634 (*Aspergillus terreus*) was used for this experiment. Finely ground quartz (< 100 μm) was used as an inducer for aggregation [Dietrich & Lampky, 1981]. The culture was grown in MEB for 2 to 3 days and harvested just before sporulation. The mycelial inoculum was homogenized using glass beads and used as inoculum for the further experiment. In a sterile plastic pouch, to 4 mL of the sediment extract, one mL of the inoculum was added. Two sets of experimental pouches were made, one with quartz and one without quartz. Both the sets were incubated under following conditions:

1. 200 bar / 30°C
2. 200 bar / 5°C
3. 1 bar / 30°C
4. 1 bar / 5°C

The bags were incubated at the above-mentioned conditions for 20 days. At the end of the incubation period, the contents of the pouches were fixed with formalin. The biomass was observed under the microscope for the presence of microaggregates. The biomass obtained was also stained to check for the humic material, polysaccharides and proteinaceous matter using benzidine [Sieberth & Julseth, 1968], alcian blue and coomassie blue staining [Long & Azam, 1996] respectively (Appendix A - X).

8.3.3 Fungal and bacterial contribution to alkaline phosphatase activity

Various types of enzymes are responsible for the degradation of organic materials present in the sediments. Every organism contributes differently, both in terms of quality and quantity, to accomplish this. One of the approaches to study this is to add growth inhibitor for specific type of organism and study the effect. To study the bacterial and fungal contributions, antibacterial antibiotic and fungicides have been used previously [Flegler *et al.*, 1974]. In the present study, antibacterial antibiotics (Penicillin and Streptomycin) and fungicide (Bavistin) were used to study the individual contribution of alkaline phosphatase activity in the sediment by fungi and bacteria with respect to. A deep-sea fungal isolate *Aspergillus terreus* along with bacterial isolates from the deep sea were used as control. Ampicillin (0.1 g in 100 mL of medium used) and penicillin (4000 U in 100 mL) were used as antibacterial

antibiotics and Bavistin™ (0.5% final concentration) as antifungal antibiotics. The concentrations of the fungicide and antibacterial antibiotic used in the study were standardized by growing different bacteria and fungi in varying concentrations of both the inhibitors. The second lowest concentration not showing cross inhibition (i.e. fungicide not inhibiting bacteria and vice versa) were used in the study. To simulate the sporadic enrichment of the deep-sea sediments with phytodetritus and dead animals, the sediment was spiked with detritus and artemia (cysts and larvae) as additional nutrient sources. The detritus was collected from shallow waters from Dona Paula Bay, using 200 µm plankton net. The detritus was a mixture of phyto-and-zooplankton.

Following combinations were used to study the exact contribution of each one to alkaline phosphatase activity:

1. Plain sediment
2. Sediment + fungicide (to suppress fungal growth)
3. Sediment + antibiotics (to suppress bacterial growth)
4. Sediment + detritus
5. Sediment + detritus + fungicide
6. Sediment + detritus + antibiotics
7. Sediment + artemia
8. Sediment + artemia + fungicide
9. Sediment + artemia + antibiotic
10. Sediment + detritus + antibiotic + fungus
11. Sediment + detritus + fungicide + bacteria

All these inoculations were done in quadruplicates in sterile plastic pouches, and incubated at following conditions for 20 days ---

1. 200 bar and 5°C
2. 500 bar and 5°C

At the end of the incubation period, the pouches were cut open and the contents centrifuged to obtain cell free supernatant, which was used for estimating the total alkaline phosphatase activity. The activity was estimated using 1mM MUF-phosphate as substrate prepared in methyl cellosolve [Hoppe, 1983; Ammerman & Glover, 2000]. The contents of the plastic pouches were transferred to 100 mL sterile conical flasks. To this, 10 mL of sterile 0.1 M tris HCl buffer pH 8.3 and the substrate, 100 µL of MUF phosphate were added. Toluene (25 µL) was added to avoid any bacterial activity and the flask was incubated in dark for 24 h at 5°C. When the enzyme hydrolyzes the substrate, equal amounts of orthophosphate and fluorescent non-phosphate moiety (methylumbelliferon) are produced. At the end of the incubation period, the suspension was centrifuged to obtain a clear supernatant. The methylumbelliferon (MUF) produced after the assay was detected as increase in fluorescence by using a Shimadzu RF-5301 spectrofluorometer, with excitation at 350 nm and emission at 450 nm. The fluorescence emitted is directly proportional to the alkaline phosphatase activity. A standard curve with MUF (Sigma) was used to quantify the amount of MUF produced by alkaline phosphatase activity (Appendix A - XI).

8.4 Results

8.4.1 Fungal biomass in the sediments of CIB

Actively growing fungi are found as filamentous hyphae in their natural habitats, in contrast to dormant spores. In order to detect such hyphae in the sediments, the sediments were stained with the fluorescent brightener, Calcofluor (Sigma, USA). Only 5.1 % (Table 8.1) of the deep-sea sediment samples examined

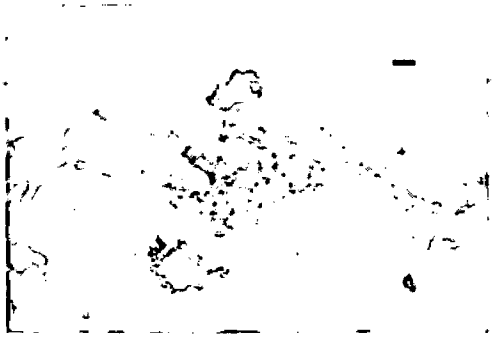
Table 8.1 Fungi in deep sea sediments detected in presence and absence of EDTA

Sub-surface depth (cm.)	Number of cores showing presence of fungi					
	Cruise # AAS 61			Cruise # ABP4		
	Total cores	Fungi observed in cores treated with EDTA	Fungi observed in cores without EDTA treatment	Total cores	Fungi observed in cores treated with EDTA	Fungi observed in cores without EDTA treatment
0 – 2	36	22 (61)	4 (11)	5	4 (80)	2 (40)
2 – 4	36	7 (19)	3 (8)	5	0	0
4 – 6	36	12 (33)	4 (11)	5	0	1 (20)
6 – 8	36	5 (14)	3 (8)	5	1 (20)	0
8 – 10	36	2 (6)	3 (8)	5	2 (40)	1 (20)
10 – 15	36	0	2 (6)	5	3 (60)	0
15 – 20	36	2 (6)	2 (6)	5	1 (20)	1 (20)
20 – 25	35	3 (9)	3 (9)	5	0	0
25 – 30	32	0	0	4	0	0
30 – 35	23	2 (9)	1 (4)	4	0	0
35 – 40	11	0	0	4	0	0
40 – 45	3	0	0	1	0	0

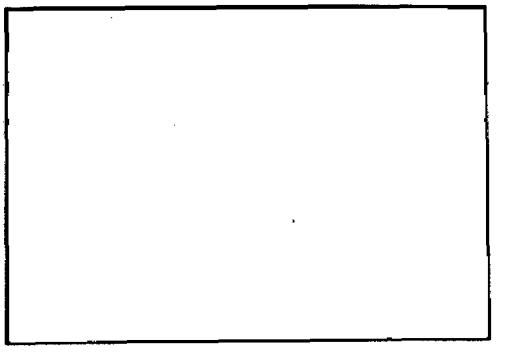
* figures in bracket indicate % cores showing presence of fungi

revealed fungal hyphae using this staining. Fungal biomass, estimated based on the total biovolume of fungal hyphae detected by epifluorescence microscopy ranged from 2.3 to 6.3 μg C g^{-1} sediment (Table 8.2). The same sediments samples treated with EDTA prior to staining with Calcofluor, revealed fungi in 12.8 % of sediment samples, an increase of two-fold over control treatments without EDTA. Fungal biomass carbon of EDTA-treated, Calcofluor stained sediment samples ranged from 2.3 to 5.1 μg g^{-1} dry sediment, which was similar to the samples without EDTA treatment. The treatment resulted in disrupting the soil to reveal numerous microaggregates, all of which contained fungal hyphae. Numerous microaggregates were connected by fungal hyphae to form a complex. Surfaces of such hyphae were encrusted with particulate matter. These encrustations stained positive for humic material (Fig. 8.1 a, b). Results obtained by this method could still be conservative estimates, since biomass estimated by hyphal length may often underestimate the true values, as compared to phospholipid estimations [Balser *et al.*, 2005].

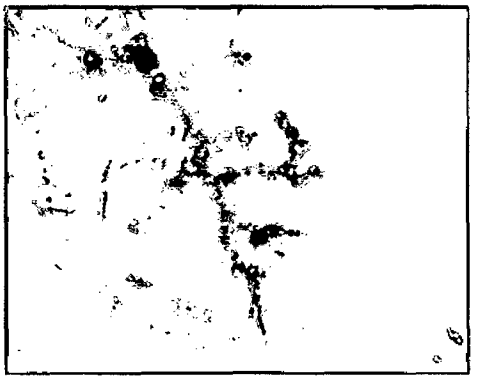
The deep-sea fungal isolate # A4634 (*Aspergillus terreus*) showed good growth in the sediment extract. The three terrestrial cultures obtained from IMTECH were also able to grow in the sediment extract, but their growth was better in MEB, whereas in the case of deep sea isolate # A 4634, the biomass produced was almost the same in sediment extract and MEB when incubated under elevated pressure of 200 bar and 5°C (Table 8.3).



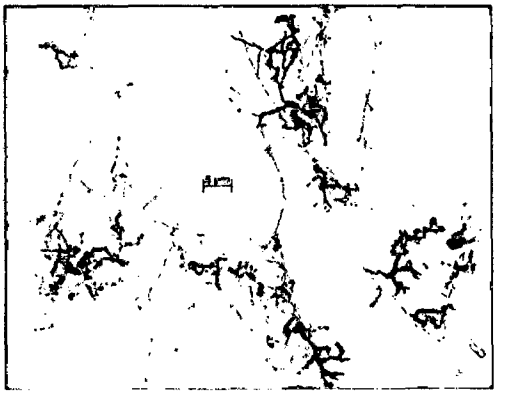
(a) Benzidine stained humic material in the vicinity of hyphae as seen under bright field microscope. Bar equals 10 μm.



(b) The same material as seen in (a) under epifluorescence microscope Bar equals 10 μm.



(c) Coomassie blue stained proteinaceous material in the vicinity of hyphae grown in sediment extract. Bar equals 5 μm.



(d) Alcian blue stained polymeric substances in the vicinity of hyphae grown in sediment extract. Bar equals 5 μm.

Fig. 8.1 The aggregates produced by *Aspergillus terreus* (# A4634) in the sediment extract stained positive for humic substances (a & b), proteins (c) and exopolymeric substances (d).

Table 8.2 Comparison of fungal biovolume and carbon contribution to sediments of different habitats

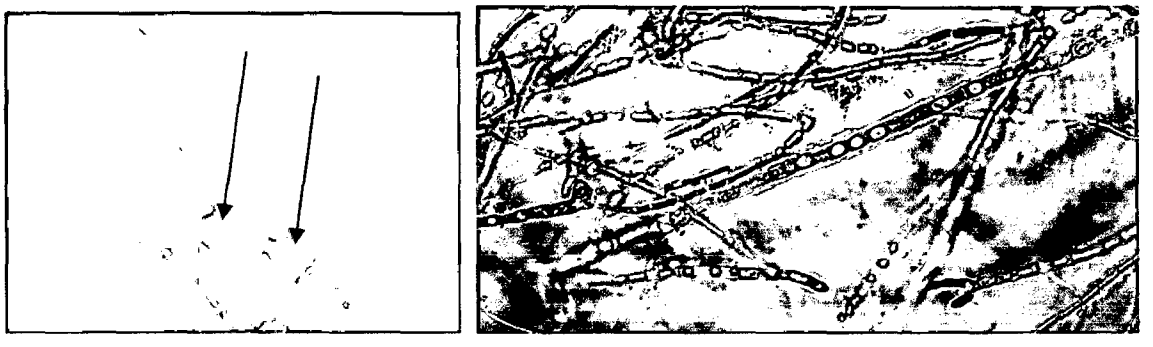
Source	Location	CFU	Biomass (fungal carbon in $\mu g\ g^{-1}$ dry sediment)	Reference
Sea water	Arctic	1000-3000 L^{-1}	---	Gunde-Cimermann *et al.*, 2003
Forest Soil	Brazil	50-75x10^4 g^{-1}	---	Viera & Nahas, 2005
Grassland soil	Denmark	---	36	Klein & Paschke, 2000
Grassland soil	Not mentioned	---	453	Chiu *et al.*, 2006
Forest soil	Not mentioned	---	3375	
Forest soil	Denmark		250-3500	Ekelund *et al.*, 2001
Deep-sea sediments (~5000 m depth)	Central Indian Basin	18-2130 g^{-1}	2.3-6.3 (bacterial carbon= 10-40)	Present work

Table 8.3 Growth of deep sea and terrestrial isolates in sediment extract

Culture	Biomass produced (mg) in			
	Sediment		MEB	
	200 bar / 5°C	1 bar / 30°C	200 bar / 5°C	1 bar / 30°C
A 4634	**24.9**	5.2	29.1	31.4
MTCC 279	8.2	28.4	17.8	28.0
MTCC 479	17.4	20.6	29.2	40.6
MTCC 635	7.4	6.2	7.8	8.3
A 344	3.8	10.2	7.4	14.2

It showed better growth in sediment extract at 200 bar and 5°C as compared to growth in MEB at 1 bar and 30°C. For rest of the cultures (except # A4634), the growth was better at 1 bar and 30°C than at 200 bar and 5°C in MEB.

Fig. 8.2 Growth of the deep-sea isolate *Aspergillus terreus* in malt extract broth(a) and sediment extract(b) grown under hydrostatic pressure of 200 bar



(a) **(b)**

Apart from the differences in the biomass produced, the deep-sea isolate also showed morphological differences when grown in MEB and sediment extract. The isolate when grown in MEB under elevated hydrostatic pressure, showed abnormal **swellings of hyphae** (Fig. 8.2 a, arrows), **which were not seen when the culture was grown under elevated hydrostatic pressure in the sediment extract** (Fig. 8.2 b).

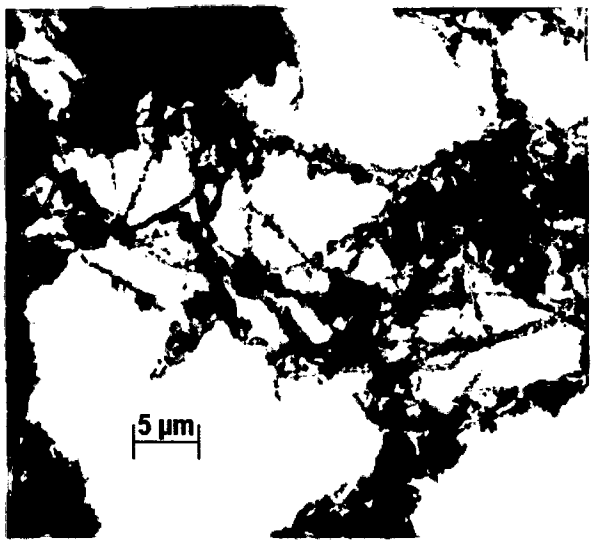
In the previous chapter, it is seen that most of the fungi showed growth under elevated pressure very easily when the inoculum was in the form of growing mycelia, and that the spores failed to germinate at low temperature, at elevated hydrostatic pressure as well as at 1 bar pressure. Therefore, the ability of spores to germinate in

the sediment extract was determined at low temperature. Although, morphology of the fungal hyphae was normal in the sediment extract (Fig. 8.2 b), the

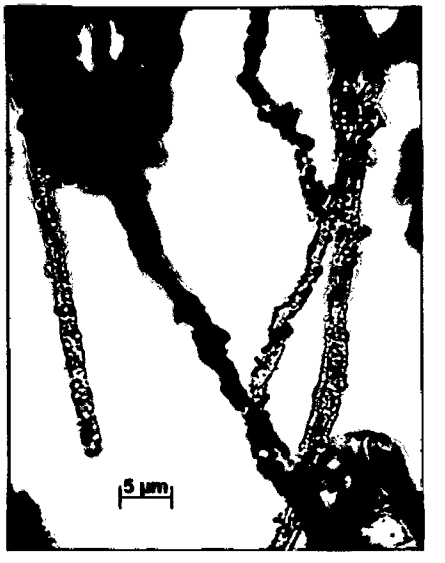
Table 8.4 Germination of spores of deep-sea *Aspergillus terreus* # A4634 in malt extract broth (MEB) and sediment extract

Incubation conditions	Spore germination in soil and MEB growth medium			
	Growth medium	Number of spores showing germination	Number of spores not showing germination	% germination (with SD)
200 bar / 30°C	MEB	145	115	56 ± 12
	Sediment	208	102	67 ± 8
200 bar / 5°C	MEB	8	212	4 ± 3
	Sediment	12	187	6 ± 4
1 bar / 30°C	MEB	190	33	85 ± 21
	Sediment	388	98	80 ± 12
1 bar / 5°C	MEB	4	245	2 ± 2
	Sediment	6	173	3 ± 2

spores showed negligible germination at 5°C at both 1 and 200 bar (Table 8.4). The spores showed 67 % germination in sediment extract at 200 bar/30°C, which was slightly better than the germination in MEB. The germination was not much different when the nutrient supply was limited (1: 100 diluted medium) as discussed in the previous chapter.



(a)



(b)

(c)

(d)

Fig. 8.3 (a – d) Stepwise microaggregate formation by *Aspergillus terreus* (# A 4634) during growth in sediment extract

8.4.2 Microaggregate formation: The culture showed micraggregate formation both in the sediment extract and MEB. There was no significant difference in microaggregation in presence or absence of the quartz. The microaggregation was seen in a step wise manner (Fig. 8.3 a - d). The fungi first showed granular precipitation around the hyphae, which acted as starter for all the further steps leading to aggregation. The end product of this process was formation of quite thick bundle-like aggregations of the fungal hyphae. The aggregates formed in the sediment extract appeared like loose, fluffy material to naked eye (Fig. 8.4). The aggregates stained positively for humic substances with benzidine (Fig. 8.1 a, b), for proteinaceous material with coomassie blue (Fig. 8.1 c) and exopolymeric substances with alcian blue (Fig. 8.1 d).

Fig 8.4 Formation of aggregate (arrow) in presence of quartz by the deep sea fungus *Aspergillus terreus* during growth in sediment extract

* The plastic pouches in which the cultures were incubated at elevated hydrostatic pressure.

8.4.3 Alkaline Phosphatase Activity (APA)

Plain deep-sea sediment showed APA responsible for hydrolysis of 5.57 and 4.64 μg L^{-1} h^{-1} substrate at 200 bar and 500 bar and 5°C respectively. Addition of antibiotic affected the activity marginally, whereas addition of fungicide affected to a greater extent (Table 8.5).

Table 8.5 Alkaline phosphatase activity (APA) in the sediment in the presence and absence of additional nutrient sources

Experimental condition		Release of MUF (μg L^{-1} h^{-1})	
		200 bar / 5°C	500 bar / 5°C
1	Plain sediment	5.57 ± 0.3	4.64 ± 0.9
2	Sediment + antibacterial antibiotic	4.52 ± 1.2	4.94 ± 0.1
3	Sediment + Fungicide	1.47 ± 0.9	0.19 ± 0.1
4	Sediment + detritus	101.21 ± 25.4	98.57 ± 21.1
5	Sediment + detritus + antibacterial antibiotic	90.01 ± 8.4	96.96 ± 15.9
6	Sediment + detritus + Fungicide	73.53 ± 34.9	70.13 ± 34.9
7	Sediment + artemia	4.99 ± 1.6	2.63 ± 0.4
8	Sediment + artemia + antibacterial antibiotic	3.88 ± 0.6	1.99 ± 0.7
9	Sediment + artemia + Fungicide	3.38 ± 1.5	2.64 ± 1.2
10	Sediment + detritus + Deep-sea fungus + antibacterial antibiotic	108.02 ± 0	93.85 ± 9.3
11	Sediment + detritus + Deep-sea bacteria + Fungicide	84.85 ± 16.6	67.41 ± 0.6

When the nutrient source was added in the form of detritus to this sediment, the APA went up drastically at both the pressures of incubation. When fungicide was added to the mixture of sediment and detritus, the APA decreased by 25 %, whereas

addition of antibacterial antibiotic decreased the APA by 10 % as compared to plain sediment with detritus and without any inhibitor. But when the nutrient source was added in the form of artemia larvae and cysts, there was no detectable increase in the APA, rather a drop in the activity was seen. When the deep sea fungal isolate # A4634 was added along with antibiotic to sediment and detritus mixture, the APA increased almost 20 times as compared to plain sediments at both 200 and 500 bar pressure, whereas addition of deep sea bacteria along fungicide, increased the APA only 15 times at both 200 and 500 bar pressure.

8.5 Discussion

The fungal biomass constitutes only a portion of the potentially mineralizable organic matter associated with aggregates in terrestrial environment, which is **mineralized only upon aggregate disruption [Gupta & Germida, 1988]. In this study** also, the fungal biomass contribution is 2.3 - 6.3 μg C g^{-1} dry sediment, which is much less than the bacterial carbon reported in the sediments of CIB, ranging from 3 to 40 **μg C g^{-1} dry sediment [Raghukumar *et al.*, 2001].**

The deep-sea isolate # A4634 was able to grow better in the sediment extract as compared to MEB at 200 bar and 5°C. It appears that these conditions are closer to the existing conditions in the sea than the rich culture media. In contrast, the terrestrial cultures failed to emulate this growth in the sediments and were more suited to grow in MEB.

The sediment extract, when extracted without heating or boiling supported better fungal growth than the extract prepared by boiling. For culturing actinomycetes

routinely in laboratory, sediment extract is prepared by boiling sediment for one hour and filtering just before use. It is possible certain essential heat labile substances are present in the sediment, which help fungi to grow better. Addition of EDTA during sediment extract preparation was found to be helpful. EDTA has been shown to be an effective mild extractant of soil organic matter, extracting as much as 30 % organic matter from soils [http://www.ar.wroc.pl/~weber/ekstrak2.htm]. Although alkali extraction (NaOH) has been shown to be best extractant, able to extract as much as 80 % of soil organic matter, it has many undesirable features, due to which EDTA extraction was favoured. Other extractants like acetyloacetone, cupferron, hydroxyquinolone, and formic acid [http://www.ar.wroc.pl/~weber/ekstrak2.htm] are also used but these were not used here, as these could be deleterious to fungal growth. The sediments of CIB contain LOM (labile organic matter) up to 3.845 mg g^{-1} dry sediment [Raghukumar *et al.*, 2001], out of which over 60 % (2.4 mg g^{-1} dry sediment) is contributed by carbohydrates. EDTA is shown to be effective in solubilising the polysaccharides [Liu *et al.*, 2002] so that it is available for further activity, for uptake by other organisms or analytical purpose.

The microaggregates stained positively for humic substances indicating that the fungal activities are responsible for the formation of humus possibly in deep-sea sediments also as described previously for terrestrial environments [Stevenson, 1982]. Reducing sugars and amino acids, formed, as by-products of microbial metabolism in terrestrial sediments are known to undergo nonenzymatic polymerization to form a series of compounds like 3-carbon aldehydes, ketones and reductones and hydroxymethyl furfurals. These highly reactive compounds readily polymerize in the

presence of amino compounds to form brown-coloured products, constituting humus [Tisdall & Oades, 1982]. The humic material combines with soil particles to form microaggregates. Fungal hyphae further act as binding agents themselves to form macroaggregates by trapping fine particles into the microaggregates [Kandeler *et al.*, 1999]. Fungi or bacteria can thus remain protected in certain particle size classes [Suberkropp & Weyers, 1996]. Microaggregates in the present study also stained positive for polysaccharides and proteins. Cations such as Si^{4+}, Fe^{3+}, Al^{3+} and Ca^{2+} form bridges between particles in terrestrial microaggregates [Bronick & Lal, 2005]. The fungi, thus present, concealed inside these microaggregates, contribute to the nutrient cycling in the deep-sea habitat, by acting upon them from inside the aggregates. Fungi can be important for both the formation as well as stabilization of soil aggregates [Lynch & Bragg, 1985]. Although fungi are generally believed to be more effective at aggregate stabilization than other soil microflora, several studies have concluded that their primary contribution to aggregation is through hyphal entanglement of soil particles [Molope & Page, 1986]. But the polysaccharide-mediated binding by fungi has also been shown equally effective [Aspiras *et al.*, 1971; Burns & Davies, 1986]. This aggregation protects the soil organic matter within water-stable aggregates, which is an important, biotically regulated mechanism for accumulation and maintenance of organic matter in soil [Elliot, 1986; Gupta & Germida, 1988; Beare *et al.*, 1994]. The results support the previous reports [Beare *et al.*, 1994] that fungi contribute significantly to the stabilization of soil aggregates, but the fungal biomass constitutes only a portion of the potentially mineralizable organic matter associated with aggregates, which is mineralized only upon aggregate

disruption [Gupta & Germida, 1988]. This could also be the reason for increased sighting of fungi after treating the sediments with EDTA as reported here (Table 8.1).

The enzyme phosphatase in sediments has received considerable attention over the last years. Previous research indicates that it plays a crucial role in the cycling of P [Ayyakkannu & Chandramohan, 1971; Degobbis *et al.*, 1984; Kobori & Taga, 1979; Silva & Bhosale, 1990]. Sabil *et al.* [1994] found, in the sediment phase of the Venice Lagoon within an area of shallow water, the Palude della Rosa, that the immobilized enzymes, including phosphatase, showed marked heat stability and an increased resistance to environmental changes. They could be useful as diagnostic factors of the ecosystem, since their presence is related to the waste products and their life span is prolonged by their insolubilization. Thus, bioassessment can reveal long term effects on sediments after the cause of the impact has passed and is itself undetectable, making the temporal scale of the enzymatic response appropriate for pollution. Most of the time, high phosphatase activity corresponds to low total P and low inorganic P, but high organic P in the sediments [Silva & Bhosale, 1990]. Alkaline phosphatase activity (APA) is one of the parameters for describing the microbial activity in the sediments. Here it was tried to differentiate the contribution of fungi and bacteria towards the APA. The plain sediment without any additional nutrients showed very low APA. When the detritus was added, the APA increased 20-fold, indicating acute increase in microbial activity, typical of a 'feast and famine' deep-sea habitat, where the nutrient supply is mostly in the form of pulse, meaning sometimes available in plenty and sometimes not available for days together. APA was higher in the presence of antibacterial antibiotic, indicating a major share of fungi towards the APA. This

indicates that fungi might play an important role in degradation of detritic material as reported in the terrestrial and mangrove environments [Newell, 1996; Ananda & Sridhar, 2004]. In contrast, addition of autoclaved artemia larvae and cysts had no effect on the APA. Protease enzyme might play a direct role in degradation of such animal detritus. The deep-sea fungal isolate #A4634, in the presence of antibacterial antibiotics showed a very high APA at both the pressures of incubation as compared to APA shown by deep-sea bacteria in the presence of fungicide, indicating that deep-sea fungus used could be contributing more towards APA than the deep-sea bacteria used.

In conclusion, these studies suggest that fungi are present in abundance in deep-sea sediments and may play following major roles.

1) By virtue of a biomass of about 25 % of that of the bacterial standing crop [Raghukumar *et al.*, 2001] as shown here, they form an important component of the microbial consortium and contribute substantially to carbon mineralization and sedimentary carbon on a global scale. The polymers of fungal cell wall melanin and chitin are not easily degradable whereas, phospholipids forming the main component of bacterial cell wall are readily degraded. Thus, in some instances, fungal C in terrestrial leaf litter is known to be about 26 times greater than that of bacterial sequestration [Okada *et al.*, 2005]. Fungal mediated C storage is expected to be more persistent in contrast to the bacterially sequestered one [Bailey *et al.*, 2002]. Understanding the relative contribution of C in the deep-sea sediments by fungi and bacteria may allow prediction of longevity of stored C in deep-sea sediments. Such a sequestration might be enhanced by the formation of aggregates composed of humic material, as demonstrated in the experiments that were carried out.

2) Fungi in deep-sea sediments might also be important in the food web involving meiofauna. The labile portion of the sediment organic matter consists mainly of simple sugars, fatty acids and proteins, which are rapidly mineralized [Fry, 1987]. Alongi [1990], analyzing the deep-sea sediment of the Solomon and Coral Seas, did not find any relationship between bacterial parameters and organic carbon content; he hypothesized that bacterial activity, even in deep sediments, may be correlated with standing amounts of specific labile compounds, such as lipids, amino acids and proteins.

3) Nematodes found in abundance in sediments from CIB are an important component of deep-sea sediment fauna. Nematodes constituted more than 25 % of the benthic macrofauna recorded in the sediments of CIB [Ingole *et al.*, 2001]. They are avid feeders of fungi in terrestrial soil [Okada & Kadota, 2003; Okada *et al.*, 2005]. The nematodes fed on fungi have been shown to exhibit a higher population growth rate. Deep-sea fungi may provide a direct food source to nematodes, contributing in a significant manner in the deep-sea food chain also. Fungal feeding habit of nematode is also a classification tool. Based on the presence of a particular type of fungus-feeding nematode, it may be also possible to predict the presence or absence of the host fungus [Okada & Kadota, 2003].

Chapter 9

Summary

The deep sea, the largest single ecosystem on earth is an extreme environment, and the least explored habitat. This is mainly due to the remoteness of the deep sea and the difficulties encountered in its exploration that have resulted in it being the least understood environments on the earth. The understanding of deep sea will unravel a lot of exciting information about the history of life existing on earth.

The deep sea was thought to be a very difficult environment for life forms to exist due to combination of different extremities in the form of low temperature, high hydrostatic pressure, low nutrient supply and absence of light. Due to these factors, deep sea was thought to be devoid of life till the mid 19th century. This assumption was first challenged in 1860 with the discovery of corals and sponges attached to a transatlantic cable that was hauled up from the seafloor for repair. Since then study of marine life forms has progressed a lot. It can be concluded safely that deep sea is one of most diverse habitat in terms of species richness on earth.

Marine sediments overlay two-thirds of the earth's surface and harbour diverse and abundant fauna. The organisms in these sediments can be divided into two categories as infauna (living within sediments) and epifaunal (living on surface of the sediment). Amongst these, bacteria are one of the most extensively studied microorganism with respect to their abundance, diversity, phylogeny, growth and enzyme profiles and different adaptations. 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) with respect of description of new species. In spite of this, fungi have remained largely neglected in the vast environment of deep sea, with some sporadic reports appearing

once in a while. With this study, it is tried to reduce this void by describing the occurrence and diversity of fungi from the Central Indian Basin.

For this, fungi were isolated from the sediments from the Central Indian Basin from an average depth of ~ 5000 m by different techniques viz. dilution plating, particle plating and pressure enrichment technique on board the vessel AA Sidorenko. The particle plating method yielded the lowest number of fungi while dilution plating and pressure enrichment resulted in almost similar recovery of fungi from the sediments. 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. However, a one-way analysis of variance (ANOVA) comparing the number of species isolated from different subsections of each core down to 30 cm depth showed that differences between the subsections were not significant. None of the media used for isolation were selective for isolating specific type of fungi.

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. Among the various Aspergilli isolated, many showed abnormal morphology on solid media during the first couple of generations, which was not seen later.

When these deep sea isolates were compared with terrestrial species, they showed a different growth, physiological and genetic patterns indicating that these are completely different strains as can be deduced from their C- source utilization and ITS RFLP profile.

Along with filamentous fungi, some yeasts were also isolated from sediments and pore water (water trapped between the sediment particles). Two distinctly different groups were isolated from sediment and water as seen from the biochemical and molecular identifications. The orange yeasts isolated from the sediments belonged to *Rhodotorula mucilaginosa* while those from the pore water to *Rhodosporidium paludigenum*.

One of the main reasons for fungi remaining neglected is that they cannot be easily visualized in the sediments. In this study, an optical brightener, Calcofluor, was used in order to facilitate easy visualization of fungi in the sediments. But even with this, fungi could be visualized in only ~5 % of the sediment subsections. But when the same samples were treated with EDTA prior to staining with calcofluor, the number of sediment samples showing fungal presence doubled to ~ 13 %. This could have been because of disruption of the microaggregates by EDTA, the former entrap fungal hyphae within themselves.

Another method that of immunofluoresence staining, was used to visualize fungi in the sediment and to prove that the fungal cultures isolated were native of the deep sea sediments. Polyclonal antibodies were produced against one of the most frequently isolated fungus, *Aspergillus terreus*, in rabbit. Sediment samples of the core subsection from which this particular culture was isolated revealed fluorescing hyphae.

Being obtained from an extreme environment of high hydrostatic pressure and low temperature, these fungi should have been able to grow in these conditions simulated in laboratory. But, a large number of fungi (109 in all) did not show growth when directly incubated under elevated pressure of 500, 400 & 300 bar. Majority of these 109 isolates that did not show direct growth initially when incubated at elevated hydrostatic pressure showed growth at 50 bar pressure. Decreasing numbers of fungi showed viability and growth when they were gradually subjected to higher pressure. Only two strains of *Aspergillus terreus* and the yeast # A 344 grew at 300 bar and only the latter at 400 bar pressure. It may be hypothesized that although several fungi reach the seafloor, most of them remain dormant and only a few show active growth.

In a comparative study, the terrestrial cultures obtained from the culture collection of IMTECH, Chandigarh also showed growth at 200 bar pressure, but the biomass produced by these was less than the similar deep sea isolate of *Aspergillus terreus* (# A 4634).

All the sporulating cultures tested for germination of spores, failed to show germination at low temperature of 5°C at both 1 bar and elevated hydrostatic pressures. 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. Contrary to hyphae, spores may be poor candidates for propagation of fungi in the deep-sea sediments. None of the four fungi that were tested, including 3 terrestrial and 1 deep-sea isolate, germinated at 5°C at 1 or 200 bar. The terrestrial isolates germinated very poorly at 200 bar 30°C, while the marine isolate showed substantial germination. Thus, pressure appears to have different effects on mycelium and spores of fungi.

At 30°C and elevated hydrostatic pressures, where germination was recorded, it was seen that the germination rate is inversely proportional to the hydrostatic pressure. The viability of the spores is also inversely proportional to the hydrostatic pressure. But here also, the deep - sea isolates showed a lot of variability amongst themselves. The viability decreased gradually from 92.3 to 12.8 % at 100 and 500 bar respectively in case of # A 4634, while in case of other deep-sea isolates, the viability was not much affected by the incubation pressure, but was on the lower side.

Not only pressure, low temperature also adversely affected the viability of the spores. The spores of the two deep sea isolates tested showed better viability than the similar terrestrial isolate and another deep sea isolate.

Due to the "Feast & Famine" conditions of the nutrients prevailing in the deep sea, effect of nutrient concentrations was also tested, but it did not show any substantial effect on the germination rates. There was a random change in the germination rates irrespective of the nutrient concentrations. This could be because of requirement of less stringent nutrient conditions for the viability and germination of spores.

Use of a known stress protectant, DMSO as reported in yeasts, did not seem to affect the growth of the deep sea fungi in this case. The biomass production or spore germination of these fungi in presence of DMSO did not increase indicating that DMSO is not playing any role here.

Apart from this external stress protectant, the deep sea yeasts were tested for trehalose (another stress protectant) production under different temperatures. Although, reported previously to be produced under the stress of low temperature, in the yeast cultures tested, maximum trehalose production was seen at higher

temperatures of 15 & 30°C, and not at 5°C. This can be further proof of these yeasts being deep sea inhabitants, hence low temperature not really proving to be a stress.

When the deep sea cultures, both the filamentous fungi and the unicellular yeast were analyzed for intracellular proteins by the means of electrophoresis, no conclusive results were obtained which could point to a specific stress protein being induced or repressed due to the elevated pressure. Although a large number of proteins were induced or repressed, these could not be assigned to any of the described stress protein families.

Along with the intracellular proteins, these deep sea fungi were also analyzed for one of the extracellular enzymes produced by them, the protease. Proteases were selected for study as these occupy a very important position in the nutrient cycling in the deep sea environment mainly because proteins and peptides constitute a substantial portion of the organic nutrients present there. This enzyme was produced by majority of the fungi isolated, in varying amounts. But, not all the cultures were producing protease active at alkaline pH and low temperature. Similar to the growth and spore germination, protease production and activity was also affected by low temperature. The number of fungi showing protease activity at 30°C was much higher than those showing at 5°C. Although, growth was fairly unaffected by elevated pressure and low temperature (inoculum in the form of mycelia), protease production was reduced 10 to 100-fold under similar conditions.

One of the deep sea cultures, *Aspergillus ustus* (# NIOCC 20) was found to produce maximum amount of protease in the primary screening. It showed a substantial biomass at elevated hydrostatic pressure (100 bar). But, when the fungus was grown under elevated hydrostatic pressure, whether at 30 or 5°C, little or

negligible amount of protease production was seen. In contrast, the enzyme produced by fungus at 30°C / 1 bar was more tolerant to elevated pressure, with almost same activity recorded up to 300 bar of pressure. However, with increasing pressure, the K_m value for the enzyme also increased indicating a low substrate affinity.

The enzyme produced at 30°C and 1 bar pressure was further characterized. The fungus produced a maximum of 1639 ACU mL^{-1} of protease by day 7. The enzyme, with molecular mass of 32 kDa showed several interesting properties. It had a broad pH range of 6–10, with an optimum at pH 9. The optimum temperature for protease activity was 45°C and approximately 10% of the activity was retained at 2°C. The enzyme was totally inhibited in the presence of 2 mM PMSF suggesting it to be a serine protease. It was active in the presence of several commercial detergents and in the presence of 0.5M NaCl, equivalent to 29 parts per thousand salinity. In the presence of stabilizing agents such as glycerol, $CaCl_2$, its thermostability at 60°C was enhanced. Heavy metal ions Cu, Hg, Fe, Ni and Zn did not inhibit the enzyme activity considerably.

While the deep sea *Aspergillus ustus* produced substantial amounts of protease enzyme as described, the terrestrial isolate of *Aspergillus ustus* obtained from IMTECH, Chandigarh (MTCC 2200) secreted negligible amounts of protease.

A conclusive proof for the existence of fungi in the deep sea sediments has been provided by us, but the question does not end here. The major question that still remains to be answered is that "are they merely present there or growing actively to play a role in the deep sea environment?" This can be answered by determining their ability to grow in the native conditions being provided to them in the laboratory. The deep sea isolate # A 4634 showed growth in the sediment extract, without any

additional nutrients suggesting that, they can not only survive but also grow normally in these conditions. The biomass produced by the culture in the sediment extract was similar to the biomass produced in laboratory culture medium. Not only the biomass production, the growth morphology was better in the sediment extract under elevated hydrostatic pressure than in malt extract broth, indicating sediment to be better environment for these deep sea cultures.

Apart from the protease production as discussed above, these fungi could also be playing a role in nutrient cycling by production of other enzymes like alkaline phosphatase. Alkaline phosphatase activity is widely used as indicator of microbial activity in the sediments. When individual contribution of fungi and bacteria towards APA was determined at elevated hydrostatic pressure, the share of fungal APA was more as compared to the bacterial APA, indicating that deep sea fungi could be contributing more towards APA than the deep sea bacteria used.

In conclusion, this study proves an active role of fungi in the deep sea sediments of the Central Indian Basin. It would be worth while to conduct similar studies in other oceans (deep-sea sediments) for comparison.

APPENDIX

A - I. DNA Extraction Method [van Burik *et al.*, 1998]

Fungal mycelium (approx. 1 g wet wt.)

↓

Ground to powder in liquid nitrogen

↓

Add 0.5mL extraction buffer (preheated to 65°C)
+ 100 µL 1% β-mercaptoethanol

↓ Mix vigorously

Incubate at 65°C for 30 min

↓

Add 100 µL of 3M sodium acetate

↓

Centrifuge at 10,000 rpm for 10 min

↓

Supernatant + equal volume of phenol (pH 8): $CHCl_3$: isoamyl alcohol (25:24:1)

↓ Mix vigorously

Centrifuge at 10,000 rpm for 15 min

↓

Supernatant + equal volume of $CHCl_3$: isoamyl alcohol (24:1)

↓ Mix

Centrifuge for 10min

↓

Supernatant + 200 µL of RNase (1 mg mL^{-1} stock)

↓

Incubate at 37°C for 30 min

↓

Equal volume of phenol (pH 8) : $CHCl_3$: isoamyl alcohol (25:24:1)

↓ Mix vigorously

Centrifuge at 10,000 rpm for 15 min

↓

Supernatant + equal volume of $CHCl_3$: isoamyl alcohol (24:1)

↓ Mix

Centrifuge for 10 min

↓

Supernatant + 2.5 volumes of chilled isopropanol

↓

Centrifuge at 10,000 rpm for 10 min at 4°C

↓

Wash with 70% cold ethanol

↓

Dissolve in minimum volume of sterile d/w

↓

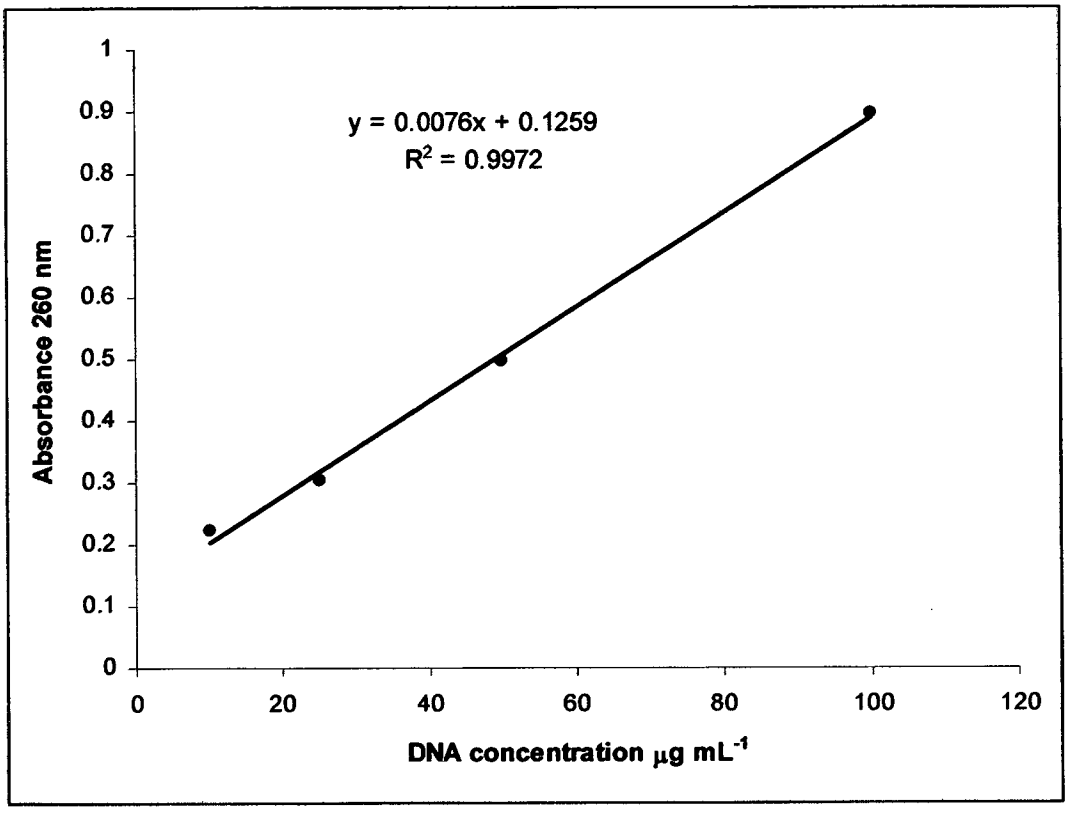
Store in –20°C

Extraction buffer:	Tris HCl	100 mM
	Na_2EDTA/Na_4EDTA	20 mM
	CTAB	2 % (w/v)
	NaCl	1.4 M
	Poly vinyl pyrolidine	1 % (w/v)

T.E buffer	Tris-HCl (pH 8)	10 mM
	EDTA	1 mM

A - II. DNA Standard

DNA (HiMedia, Cat. RM 511) was dissolved in sterile distilled water to get the desired concentration and the absorbance was read at 260 nm.



A – III. Protocol for the intracellular protein extraction using Sigma total protein extraction kit [Cat. PROT-TOT]

Fungal biomass

↓

Grinding in liquid nitrogen in a mortar and pestle to a fine powder

↓

100 mg crushed biomass

+

2 mL Chaotrophic Extraction Reagent 1 (Cat. C 0481)

+

0.2 mL Protease inhibitor cocktail (Cat. P 2714)

Incubate for 30 min. at 30°C

↓

Centrifuge at 10,000 rpm for 30 min at 15°C

↓

Decant supernatant into a clean tube and discard the insoluble pellet

↓

Supernatent + 50 µL of Tributylphosphine solution (200 mM) (Cat. T 7567)

Incubate at room temperature for 1 hour

↓

Add 60 µL of Iodaacetamide (500 mM) (Cat. A 3221)

Incubate at room temperature for 1.5 hours

↓

Centrifuge at 10,000 rpm for 5 min.

↓

Supernatant

↙ ↘

Protein estimation Electrophoresis

A - IV. Urea buffer protein extraction method

[Nir Osherov & Gregory May, Webpage – Department of Cell Biology, Baylor college of Medicine, Houston, Texas, 77030 USA; *Optimization of protein extraction from Aspergillus nidulans for gel electrophoresis*]

Lyophilized fungal biomass powder in 0.2 mL urea sample buffer

↓

Mix vigorously

↓

Boil for 2 min.

↓

Vortex for 1min.

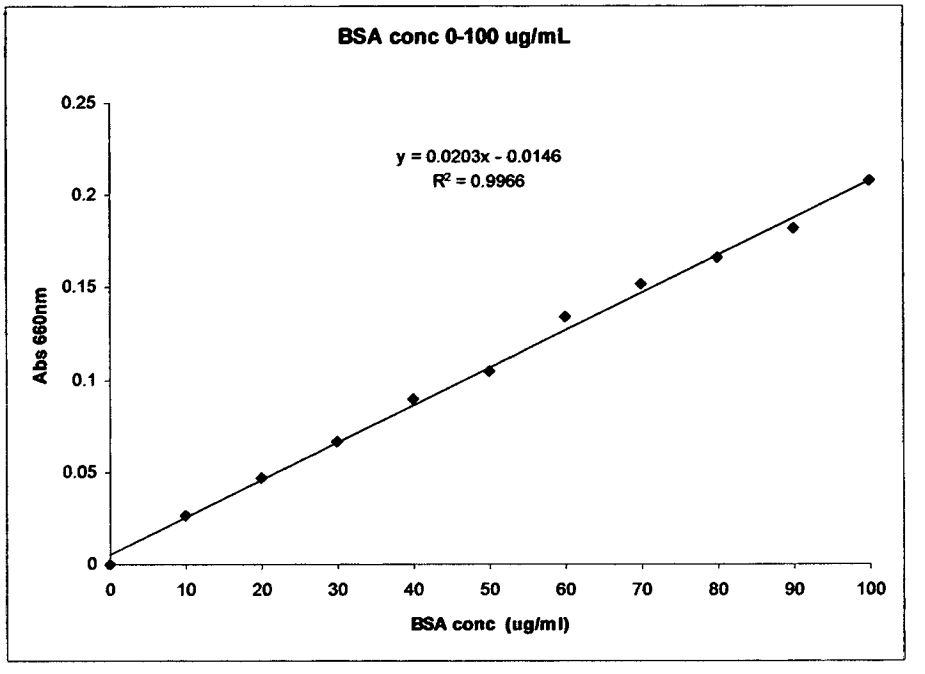
↓

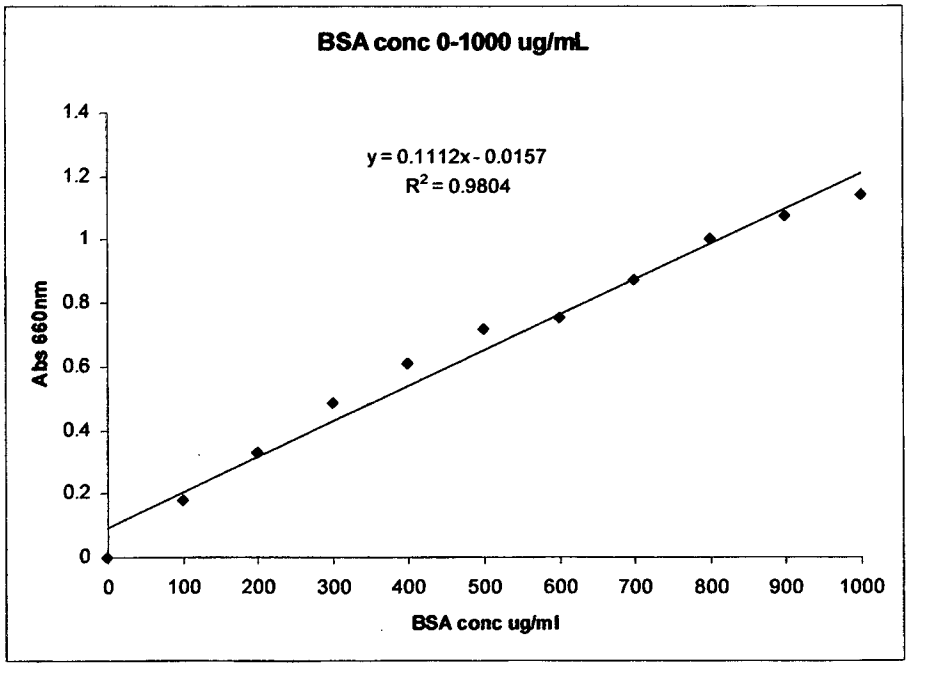
Boil for 1 min.

Urea buffer – 1 % SDS
9 M Urea
25 mM Tris-HCl pH 6.8
1 mM EDTA
0.7 M mercaptoethanol

A – V. Protein standard [Lowry *et al.*, 1951]

Bovine Albumin (BSA) (SRL chemicals, Cat. 0140299) dissolved in d/w was used as standard for protein. The dilutions required were done using d/w and the absorbance was read at 660 nm on a Shimadzu spectrophotometer (Model UV 1201V)





A – VI. SDS PAGE ----- Reagents and Gel Compostions [Laemmli, 1970]

Acryalmide / Bis : Acrylamide - 29.2 %
N'N'-bis-methylene-acrylamide - 0.8 % in d/w

10% (w/v) SDS in d/w

Resolving buffer : 1.5 M Tris-HCl pH 8.8 (adjust pH with 6 N HCl)

Stacking buffer : 0.5 M Tris-HCl pH 6.8 (adjust pH with 6 N HCl)

% gel	D/w mL	Acrylamide/bis mL	Gel buffer mL	10% SDS mL
4	6.1	1.3	2.5	0.1
5	5.7	1.7	2.5	0.1
6	5.4	2.0	2.5	0.1
7	5.1	2.3	2.5	0.1
8	4.7	2.7	2.5	0.1
9	4.4	3.0	2.5	0.1
10	4.1	3.3	2.5	0.1
11	3.7	3.7	2.5	0.1
12	3.4	4.0	2.5	0.1
13	3.1	4.3	2.5	0.1
14	2.7	4.7	2.5	0.1
15	2.1	5.0	2.5	0.1
16	2.1	5.3	2.5	0.1
17	1.7	5.7	2.5	0.1

10% Ammonium persulphate : freshly prepared

For 10 mL gel volume: for resolving --- 50 µL 10% APS & 5 µL TEMED
for stacking --- 50 µL 10% APS & 10µL TEMED

Sample buffer (SDS reducing buffer)

D/w	3.55 mL
0.5M Tris-HCl, pH 6.8	1.25 mL
Glycerol	2.5 mL
10% SDS	2.0 mL
0.5% (w/v) bromophenol blue	0.2 mL
Total volume	9.5 mL

10X electrode running buffer, pH 8.3

Tris base	30.3 g/L
Glycine	144 g/L
SDS	10 g/L

Use 1X buffer for the electrophoresis run

A – VII. **Silver staining protocol** [Heukeshovan & Dernick, 1985]

Electrophoresed gel

↓

Fix* the gel in Fixative solution for at least 1 h

↓

Wash* gel with methanol (20 min x 2-3 times)

↓

Treat* with 0.02 % $Na_2S_2O_3$ for 1 min.

↓

Rinse the gel with d/w

↓

0.2 % Silver Stain solution* for 30 min. (place in dark)

↓

Rinse the gel with d/w to wash off excess silver stain solution

↓

Add developer solution till proteins bands appear

↓

Fixative for 10 min

↓

Wash the gel with d/w

* The gel was placed on gel rocker during these steps.

A – VIII. Reagents for Western blotting

Transfer buffer:

Tris	-	3.03 g
Glycine	-	14.4 g
Dist. Water	-	800 mL
Methanol	-	200 mL

TBST buffer :

Tris	-	2.42 g
NaCl	-	8 g
Tween 20	-	500 µL
Dist. Water	-	200 mL

Adjust pH to 7.2 to 7.5 and make up volume to 1000 mL using dist. Water

A – IX. Czapek Dox (CD) medium composition (with modification)

Glucose*	-	10 g
Sodium nitrate	-	3 g
Dipotassium hydrogen phosphate	-	1 g
Magnesium sulphate	-	0.5 g
Potassium chloride	-	0.5 g
Ferric sulphate	-	0.02 g
Sea water	-	1000 mL

Wherever required, milk solution (prepared using skimmed milk preapered in d/w) was added to this basal medium, after autoclaving separately

* in original composition, sucrose is used instead of glucose.

A – X. Staining reagents used for visualization of humic substances, proteins and exoploysaccharides (TEP) in sediment extracts

1. Benzidine reagent for humic substances [Sieburth & Jensen, 1968]

   Equal volumes of 10 % aq. sodium nitrite and 5 g of benzidine in 25 mL conc, HCl diluted to 1 : 1

2. Coomasie brilaiiant blue stain for protein particles [Long & Azam, 1996]

   Stock solution - 1 % (w/v) in sterile d/w
   Working solution - Dilute the stock 25-fold in 0.2 μm filtered seawater to 0.04% concentration and pH 7.4

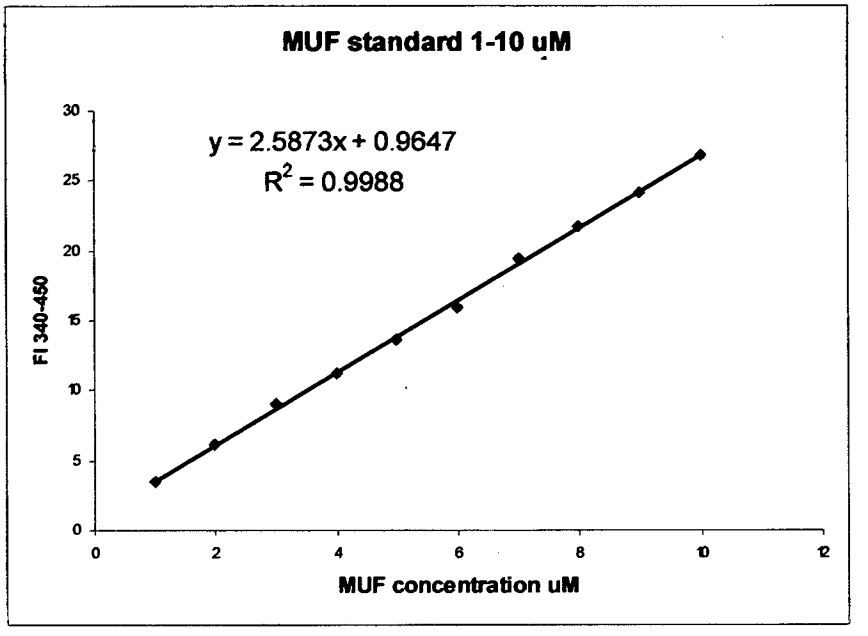
3. Alcian blue stain for TEP [Long & Azam, 1996]

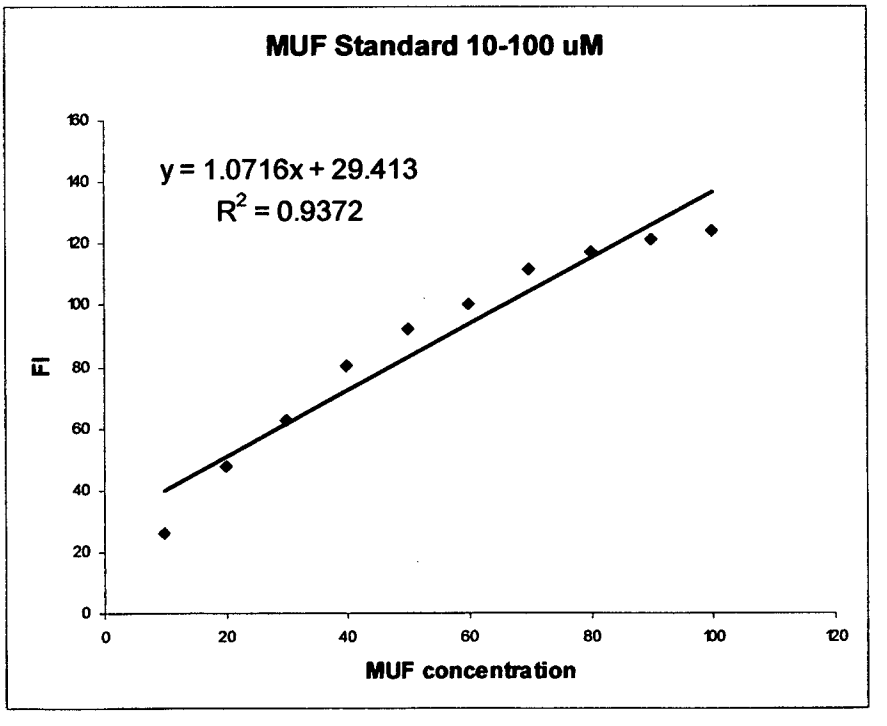
   0.02 % alcian blue in 0.06 % acetic acid (pH 3.3)

A – XI. **Methylumbelliferone (MUF) standard** [Hoppe Hans - Georg, 1983]

4 - Methylyumeblliferone (Sigma Chemicals, USA, Cat. M 1381) was dissolved in methycellosolve to get the desired concentrations. The fluorescence was measured at 340 nm exciation and 450 nm emission on a Shimadzu spectrofluorimeter (Model RF1501).

The standards were done for 1-10 mM and 10 – 100 mM MUF concentration range.





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- **Samir Damare**, Chandralata Raghukumar, S. Raghukumar (2006). *Fungi in deep-sea sediments of the Central Indian Basin*. Deep-sea Research I, Vol. 53, 14-27.
- **Samir Damare**, Chandralata Raghukumar, Ushadevi Muraleedharan, S. Raghukumar (2006). *Deep-sea fungi as a source of alkaline and cold-tolerant proteases*. Enzyme and Microbial Technology, Vol.39, 172-181.
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- **Samir Damare**, Manju Nagarajan, Chandralata Raghukumar. "*Induction of Spore Germination Under Elevated Hydrostatic Pressure in Deep-sea and Terrestrial Fungi*"
- **Samir Damare** and Chandralata Raghukumar. "*Stress Response of Deep-sea Fungi as Elucidated by Electrophoresis*"
- **Samir Damare**, Varada Damare, Pankaj Verma, Yogesh Shouche, Chandralata Raghukumar. "*Diversity of Yeast from the Water Column of Equatorial Indian Ocean*"

Patent

Chandralata Raghukumar, **Samir Damare**, Ushadevi Muraleedharan. "*A Process for Production of Low Temperature Active Alkaline Protease from Deep-sea Fungus*' has been filed in India (No. 0271NF), US and WO. European patent – **EP 1692296**.

Presentations at Symposia and Conferences

- Presented a paper (oral presentation) titled '*Deep-sea Fungi as a Source of Extremophilic Enzymes for Industrial Applications*' at the National Seminar on New Trends in Biotechnology at Goa, India, 11th & 12th Jan. 2007.
- Presneted a paper (oral presentation) titled '*Production of Alkaline Proteases from Deep-sea Fungus by Solid Substrate Fermentation*' at the National Seminar on Recent Trends

in Mycological Research at J.J. College of Arts and Science, Tamilnadu, India, Dec. 28th & 29th Dec. 2006.

- Presented a paper (oral presentation) titled '*Effect of Deep-sea Conditions on Fungal Spores*' at the 7th Asia Pacific Marine Biotechnology Conference at Cochin, India, Nov. 2 - 5, 2006.
- Presented a paper (oral presentation) titled '*Deep-sea Fungi as a Source of Cold-tolerant Proteases*' in the Conference "From Functional Genomics to Natural Products of Marine Microorganisms" at Greifswald, Germany, June 21-24, 2006.
- Presented a paper (oral presentation) titled ' *Deep-sea fungi as a source of alkaline, cold-tolerant proteases*' in the Conference on Microbiology of the Tropical Seas held at National University of Oceanography, Goa during Dec.13-15, 2004.
- Presented a paper (oral presentation) titled '*Occurrence of fungi in deep sea sediments of the Central Indian Basin*' in the National Seminar on Recent Advances in Mycology held at Mangalore University, Dec. 2-3, 2004.
- Poster titled '*Barotolerant Deep-sea Fungi and their Proteases*' was presented in National Symposium on Prospecting of Fungal Diversity and Emerging Technologies at 29th Annual Meeting of Mycological Society of India at Agharkar Research Institute, Pune in Feb. 2003, and the abstract published in the proceedings of the same.

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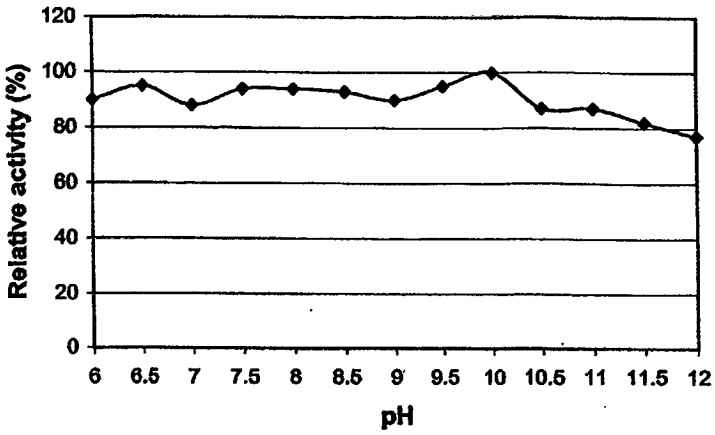
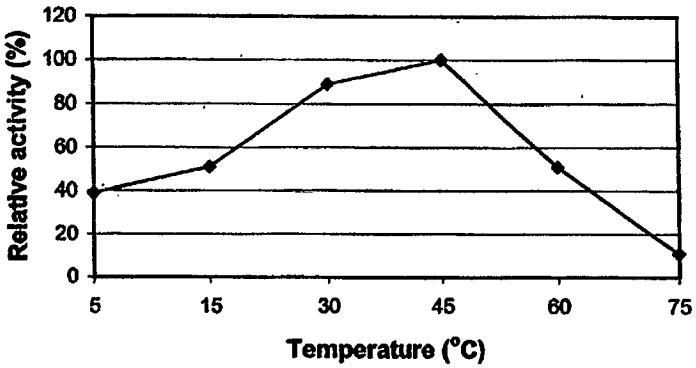
(54) Title: A PROCESS FOR PRODUCING AN ALKALINE PROTEASE FROM A DEEP-SEA FUNGUS





(57) Abstract: Present invention particularly relates to production of alkaline protease for various industries and especially for "enzyme detergents" by a fungus *Aspergillus sp.* deposited in the microbial type culture collection of Institute of Microbial Technology, Chandigarh, India, under the accession number MTCC 5102 and the said fungus can be grown in conventional media with commercial brands of milk powder using distilled water at pH 7.0 at room temperature (28oC±2°C), however, the said fungus grows well in seawater media too and also at 5°C; further, the protease enzyme produced by this fungus acts equally well in the pH range of 6 to 11 and shows 100% activity at temperature of 42-47°C but almost 90% of the activity is present at 30°C, 50% of the activity at 15°C and at 60°C The enzyme is thermostable up to 45 min at 450C.

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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°S and 73–76°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° as well as at 5 °C. Many fungi showed abnormal morphology at 200 bar/5 °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 *A. terreus* (# A 4634), but not the terrestrial ones, showed germination at 200 bar pressure and 30 °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.

Keywords: Deep-sea fungi; Hydrostatic pressure; Diversity; Central Indian Basin

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

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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 calcarcous 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°S and 73–76°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

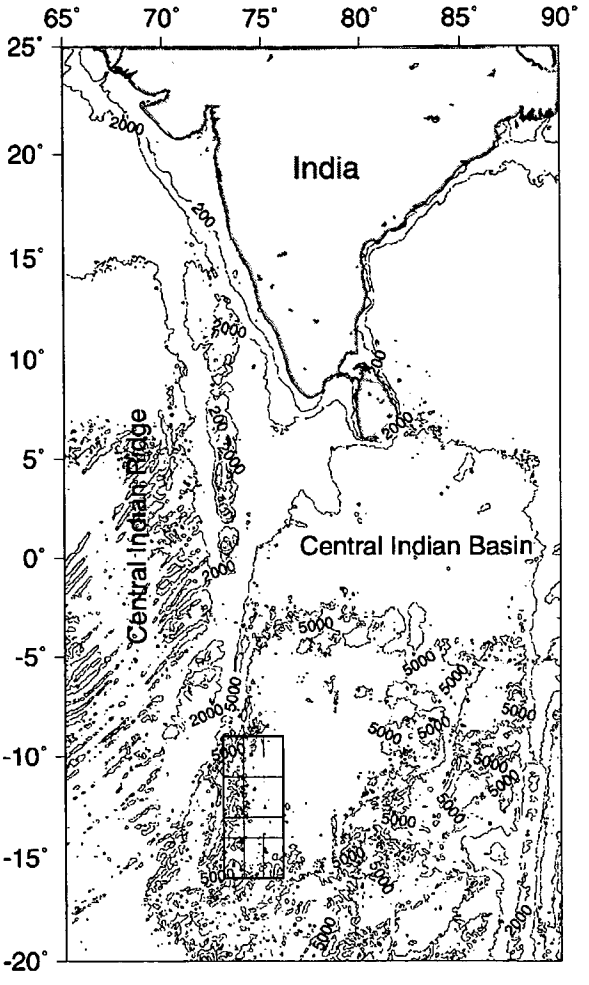

Fig. 1. Map of the Central Indian Basin showing within inset location of the sampling sites (9–16°S and 73–76°E) during cruises # AAS 34, # AAS 46 and # AAS 61.

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″–10°10′364″ S; 75°21′000″ -76°05′160″E), 7 during Cruise # AAS 46 in June 2002 (10°00′237″–10°02′661″S; 75°59′498″–76°09′822″ E) and 38 during cruise # AAS 61 in March 2003 (9°59′861″–16°00′047″S; 73°29′819″ 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 sea water, 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 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 °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 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 °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 °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 min 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 °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 (log 2). 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°35′N and 72°39′E) in the Arabian Sea by the particle plating technique. Two isolates of *Aspergillus terreus* (MTCC # 279 and 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 °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 330–385 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 # A 4634) 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 *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°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 °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.

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 °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 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° and 5 °C. Similarly prepared pouches were incubated at 1 bar pressure at 30° and 5 °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 *A. terreus* (MTCC # 279 and MTCC # 479) and one *A. 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 °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° and 5 °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° and 30 °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).

Table 1
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
Unidentified 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	P1	MEB
A 4636	*A. terreus*	AAS 46	BC 12	10° 01′	76° 00′	5400	0–2	P1	MEB
A 4634	*A. terreus*	AAS 46	BC 12	10° 01′	76° 00′	5400	15–20	P1	MEB
A 4633	*A. terreus*	AAS 46	BC 12	10° 01′	76° 00′	5400	10–15	P1	MEB
A 4630	*Aspergillus* sp.	AAS 46	BC 7	10° 02′	76° 00′	5296	4–6	P1	MEB
A 4628	*A. terreus*	AAS 46	BC 12	10° 01′	76° 00′	5400	8–10	P1	MEB
A 61 P10	*A. terreus*	AAS 61	BC 14	14° 00′	75° 30′	5145	20–25	P1	MEB
A 61 P4	Unidentified	AAS 61	BC 3	11° 59′	76° 29′	5280	0–2	P1	Artemia
A 4625	Unidentified	AAS 46	BC 7	10° 02′	76° 00′	5296	8–10	P1	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	P1	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	P1	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

P1—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.

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.

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 °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 and b). 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° and 30 °C.

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 300 bar pressure and 5 °C. Bar represents 10 μm.

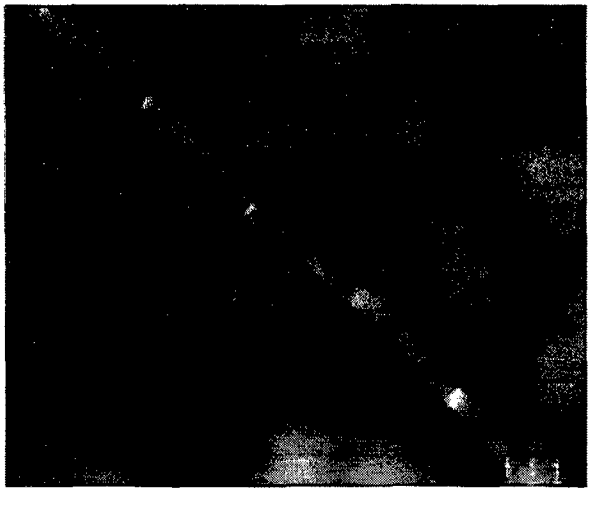

Fig. 4. An epifluorescence microscopy photograph of a fungal hypha from a deep-sea sediment sample stained with Calcofluor. Bar represents 10 μm.

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

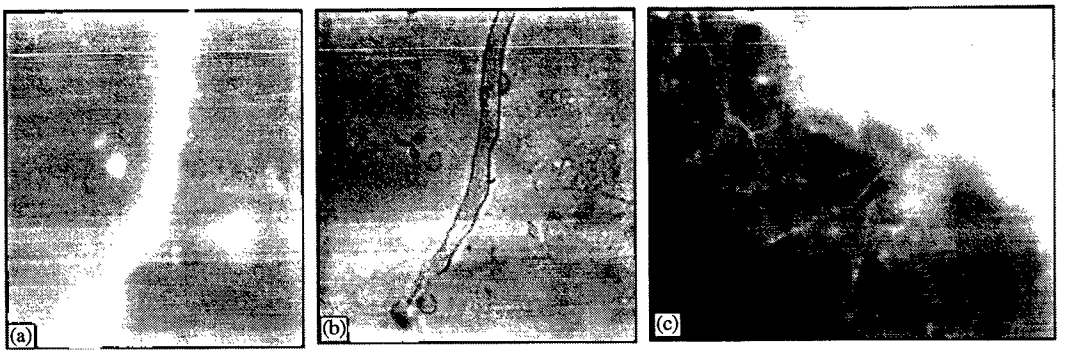

Fig. 5. (a) Immunofluorescence detection of a hypha of *Aspergillus terreus* (isolate # A 4634) from 15 to 10 cm depth of a core of the deep-sea sediment treated with FITC-tagged polyclonal antibodies raised against the fungus. Bar represents 10 μm. (b) Bright-field photomicrograph of the same hypha as in (a). Bar represents 10 μm. (c) Immunofluorescence detection of a dense cluster of hyphae of *Aspergillus terreus* on an organic particle from 15 to 20 cm core of a deep-sea sediment treated with FITC-tagged polyclonal antibodies raised against the fungus. Bar represents 10 μm.

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). Eleven 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, b). The conidia of *Penicillium* sp. (isolate # 4615) showed regular with germination at 30 °C/100 bar pressure, while those of *Aspergillus* sp. (isolate # 3454) showed swollen conidia (Figs. 8 and 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 *A. terreus* and *A. 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).

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[a]	134.5
A 4636	*A. terreus*	38.4	7.7	132.7
A 4634	*A. terreus*	18.3	19.6[a]	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[a]	118.3
A 61 P10	*A. terreus*	20.6	12.9	125.7
A 61 P4	Unidentified	17.8	27.1[a]	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[a]	228.0
A 3449	*Fusarium* sp	14.2	10.1	125.8
A 3441	Unidentified	31.5	38.9[a]	178.0
A 348	Non-sporulating	4.7	5.4[a]	64.3
A 3428	*Curvularia*sp.	4.5	19.3[a]	175.7
A 61 P63	Yeast	23.4	9.1	249.2
A 3426	Unidentified	21.0	22.2[a]	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[a]	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[a]	105.3
A 3412	*Aspergillus* sp.	3.1	1.7	140.0

[a]Fungi showing better growth at 5 °C than at 30 °C under 200 bar pressure.

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

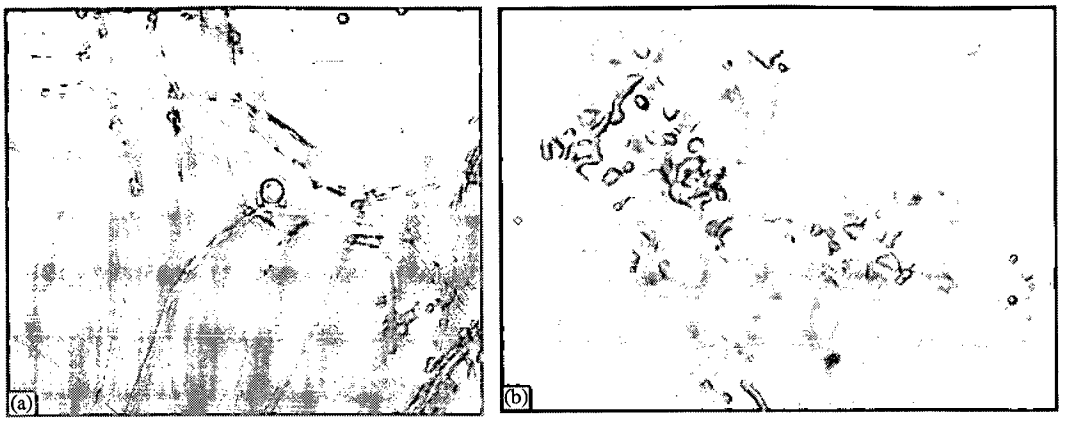

Fig. 6. Photomicrographs of *Aspergillus terreus* (isolate # A 4634) showing (a) normal hyphae during growth at 1 bar/30 °C, and (b) abnormal hyphae with swellings during growth at 200 bar/5 °C. Bars represents 10 μm (b) was photographed under phase contrast.

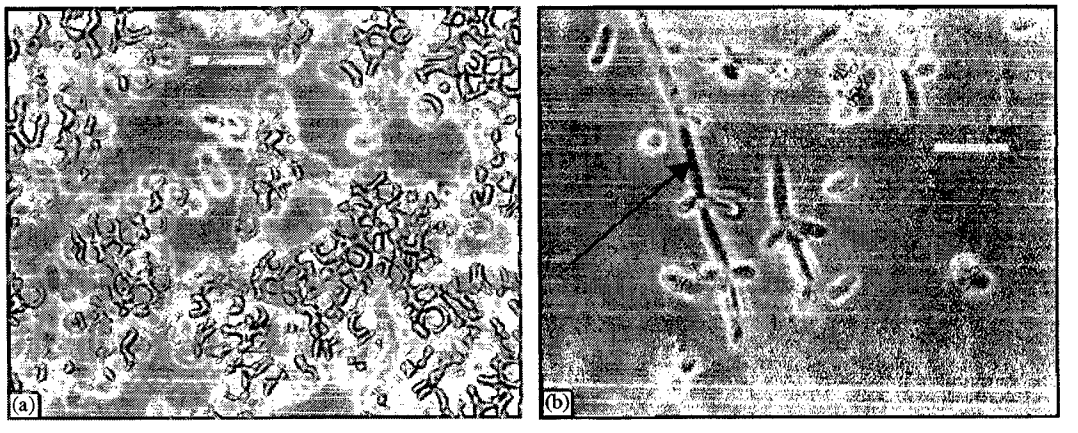

Fig. 7. Nomarski Differential Interference Contrast photomicroagraphs of the yeast (isolate # A 344) showing (a) normal growth at 1 bar/30 °C, and (b) normal growth with abundant pseudomycelia at 200 bar/5 °C (arrow). Scale 10 μm.

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

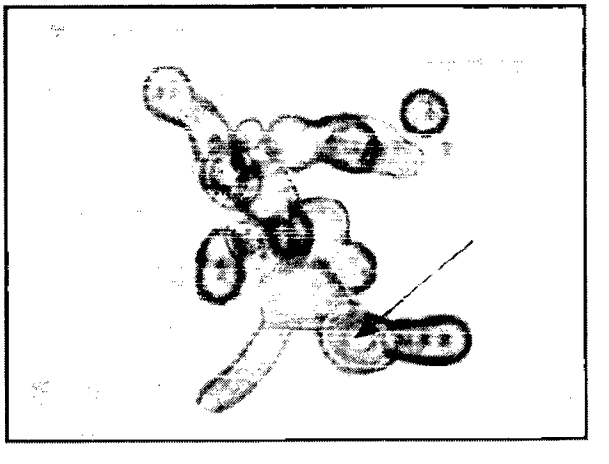

Fig. 9. Spores of *Aspergillus sp.* (isolate # 3454) germinating at 300 bar/30 °C. Note the swollen conidia (arrow). Scale 10 μm.

Table 5
Germination of spores of the deep-sea isolate *Aspergillus terreus* (# A 4634) 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

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 61 P63)	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

Mycelial inocula were used.

4634), *Aspergillus* sp. (# A 61 P4) and the unidentified isolate # A 3415 did not show significant difference in biomass production at 30 °C/1 bar or 200 bar pressure. The orange yeast (# A 61 P63) 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).

3.6. 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 # A 344 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 Section 2. One of the culture techniques that we used for enhancing recovery of native fungi was the particle plating method, which employs culturing

Table 7
Number of fungi surviving and showing active growth after exposure to sequential increase of hydrostatic pressure

Incubation pressure (bar)	Total fungi tested—109	
	Viable, but not growing[a]	Actively growing
50	32	74
100	39	42
200	6	16
300	8	3
400	2	1

All experiments were carried out at 30 °C.
[a]Viability tested by plating on MEA medium.

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 4–12 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 TOM and LOM 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. *A. 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, b) 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 °C at 1 or 200 bar. The terrestrial isolates germinated very poorly at 200 bar/30 °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 # A 344 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)

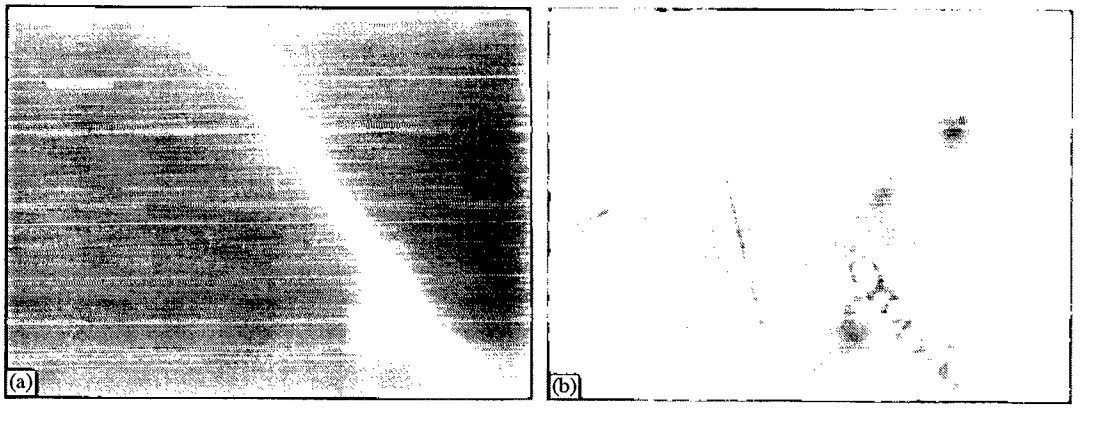

Fig. 10. (a) Epifluorescence microscopy of a fungal hypha in a transparent exopolymeric particle (TEP) collected from 30 m depth in the Arabian Sea. TEP was stained with Calcofluor and photographed under blue light with an epifluorescence microscope. Scale 10 μm. (b) The same hypha photographed under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

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 °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 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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Rapid communication

Deep-sea fungi as a source of alkaline and cold-tolerant proteases

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Abstract

Fungi from coastal environments have been widely studied with respect to the production of secondary metabolites and biotechnologically useful lignocellulolytic enzymes. A few studies on mycology of deep-sea sediments, however, have been carried out. This paper reports a study on alkaline, cold-tolerant proteases from deep-sea fungi. A total of 221 deep-sea isolates of fungi from 5000 m in the Central Indian Basin were screened for the enzyme. Many of these grew and produced alkaline protease at 5 and 30 °C and 1 bar pressure. *Aspergillus ustus* (NIOCC #20) producing the highest amounts of the enzyme was selected for further studies. The growth yield was substantial at 30 and 5 °C at 1 bar and elevated hydrostatic pressures. The fungus produced alkaline, cold-tolerant protease when grown at 30 °C and 1 bar pressure. The enzyme was active at combinations of 30, 5 °C and 50 and 300 bar pressure. However, protease production was negligible when the fungus was grown at 5 °C, under 1 bar or elevated hydrostatic pressures. The enzyme produced at 30 °C and 1 bar pressure was further characterized. The fungus produced a maximum of 1639 ACU mL^{-1} of protease by day 7. The enzyme, with molecular mass of 32 kDa and p*I* values of 6.6 and 6.9 showed several interesting properties. It had a broad pH range of 6–10, with an optimum at pH 9. The optimum temperature for protease activity was 45 °C and approximately 10% of the activity was retained at 2 °C. The enzyme was totally inhibited in the presence of 2 mM PMSF suggesting it to be a serine protease. It was active in the presence of several commercial detergents at 2 g L^{-1} concentration and in the presence of 0.5 M NaCl, equivalent to 29 parts per thousand salinity. In the presence of stabilizing agents such as glycerol, $CaCl_2$ its thermostability at 60 °C was enhanced. Heavy metal ions Cu, Hg, Fe, Ni and Zn did not inhibit the enzyme activity considerably. This study indicates that fungi from deep-sea sediments could be a useful source of proteases.

Keywords: Central Indian Basin; Sediments; Hydrostatic pressure; Deep-sea fungi; Proteases

1. Introduction

Fungi from marine habitats have received much attention in recent years for the production of useful secondary metabolites [1–3]. Research on marine fungi and biotechnologically useful enzymes produced by them, however, has been restricted to those isolated from coastal habitats and to lignocellulose degrading enzymes for application in bioremediation and paper industries [4,5]. Fungi and their enzymes from the deep-sea environment have received scant attention. Proteins and peptides constitute a substantial portion of the organic nutrients present in the deep-sea sediments as well as suspended particulate matter [6,7]. Therefore, extracellular proteases would play a pivotal role in the physiology of deep-sea fungi and in remineralization processes. Raghukumar and Raghukumar [8] reported production of protease enzyme under simulated deep-sea conditions by two filamentous fungi isolated from deep-sea calcareous sediments. About 25 and 75% of fungi isolated from the deep-sea sediments of the Central Indian Basin showed hydrolysis of casein and gelatin [9]. These studies suggested that deep-sea fungi could be a potential source of proteases.

Proteases are of immense interest in food, dairy, detergent, pharmaceutical and leather industries [10]. More than 25% of the worldwide sale of enzymes is contributed by proteases alone, where mainly alkaline proteases are used [10]. Alkaline proteases of bacteria from a variety of marine substrates, such as the hemolymph of a polychaete [11], the stomach of Antarctic krill [12], marine crab [13] and deep-sea sediments [14,15] have been described in recent literature. Extremophiles are an important source of enzymes and their specific properties are expected to be useful for novel applications [16]. In the light of the above, we have carried out a study on the production of alkaline, cold-tolerant proteases from fungi isolated from deep-sea sediments of the Central Indian Basin. The enzyme produced by one of the

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isolates was characterized in detail, and the results are presented in this paper.

2. Materials and methods

2.1. Isolation of fungi

Sediment samples were obtained from depths of around 5000 m in the Central Indian Basin (9–16°S and 73–76°E) on board the Russian research vessel AA Sidorenko, using USNEL-type box corer of 50 cm^3 size. Using alcohol-sterilized PVC cylinders of 5 cm diameter, subcores of sediments were collected from the center of the box corer. Subsections of 2 cm were extruded from these sediment cores of ~30 cm length directly into sterile plastic bags to avoid any aerial contaminants. The bags were closed with rubber bands and transported to the laminar flow hood in the laboratory on board. A portion of the sediment from the middle of each sub sample that had not been in contact with the walls of the PVC cylinder was removed using an alcohol flame sterilized spatula and placed in sterile vials for isolation of fungi [17]. Fungi were isolated using different techniques [18].

For a comparison between deep-sea fungal isolates and shallow-water ones, fungi were also isolated from sediments collected from 30 m depth of a coral lagoon of the Lakshadweep Islands in the Arabian Sea (11°N and 71°E) using similar technique [18].

2.2. Protease production

All the 221 deep sea and 22 shallow water fungal cultures isolated during the study were tested for their ability to produce alkaline protease using a qualitative plate assay on Czapek Dox agar (CDA) supplemented with 1% skimmed milk powder (Trade name Sagar, India). Clearance zone produced around the fungal colonies in plates indicated protease positive reaction [19]. The protease positive cultures were used for further quantification of the enzyme. This was done by growing the cultures in CD broth (Czapek Dox broth without agar) with 0.3% skimmed milk powder. Protease production was compared at 5 and 30 °C by growing the cultures at these temperatures. The culture supernatants collected by centrifugation at 10,000 rpm and 5 °C were used to assay the protease enzyme.

2.3. Protease assay

The protease activity was assayed with 150 μL of crude culture filtrate and 250 μL of the substrate azocasein (Sigma Chemicals, USA) at 2% concentration prepared in 0.1 M boric acid–borax buffer at pH 9. Protease activity was measured by incubating the reaction mixture at 30 and 5 °C for 30 min. The reaction was stopped by addition of 1.2 mL of 10% trichloroacetic acid solution. The contents were centrifuged at 8000 rpm for 10 min. To the supernatant, 1.4 mL of 1N NaOH was added and the absorbance read immediately at 440 nm against appropriate blanks in a spectrophotometer (Shimadzu, Model 1210, Japan). One ACU (Azocasein Digestion Unit) is defined as the increase in absorbance by 0.001 min^{-1} under the assay conditions [14]. One tyrosine unit is equivalent to μg tyrosine released mL^{-1} min^{-1} [20]. Accordingly, 1 ACU is equivalent to 0.05 tyrosine units.

Protease activity under elevated hydrostatic pressures was assayed in Eppendorf tubes of 0.5 mL capacity. The lids of the tubes were snapped off and sealed with parafilm after adding the reaction mixture, which contained the substrate azocasein, buffer at pH 9 and appropriately diluted enzyme. Care was taken to avoid trapping of air bubbles while sealing. The tubes were suspended in deep-sea culture vessel pressurized to the desired hydrostatic pressure and incubated under desired temperature for 30 min. At the end of the incubation period, the vessels were depressurized and without any delay the enzyme activity was arrested by adding TCA. Protease activity was measured as described above.

2.4. Characterization of enzyme

One of the cultures NIOCC #20, identified as *Aspergillus ustus* (Bain.) Thom and Church (deposited at the Institute for Microbial Technology, Chandigarh, India under the reference number MTCC 5102) showed the highest protease activity at pH 9, both at 5 and 30 °C temperatures among all the deep-sea fungal cultures and therefore was selected for further studies. The conditions for obtaining maximum protease production were partially optimized with reference to the pH of the medium, protein sources and the time of harvesting. On the basis of these results, the culture filtrate was collected by filtering first through glass fibre (GF/F) filters (Whatmann, USA) and then by durapore 0.22 μm (Millipore, USA) on day 7 and was concentrated in a speed vacuum concentrator (Biotron, Korea). The concentrate was passed through an anion exchange column 'Resource Q' (Amersham Biosciences, Uppsala, Sweden). It was eluted using a gradient of NaCl (0–0.25 M) prepared with 10 mM phosphate buffer at pH 7. The flow rate was adjusted to 0.1 mL min^{-1} and the eluted fractions of 1 mL each were collected. Fractions showing protease activity were pooled, concentrated and was further subjected to gel filtration column chromatography using Superdex 200 column (Amersham Biosciences, Uppsala, Sweden). The enzyme was eluted with 0.2 M NaCl prepared in 100 mM acetate buffer at pH 4.5. The flow rate was adjusted to 0.5 mL min^{-1} and the eluted fractions of 2 mL each were collected. Fractions showing protease activity were pooled and used for the characterization of the enzyme. The homogeneity of the fractionated enzyme was confirmed by running a native PAGE with 10% acrylamide at 60 V. The gel was blotted on 1.5% agarose containing 1% casein for 1 h. The agarose blot was stained with 0.15% amido black (Sigma Chemicals, USA) and 0.3% coomasie blue in methanol:acetic acid:water at a ratio of 4:1:5 [21]. The molecular weight of the purified enzyme was determined using SDS-PAGE. The electrophoresis was carried out on 12% resolving gel at a constant voltage of 60 V. The protein was detected using silver staining method [22]. The p*I* of the purified enzyme was estimated by running an isoelectric focussing tube gel. Broad range ampholytes of pH 3–10 were used. The pH gradient was formed by running the gel at 250 V for 30 min following which the sample was loaded onto the gel. The isoelectric focussing was carried out at 500 V for 4 h.

The optimum pH of the purified enzyme activity was determined using eight different buffers (100 mM): sodium acetate (pH 5), citrate phosphate (pH 5–7), phosphate (pH 6–8), Tris–HCl (pH 8 and 9), borax–boric acid (pH 8 and 9), glycine–NaOH (pH 9 and 10), borax–NaOH (pH 10) and carbonate–bicarbonate (pH 10 and 11) at 45 °C. Optimum temperature of the protease activity was determined at pH 9 by assaying at 2, 10, 15, 30, 45, 50, 60, 70, 80 and 90 °C. The K_m constant and V_{max} were determined from Lineweaver Burke plot at pH 9 and 45 °C using the substrate azocasein with purified enzyme. Thermostability of protease at different temperatures was assayed by incubating the enzyme samples at 40, 50, 60, and 70 °C for 10 min at pH 9 and then the residual activity was measured. Thermostability of purified enzyme at its optimum temperature (45 °C) was estimated by incubating the enzyme at 45 °C and the residual activity was assayed at an interval of 10 min. The stability of purified enzyme at different pHs was carried out by incubating 25 μL of enzyme with 75 μL of corresponding buffer for 1 h and then the residual protease activity was measured. Different additives were used to increase the thermostability of the enzyme at 60 °C. The effect of various inhibitors, heavy metals, reducing agents, ionic strength, bleaching agents and detergents on protease activity of this isolate was carried out at pH 9 and 45 °C. All chemicals used were of ultrapure quality (Sigma Chemicals from USA, Merck and Qualigens from India).

2.5. Growth and protease production under elevated pressures

NIOCC #20 was also tested for its ability to grow and produce protease at elevated hydrostatic pressures. Sterile plastic pouches containing 45 mL of Czapek Dox broth with 0.3% skimmed milk solution were inoculated with 5 mL of culture suspension (finely broken mycelia and spores in seawater). These were suspended in deep-sea culture vessels (Tsurumi & Seiki Co., Japan) filled with sterile distilled water. These vessels were pressurized at 50 and 100 bar (10 bar = 1 MPa) and incubated at 30 and 5 °C for 20 days. Three replicates were maintained for each treatment. After 20 days, the biomass was separated from culture broth by centrifugation and further filtered through sterile 0.22 μm hydrophilic durapore membrane filters (Millipore, USA). Protease activity of the filtrate was estimated using azocasein as substrate. The assay was carried out at the optimal conditions of the enzyme (pH 9; 45 °C and 1 bar pressure). The activity was also assayed under 50 and 100 bar pressure at pH 9 and 30 °C.

The extracellular protease produced by the culture when grown under atmospheric pressure (1 bar pressure) and 30 °C and 50 bar/30 °C was compared by assaying its activity under elevated hydrostatic pressure of 50, 100, 200 and

Table 1
Growth and alkaline (pH 9) protease production by deep-sea and shallow water isolates of fungi

Source of fungi	Total number isolated	Culture condition	Number and (%) isolates showing growth	Cultures with protease activity at 30 °C[a]	Cultures with protease activity at 5 °C[a]
Deep sea	221	1 bar and 30 °C	221 (100%)	105 (48%)	73 (33%)
		1 bar and 5 °C	113 (51%)	15 (13%)	12 (11%)
Shallow water	22	1 bar and 30 °C	22 (100%)	10 (45%)	3 (14%)
		1 bar and 5 °C	7 (32%)	2 (29%)	2 (29%)

[a] Percentage of cultures with protease activity was calculated from the total number of cultures showing growth (numbers in the 4th column).

300 bar at pH 9 and 30 °C. The K_m constants of the protease activity from cultures grown at 30 °C and 1 bar pressure were measured at 1 bar/5 °C and 50, 100, 200 and 300 bar/45 °C using the substrate azocasein at pH 9.

3. Results

A total of 221 and 22 fungi from deep-sea and shallow depth sediments respectively were obtained by employing different techniques of isolations (Table 1). Among these, 33% of the deep-sea and 14% of shallow water fungal isolates when grown at 30 °C and 1 bar pressure produced protease, that was active at alkaline pH and low temperature of 5 °C. Up to 51% of the deep-sea isolates and only 32% of the shallow water isolates showed ability to grow at low temperature of 5 °C. Among the cultures showing growth at low temperature, 11% of the deep-sea isolates and 29% of the shallow water isolates produced protease that was active at alkaline pH (9.0) and 5 °C (Table 1). The number of isolates showing protease activity at 30 °C was much higher than those showing activity at 5 °C. A large number of isolates showed alkaline protease activity within a range of 1–10 ACU mL^{-1} (Fig. 1). Measurement of protease activity using azocasein was found to be very sensitive because as low as 1 ACU could be detected.

A culture of *A. ustus* (NIOCC #20) from deep-sea sediments, with maximum protease production initially in the range of 30–40 ACU mL^{-1} when assayed at 30 °C and 1 bar pressure, and showing growth at elevated hydrostatic pressure and 5 °C was selected for further characterization of the enzyme. The culture showed growth under elevated hydrostatic pressures at 30 and 5 °C (Table 2). Protease production by this fungus was assayed both at 30 and 5 °C. The latter temperature revealed negligible or non-detectable levels of protease in all the assays. Therefore further comparisons of protease production under different growth conditions of elevated hydrostatic pressures were made by assaying at 30 °C.

Table 2 presents that only 12.35% of protease activity was detected when the fungus was grown at 5 °C and 1 bar pressure, with respect to the amount produced at 30 °C and 1 bar pressure. At 50 and 100 bar of pressure, a very low amount of protease was produced both at 5 and 30 °C. The protease synthesized by the culture grown at 1 bar pressure and room temperature, retained 100% of the activity when assayed at elevated hydrostatic pressure (up to 300 bar of pressure). Similarly the enzyme synthesized at 50 bar and room temperature retained 100% of its activity up to 300 bar pressure (Table 3).

NIOCC #20 showed maximum biomass production on day 3, when grown at 30 °C and atmospheric pressure while the maximum protease production was observed on day 7 in Czapek Dox broth with 0.3% skimmed milk powder (Fig. 2). The enzyme production was maximum on day 7 whether assayed at 5 or 30 °C

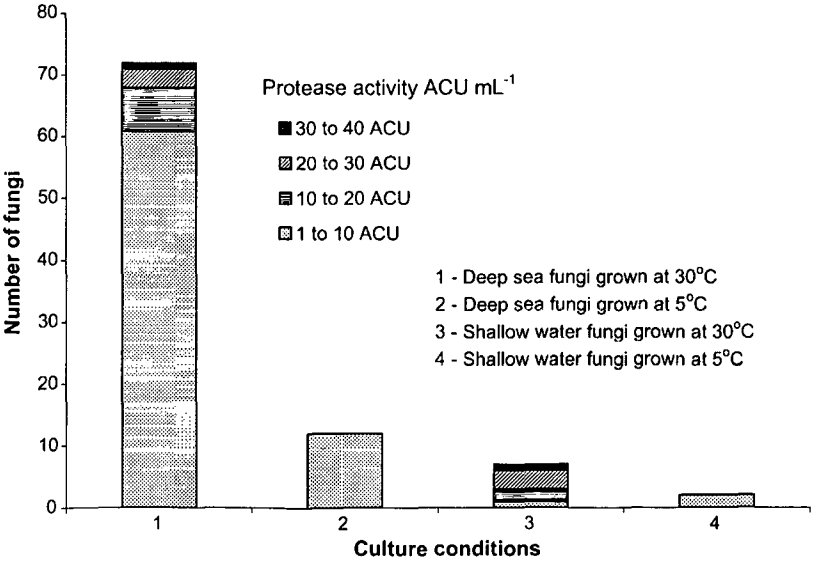

Fig. 1. Number of fungi isolated from deep-sea and shallow-water sediments showing protease activity in the range of 1–40 ACU mL^{-1} when grown at 5 and 30 °C. The NIOCC #20 belonged to the group showing 30–40 ACU mL^{-1}.

Table 2
Characteristics of the deep-sea isolate NIOCC #20 when grown for 20 days in CD broth with 0.3% skimmed milk powder under various pressure and temperature conditions

Growth temperature (°C)	1 bar	50 bar	100 bar	Parameters
30	208.2	69.4	44.1	Biomass produced (mg dry weight)
5	121.4	93.7	85.8	
30	36.02	3.67	0.78	Protease activity (ACU mL^{-1}) assayed at 30 °C and 1 bar
5	4.45	0.45	0.22	
30	100	10.2	2.17	Percentage activity against the highest value
5	12.35	1.25	0.61	
30	0.173	0.15	0.014	Protease activity/mg dry biomass
5	0.037	0.013	0.007	

The biomass represents the dry weight of the fungal mycelium. The liquid culture was filtered over a pre-weighed Whatmann No. 1 filter paper, dried to a constant weight at 50 °C and the weight determined. The values are mean of two replicates.

Table 3
Activity of the protease of the deep-sea fungus NIOCC #20 assayed under elevated hydrostatic pressures and 30 °C

Pressure (bar)	Protease activity (ACU mL^{-1})	
	A	B
1	427.33 ± 4.51	ND
50	356 ± 2.12	3.67 ± 0
100	363.33 ± 4.72	3.33 ± 0
200	417.33 ± 10.24	4.33 ± 0.24
300	428.33 ± 4.95	4.67 ± 0

A: protease produced when grown at 1 bar pressure/30 °C; B: protease produced when grown at 50 bar pressure/30 °C.

(Fig. 3). The culture showed maximum enzyme production in the medium adjusted to pH 9. Protease was produced in Czapek Dox medium without an organic nitrogen supplement (Fig. 4). Addition of milk, soy meal, malt extract, Tween 80, and corn steep liquor (CSL) increased the enzyme production by several folds (Fig. 4).

The protease was purified from 980 mL of culture supernatant using a three-step procedure that included speed vacuum concentration, anion exchange and gel filtration column chromatography. The enzyme eluted out as unbound fractions from Resource Q anion exchange column with 0.27-fold purification (Table 4). After gel filtration on superdex-200 column, the purity of the enzyme was 4.05-fold (Table 4). The homogeneity of the enzyme was further confirmed by activity staining on non-denaturing-PAGE. One distinct protease band was visualized on native gel (Fig. 5a). The molecular weight of the purified enzyme fraction obtained by gel filtration chromatography was 32 kDa as determined by SDS-PAGE (Fig. 5b). The purified enzyme had p*I* of 6.6 and 6.9 (Fig. 5c). This might be isozymes of protease which were resolved due to the presence of ampholytes in the gel. They might have been merged in the native gel where only one activity band was noticed.

The enzyme exhibited pH optima of 9 with >80% of activity between pH 6 and 10 at 45 °C (Fig. 6a). The protease activity was tested at temperatures ranging from 5 to 90 °C at pH 9 (Fig. 6b). The maximum activity was at 45 °C and about 45% of the maximum activity was detected at 20 °C and about 10% at 2 °C (Fig. 6b). Thermostability studies showed that the enzyme retained 80 and 55% of its activity at 50 and 60 °C respectively for 10 min at pH 9 (Fig. 6c). At its optimum temperature of activity (45 °C), the enzyme retained 100% of its activity for 30 min after which it steadily decreased (data not shown). The

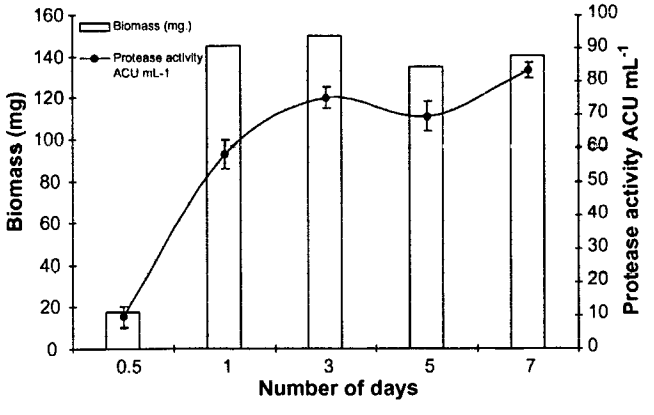

Fig. 2. Temporal production of biomass and protease by NIOCC #20 grown in CD broth with 0.3% skimmed milk powder. The protease assay was carried out at pH 9 and 45 °C with azocasein as substrate. The biomass was determined by filtering the cultures (in triplicates) on specified days over pre-weighed Whatmann filter papers and dry weight of the fungal mycelium was determined.

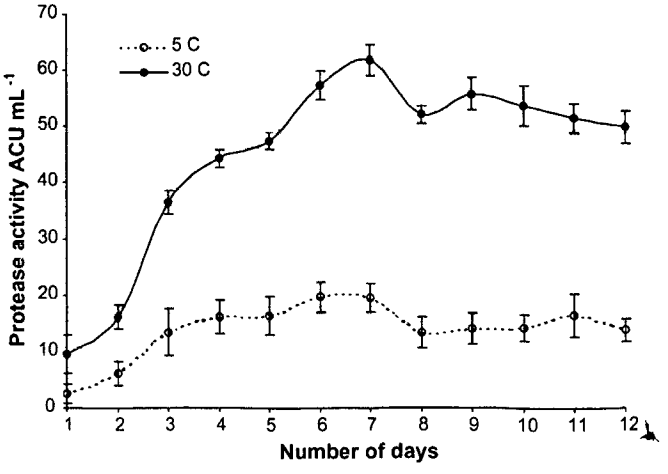

Fig. 3. Protease production by NIOCC #20 when grown at 5 °C (broken line) and 30 °C (solid line). Protease was assayed as described in Fig. 2.

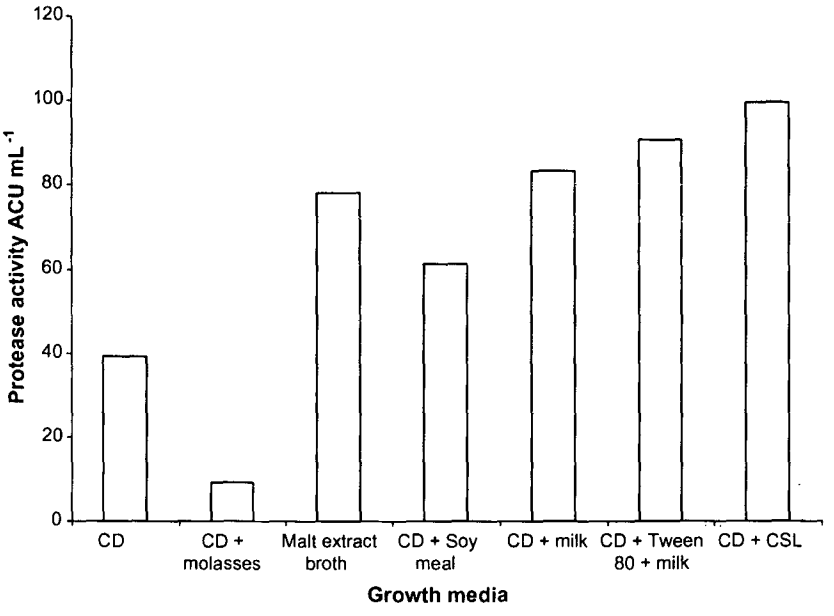

Fig. 4. Protease production by NIOCC #20 when grown at 30 °C in different media. CD = Czapek Dox broth, CSL = corn steep liquor.

Table 4
Purification of protease of the deep-sea fungus NIOCC #20 grown at pH 9.0 and 30 °C

	Total enzyme units (ACU)	Total protein (mg)	Sp. activity (ACU mg^{-1} protein)	Purification (fold)	Recovery (%)
Crude filtrate	981000	131.08	7484	1	100
Vacuum concentrated	205800	93.06	2212	0.3	21
Resource Q	166786	82.61	2019	0.27	17
Superdex-200	206800	6.82	30323	4.05	21

Protein was estimated using Folin phenol reagent [28].

enzyme was most stable at pH 4.5 in the absence of the substrate (Fig. 6d).

The protease activity was totally inhibited by 2 mM PMSF suggesting it to be a serine protease. EDTA at 5 and 100 mM concentration hardly inhibited the protease activity (Table 5) indicating that it was not a metalloprotease. The protease of NIOCC #20 is thiol-independent because reducing agents as DTT and mercaptoethanol did not affect the activity considerably (Table 5). At 1 mM concentration of Hg only 45% inhibition of the enzyme was observed. Addition of Fe, Ni and

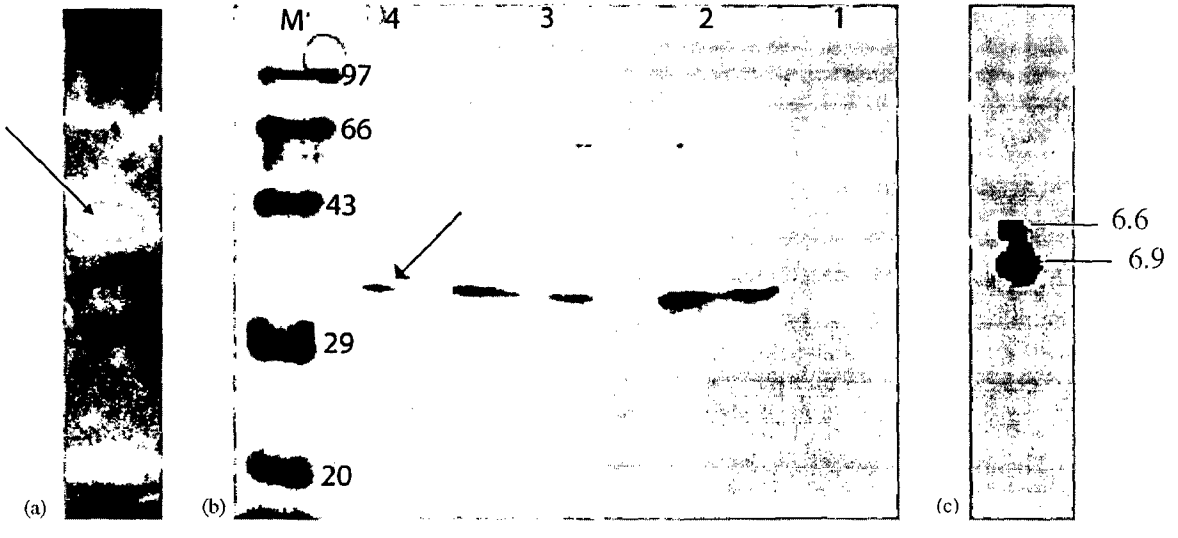

Fig. 5. (a) Zymogram of the active fraction on native page showed activity staining for protease. The clear zone in casein as a result of protease activity appears as a band. (b) The SDS-PAGE of the protease: lane 1 = crude culture filtrate; lane 2 = vacuum-concentrated culture filtrate; lane 3 = Resource Q-pooled fraction; lane 4 = pooled active fraction obtained by gel filtration using Superdex 200 showing single band of 32 kDa (arrow). (c) IEF gel showing isozymes with p*I* values of 6.6 and 6.9.

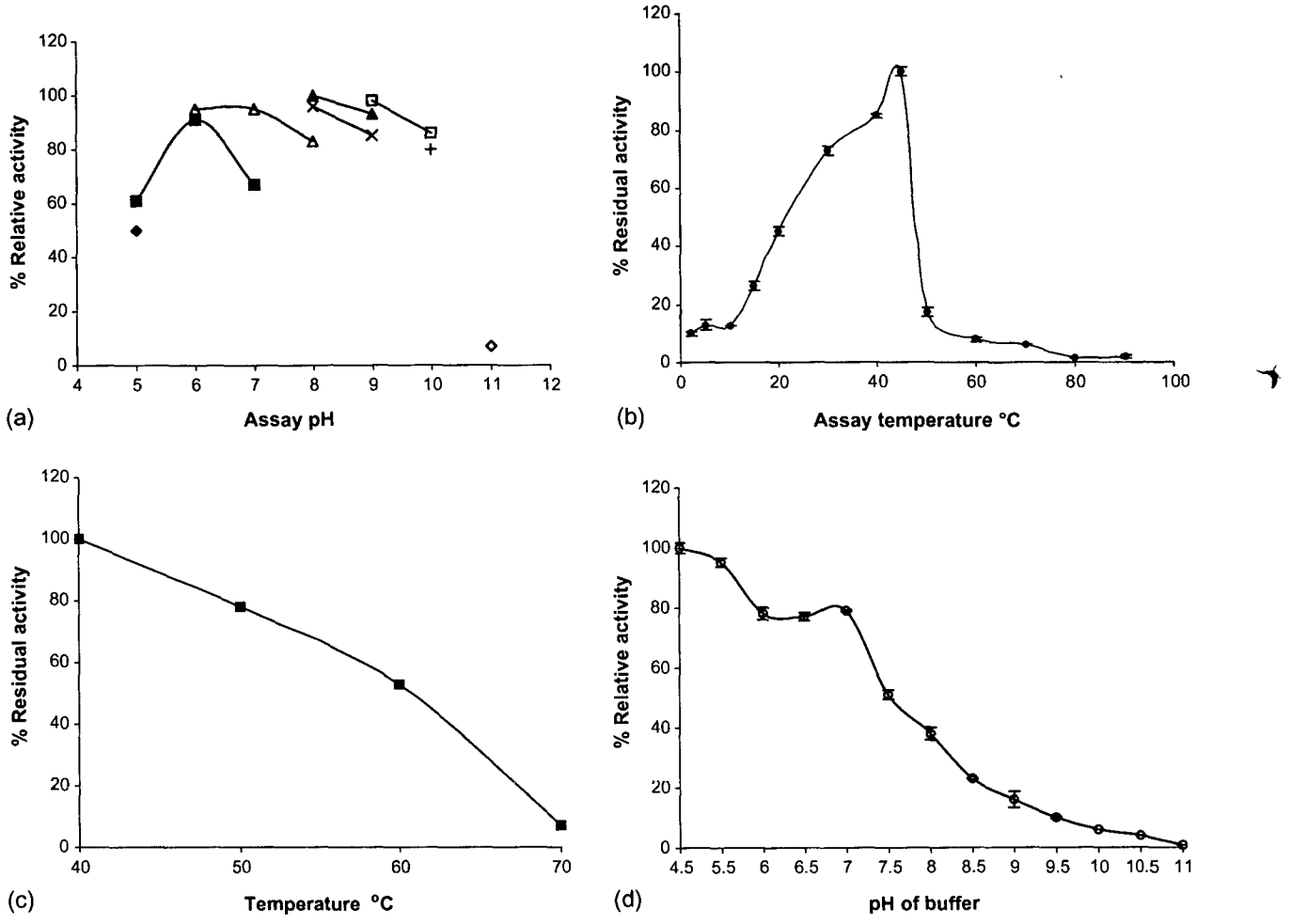

Fig. 6. (a) Relative activity of protease of NIOCC #20 at different pHs and 45 °C. Protease activity was measured as mentioned under Section 2: (▲) acetate buffer; (■) citrate phosphate buffer; (△) phosphate buffer; (×) Tris–HCl buffer; (▲) boric–borax buffer; (□) Glycine–NaOH buffer; (+) borax–NaOH; (◇) carbonate-bicarbonate buffer. (b) Relative activity of protease at different temperatures measured at pH 9. (c) Residual activity of protease was measured after incubating the enzyme sample at pH 9 for 10 min at different temperatures. (d) Stability of protease at different pHs was carried out by incubating the enzyme at specified pHs without the substrate for 60 min. Activity was measured at its optimum temperature (45 °C) and expressed as percent residual activity.

Zn at 1 mM hardly inhibited the enzyme activity. On the other hand, Cu at 1 mM concentration inhibited the protease activity totally (Table 5). About 70% of the activity was retained in the presence of sodium chloride of 0.5 M concentration which equals to 29 ppt salinity of seawater (Table 5). Chemical surfactants like Triton X-100 and Tween 80 (both at 1.0%) and commercial detergents at 2 g L^{-1} concentrations did not reduce the protease activity, although SDS (0.1%) totally inhibited its activity (Table 6). About 50% of the enzyme activity was lost after treatment at 60 °C for 10 min. However, thermostability of the enzyme at 60 °C increased on addition of glycerol, $CaCl_2$, PEG 6000, sucrose, mannitol, sorbitol and starch during enzyme assay (Table 7). The protease of the culture grown at 1 bar pressure showed a K_m constant of 2 mg mL^{-1} when measured at pH 9 and 45 °C and this increased marginally at elevated hydrostatic pressures of 50 and 100 bar (Table 8). There was a marked increase in K_m constant at 5 °C/1 bar, 45 °C/200 and 300 bar pressure. The V_{max} values were not much affected by the elevated hydrostatic pressures except a substantial drop at 50 bar pressure from that at 1 bar pressure (Table 8).

4. Discussion

The major aim of this study was to explore deep-sea fungi for production of cold-tolerant and alkaline protease. The isolate NIOCC #20 obtained after the screening produced substantial growth at 5 °C, under 1 and 100 bar pressure (corresponding to 1000 m). Under the above two conditions, it attained a biomass equivalent to 58 and 41% respectively of that produced at 30 °C/1 bar pressure (Table 2). However, when the fungus was grown at 5 °C under 1, 50 or 100 bar and the enzyme activity assayed at 5 or 30 °C under different hydrostatic pressures, little or negligible amount of cold-tolerant protease was detected. In contrast to the above, the enzyme produced by the fungus at 30 °C and 1 bar was much more versatile and retained about 10% of activity at 2 °C at 1 bar compared to its activity at optimum temperature (Fig. 6b). The enzyme produced at

Table 5
Effect of various inhibitors, heavy metals, varying ionic strengths (sodium chloride) and reducing agents on protease activity of the deep-sea fungus NIOCC #20

Inhibitors	Residual activity (%)
Protease inhibitors (mM)	
None (control)	100
PMSF (2)	0
EDTA (5)	99
EDTA (100)	93
Heavy metals (mM)	
$NiCl_2$ (1)	72
$ZnCl_2$ (1)	81
$HgCl_2$ (1)	55
$CuCl_2$ (1)	0
$FeCl_3$ (1)	74
Sodium chloride (M)	
0	100
0.1	88.6
0.25	80
0.50	70
0.75	61.4
1.0	40
Reducing agents (%)	
None	100
DTT dithiothereitol (0.1)	91
DTT (0.5)	73
β-Mercaptoethanol (0.1)	77
β-Mercaptoethanol (0.5)	75
Sodium thioglycolate (0.1)	45
Sodium thioglycolate (0.5)	27
Sodium thioglycolate (1.0)	27
Glutathione (0.1)	0

30 °C under 1 or 50 bar also retained its activity when assayed under elevated hydrostatic pressures up to 300 bar (Table 3). However, the increasing K_m constants of protease at elevated hydrostatic pressures of 200 and 300 bar (Table 8) indicates low enzyme-substrate affinity under these extreme conditions. The total enzyme activity per se is not affected by elevated pressures (Table 3), which is also indicated by the almost constant V_{max} values (Table 8).

Table 6
Effect of detergents and bleaching agents on protease activity of the deep-sea fungus NIOCC #20

Compound (concentration in %)	Residual specific activity (%)
None	100
Tween 80 (1.0)	142
Triton X-100 (1.0)	139
SDS (0.1)	0
Ala (commercial fabric bleach) (2.0)	20
Sodium hypochlorite (1.0)	2
H_2O_2 (2.0)	40
Commercial detergent wheel (2 g L^{-1})	91
Mr. White	85
Rin Shakti	64
Tide	62
Surf Excel	161
Ariel Compact	73

Table 7
Effect of additives on thermostability of the crude enzyme of the deep-sea fungus NIOCC #20 with reference to 10 min of heat treatment at 60 °C without any additive

Additive present during the heat treatment	Residual activity (%)
Control (untreated)	100
No additives	51
Glycerol (1%)	101.5
$CaCl_2$ (1 mM)	108.5
$CaCl_2$ (5 mM)	110.6
$CaCl_2$ (10 mM)	102.4
PEG 6000 (1%)	111.1
Sucrose (1%)	84.8
Mannitol (1%)	79.2
Sorbitol (1%)	85.6
Starch (1%)	98.8

Thus, although growth was fairly unaffected by elevated pressure and low temperature, protease production under similar conditions was reduced 10–100 folds. This appears to be a general trend with all the deep-sea isolates that we obtained (Table 1). Thus, a higher percentage of deep-sea isolates (51%) were capable of growing at 5 °C, when compared to those isolated from shallow waters (32%). Besides, a high number of deep-sea isolates produced low-temperature active protease when grown at 1 bar and 30 °C, but very few of these did so when grown at 1 bar and 5 °C (Table 1). Compared to the deep-sea isolates, fewer isolates from shallow water produced cold-tolerant protease when grown at 1 bar pressure and 30 °C and their growth was also poor at 5 °C. NIOCC #20 appears to be a strain adapted to deep-sea conditions, since it grew better at 5 °C/100 bar than at 30 °C/100 bar, although its production of protease under the former conditions was low (Table 2). This is in contrast to the report on a deep-sea bacterial strain DB6705, which grew better at higher rather than low temperatures when cultured at 500 bar hydrostatic pressure [23]. Growth and metabolic activities under extreme conditions are known to be low [24]. Our results with NIOCC #20 also show similar trends.

Enzymes from cold-adapted organisms have been classified into three groups [10]. Group I enzymes are heat-sensitive, but the other enzymatic characteristics are similar to mesophilic enzymes. Group II enzymes are heat-sensitive and relatively more active than mesophilic enzymes at a low temperature. Group III enzymes have same thermostability as mesophilic enzymes but are more active than mesophilic enzymes at a low

Table 8
K_m constant and V_{max} values of the purified protease of the deep-sea fungus NIOCC #20 measured under different conditions

Enzyme activity assayed at	K_m constant (mg mL^{-1} azocasein)	V_{max} (ACU mL^{-1})
1 bar and 45 °C	2.0	26.3
50 bar and 45 °C	2.2	19.2
100 bar and 45 °C	2.4	24.4
200 bar and 45 °C	3.3	25.0
300 bar and 45 °C	5.0	25.0

Table 9
Comparison of NIOCC #20 with other alkaline and low-temperature active proteases

Organism	Source of isolation	Substrate used	Maximum U	Definition of U	Optimum pH	Optimum temperature (°C)	Other properties	Reference
Alteromonas haloplanktis	Marine bacterium	MCA		nmol substrate released min^{-1}	8–9	20	74 kDa, thiol protease	[15]
Vibrio sp.	Deep-sea sediments	Casein	353 U mL^{-1}	μg tyrosine min^{-1}	8.5–9.0	40	35 kDa	[14]
Azospirillum sp.	Cold mountain soil	Casein	2 U mL^{-1}	Increase in OD of 0.1 min^{-1}	8.5	40	48.6 kDa, cysteine	[21]
Vibrio sp.	Cold marine source in Iceland	Azocasein					47 kDa, serine	[13]
Paecilomyces marquandii (fungus)	Not known	Casein, haemoglobin	0.016–0.018 U mL^{-1}		12	45	38 kDa	[29]
Nocardiopsis dassonvillei	Not known	Casein, haemoglobin	0.053 AU mL^{-1}	CPU = mM serine min^{-1}	8–9	60	Serine	[30]
Sphingomonas paucimobilis	Stomach of Antarctic krill	A variety of substrates		Tyrosine released	6.5–7.0	30 (47% of the activity at 0 °C)	Metalloprotease	[12]
Conidiobolus coronatus	Soil	Casein	30 U mL^{-1}	1 Abs mL^{-1} min^{-1} = U	9.7	37		[31]
Rhizophus oryzae	Not known	Azocasein	3370 U mL^{-1}	0.1 Abs h^{-1}	10.5	60		[32]
Bacillus mojavensis	Soil	Casein		μg mL^{-1} min^{-1} of tyrosine	10.5	60		[20]
Nocardiopsis sp.	Not known	Azocasein	50 U mg^{-1} protein	Abs h^{-1}	8.0	50	Serine protease	[33]
Paecilomyces lilacinus	Biocontrol agent	Azocasein						[34]
Bacillus sp.	Korean polychaete	Casein		1 μg tyrosine min^{-1}	10.0	45–50	Serine protease	[11]
A. ustus NIOCC #20	Deep-sea sediment	Azocasein	1639 ACU mL^{-1}	Abs 0.001 min^{-1}	6.5–10	45 (45% of the activity at 20 °C)	32 kDa, serine protease	The present work [35]

temperature. Accordingly, the protease of our isolate falls into the group II. The protease of mesophilic *Penicillium* sp. did not show any activity below 20 °C [25]. The alkaline protease from another mesophilic species of *Penicillium* did not show any activity below 35 °C [26]. On the other hand, NIOCC #20 showed about 45, 26 and 12% of the maximum activity at 20, 15 and 5 °C. It looses about 50% of its activity at 60 °C (Fig. 6c). Moreover, at its optimum temperature of activity (45 °C), it is stable only for 30 min after which it rapidly looses activity (data not shown). The enzyme is stable for more than 24 h at 5 °C and pH 4.5. Its half-life under these conditions is about 196 h. These results suggest its placement in the group II. The effect of temperature on the enzyme activity is determined by the temperature coefficient Q_{10} [27]. It is the factor by which the rate increases when the temperature is raised by 10°. If the rate of the reaction is completely temperature-independent, the resulting Q_{10} will be 1.0. If the reaction rate increases with increasing temperature, Q_{10} will be greater than 1. Thus, the more temperature-dependent a process is, the higher will be its Q_{10} value. The calculated Q_{10} value of 1 between 2 and 10 °C in the present work indicates temperature-independent enzyme activity at lower temperatures. On the other hand, the increased Q_{10} values (above 1) at 15–45 °C indicate direct relation to increasing temperatures. Above 45 °C, the Q_{10} value is less than one suggesting negative effect of temperature on the enzyme activity.

The present strain produced a maximum of 1639 ACU mL^{-1} of the enzyme, as estimated by the azocasein method. Application of different methods and the definition of enzyme units to estimate the enzyme by various authors using different substrates such as casein, haemoglobin, methylcoumarylaminoacid (MCA), azocasein or gelatin make the comparison difficult. However, our estimations have shown that the protease activity per mL in the crude culture filtrate of NIOCC #20 corresponds to 84 μg of tyrosine released. The protease production by NIOCC #20 appears to be as good as or even better than those reported (Table 9).

The protease of NIOCC #20 is a serine protease because it was totally inhibited by PMSF which is a known inhibitor of serine protease. It was not inhibited by EDTA and therefore it is not a metalloprotease. It was not substantially inhibited by the heavy metals Ni and Zn which are the inhibitors of cysteine protease. Its activity did not increase in the presence of thiol compounds, indicating that it was thiol-independent serine protease. Protease produced by this isolate showed several special features. It was active in the presence of several commercial detergents when assayed under its optimum conditions. The enzyme was active in the presence of 0.5 M NaCl equivalent to 29 ppt of seawater salinity. Its stability at 60 °C increased in the presence of various stabilizing agents. Such features in proteases are often much sought after for use as additives in detergents for low-temperature wash [10]. Most alkaline proteases have been reported to be significantly stabilized in the presence of additives such as those used by us [20].

NIOCC #20 secreted maximum extracellular protease among all the deep-sea fungi isolated and when grown in a medium prepared with seawater at pH 8 and 9 and thus is truly alkalophilic. Protease production occurred in Czapek Dox medium without an organic nitrogen source also and thus the enzyme appears to be constitutive (Fig. 4). Its production, however, could be enhanced in the presence of milk, soy meal, and corn steep liquor.

Protease of *A. ustus* (NIOCC #20) with an optimum pH of 9 and showing about 45 and 25% of its activity at 20 and 15 °C respectively is an alkaline protease. Its performance in terms of activity can be further improved by optimization of growth and other parameters. Terrestrial isolate of *A. ustus* (isolate # MTCC 2200) obtained from IMTECH culture collection showed extremely low protease production (23 ACU mL^{-1}) under similar assay conditions. This study shows that deep-sea fungi are useful candidates in the search for alkaline proteases.

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