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Fungi in cold deep seas: a hot topic*

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ABSTRACT

Deep-sea is characterised by low temperatures, elevated hydrostatic pressures, low nutrients, total darkness and constant salinity. Presently numerous workers are engaged in this topic resulting in several publications, newer insights and contributions to biodiversity and understanding the physiology of deep-sea fungi. We used several techniques to isolate and culture fungi from deep sea sediments collected at ~ 5000 m water depth in the Central Indian Basin (CIB). These were identified by conventional, morphological taxonomy as well as by molecular taxonomy. Fungi were also directly detected in formalin-preserved deep-sea sediments. We also studied culture-independent fungal diversity at several locations in the CIB. Several environmental gene libraries were constructed after amplifying the sediment DNA using universal and fungal-specific internal transcribed spacers (ITS) and universal 18S rDNA primer pairs. This approach resulted in the recovery of several fungal operational taxonomic units (OTUs). A few culturable-phylotypes and several fungal OTUs demonstrated high divergence from the existing sequences in the GenBank. Several of them were new reports for deep-sea sediments indicating hidden treasure of fungal diversity. Physiological characterization of the cultured fungi revealed that most of the fungi were mesophiles, psychrotolerants and barotolerant. Several of them produced cold-active enzymes having tolerance to high salinity. Thus it appears that deep sea sediments are repository for novel fungi. Their role in deep-sea ecosystem needs to be studied in future.

KEYWORDS : Culture dependent, culture independent, deep-sea, fungal diversity, ocean drilling program

INTRODUCTION

Microbial diversity in cold deep sea waters was a mystery a few decades ago, but it is not so anymore with advent of new sampling devices, culturing and identification tools. This was more so for the presence of fungi. Low temperatures, elevated hydrostatic pressure and low nutrient availability mark the deep sea conditions. Barghoorn and Linder (1944) demonstrated the presence of marine fungi in submerged wood. Fungi from water samples from surface to 4500 m in subtropical oceanic waters (Roth *et al.*, 1964), in shells from deep sea waters of, 4610 m depth (Höhnk, 1969) and in submerged wooden panels at 1615-5315 m depth (Kohlmeyer, 1977) were the earliest reports in deep sea. Poulicek *et al.* (1986) demonstrated mycelial fungi growing inside shells of molluscs at 4800 m depth in the Atlantic. Presence of mycelial fungi in calcareous fragments of shells collected from depths of 300-860 m in the Bay of Bengal was demonstrated (Raghukumar and Raghukumar, 1998). After demonstrating their presence and detection, came the era of culturing them under simulated deep-sea conditions and identifying them by morphological taxonomy and molecular techniques. This was followed by directly isolating DNA from deep-sea sediments and water and amplifying using fungal specific markers. Thus these rapid strides led to several reports on fungi in deep-sea sediments from various oceanic sites.

COLLECTION OF SAMPLES FROM DEEP-SEA

Sterilized Niskin bottles are used for collecting water samples from oceanic depths. Sediments are collected by box corers or multiple corer or long gravity cores (Raghukumar *et al.*, 2010). Box corer are useful when the topography of the ocean floor is flat. Sub-cores are collected from the centre of such a box core with sterile PVC cylinders. Subsections of various lengths can be extruded from these sub-cores using sterilized scalpel into sterile plastic bags to avoid aerial contaminations **(Fig. 1).** Long gravity cores are also similarly used for getting subsections. Precaution to avoid contamination is of utmost importance. This can be monitored on board a research vessel by washing the samplers with a jet of hot water or steam followed by alcohol sterilization. Samples from hydrothermal vent sites are collected with titanium syringes or robotic arms on submersibles (Burgaud *et al.*, 2010).

DIRECT DETECTION AND CULTURING OF DEEP-SEA FUNGI

Seeing is believing. This holds true for proof of fungi in deep-sea, be it sediment, water or calcareous substrata, coral or any other animal shells. Fungal filaments were directly detected in calcareous fragments obtained from deep sea after treating them with EDTA to dissolve calcium carbonate partially followed by staining with fluorescent brightner, Calcofluor white. They were observed under an epifluorescence microscope (Raghukumar and Raghukumar, 1998). Detection of fungi and germinating spores in deep-sea sediments by employing this technique was reported subsequently (Raghukumar *et al.*, 2004; Damare *et al.* ,2006b). Damare *et al.* (2006b) further demonstrated presence of the commonly isolated fungus, *Aspergillus terreus* in deep-sea sediments by immunofluorescence probes developed using polyclonal antibodies. Burgaud *et al.* (2010) reported presence of yeasts in animals from deep-sea hydrothermal vents by using fluorescent *in situ* hybridization (FISH) technique. These studies indicated that detection of fungi in deep-sea materials might remain undetected by conventional microscopy.

ISOLATION OF FUNGI FROM DEEP-SEA SEDIMENTS

It is recommended that fungi are isolated on board the research ship immediately after retrieval of samples from deep-sea to minimize contamination chances from land-based mycota. Strict procedures to avoid aerial contamination

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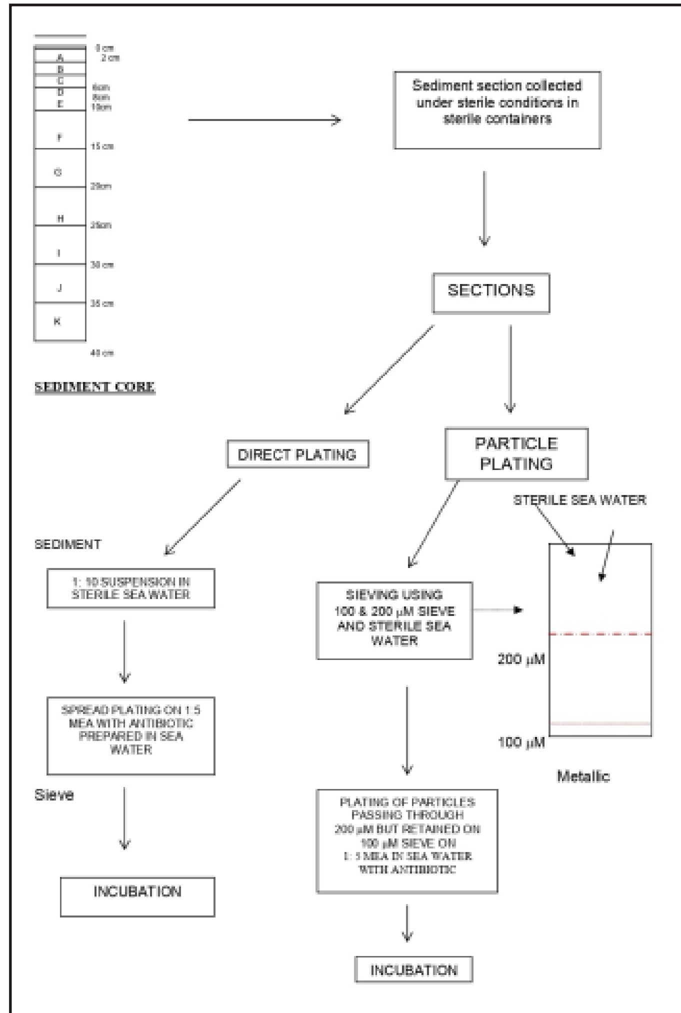

Fig. 1. Schematic diagram showing collection of sediment sample in a core, cutting sections of this core of sediment, collection in sterile bag, plating of sediments in different ways to isolate fungi. Courtesy Samir Damare.

during isolation on board are to be followed. The laboratory should be draft free and working benches or laminar flow hood sterilized with alcohol and all handling of sediments and culture media should be done using laboratory grade hand gloves and around spirit lamp/Bunsen burner. Petri plates with media should be exposed at all handling spaces to check for aerial contaminations. Contaminants that appear on these control plates are to be identified and such strains with identical morphology seen in sampling plates are to be eliminated.

Fungi are isolated by conventional dilution plating method wherein ~0.1 g of sediment is suspended in ambient sterile seawater, vortexed and 100 µl aliquots are spread plated on different media prepared with ambient seawater. Another method employed is particle plating method wherein 1 g of sediment slurry is passed through 200 and 100 µm mesh size screens and particles retained on 100 µm mesh are spread-plated on various media prepared with ambient seawater. For both these techniques the plates are incubated at 5-10º C and 0.1 MPa (1 bar) pressure. Pressure incubation technique

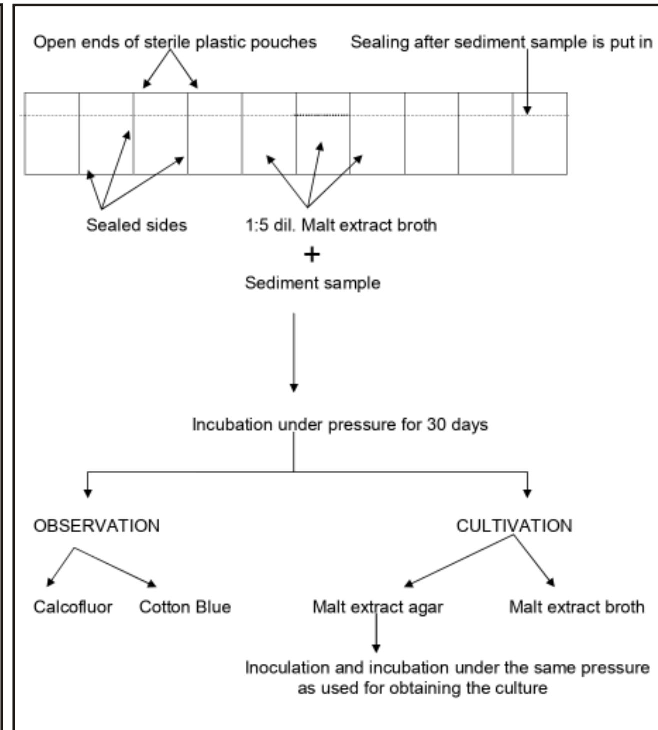

Fig. 2. Schematic diagram showing culturing of fungi in deep-sea culture vessel. Direct observation for the presence of fungi in deep-sea sediments was carried out by staining with Calcoflour or cotton blue. Courtesy Samir Damare.

involves suspending 0.5 g of sediment in 2 ml of seawater malt extract broth in sterile plastic pouches of 4x4 cm size. The open ends of pouches are sealed and suspended in water column in deep-sea culture vessel (Tsurumi &Seike Co., Japan) and pressurised to the required pressure. These are incubated at 5º C for 25-30 days. At the end of incubation period the pressure vessels are depressurized extremely slowly and 100 µl of aliquots are spread-plated in various media and incubated further for 8-10 days at 1 bar pressure, at 5 and 25º C **(Fig. 2)**. Fungal colonies appearing are isolated in pure cultures and maintained for further work. Pressure incubation technique yielded higher percentage of culturable fungi in comparison to other methods **(Table 1).** Newer techniques as discussed by Raghukumar *et al.* (2010) need to be tested for tapping this great reservoir of deep-sea for fungal diversity.

Table 1. Number of fungi isolated by different methods during three cruises in the Central Indian Basin (CIB).

Method used for isolation	Total No. of Sediments samples used	Number of fungi recovered###	Percentage Recovery
Particle plating	532	101	19
Dilution plating	260	50	19
Pressure incubation#	263	94	36
Direct incubation in MEB## at 5° C	153	3	2

#Incubated at 30 MPa pressure; ##malt extract seawater broth; ### Number of distinct morphological types obtained during 3 cruises in the CIB.

GROWTH OF FUNGI UNDER SIMULATED DEEP-SEA CONDITIONS

Pressure vessels were used by Raghukumar and Raghukumar (1998) for culturing fungi from deep-sea sediments. Use of oxygen-permeable polypropylene bags for suspending cultures made oxygen available to cultures during its growth in pressure vessels. Under such conditions several filamentous fungi and yeast from deep sea sediments showed good biomass build up **(Table 2).** A few of the terrestrial fungi also showed biomass build up under hydrostatic pressure of 20 MPa at 5 as well as 30º C (Damare *et al.*, 2006b).

Table 2. Number of fungi and yeast that showed growth under various growth conditions.

Growth conditions	No. of filamentous fungi	No. of yeast
0.1 MPa/30° C	16	12
0.1 MPa/5° C	16	12
20 MPa/30° C	16	12
20 MPa/5° C	16	12
Although all the fungi and yeast showed growth under all the culture conditions, the biomass produced varied from 5 to 55 mg dry weight in 20 ml of culture medium.		

Some of the fungi showed abnormal morphology immediately after initial isolation from deep-sea sediments (Damare *et al*., 2006b). They showed extremely long conidiophores and vesicles covered by long hyphae, instead of phialides of metulae or conidia, as is typical of the genus *Aspergillus*. These features disappeared after few subculturing. Several non-sporulating fungi formed spores after a few subculturing. Some of the fungi showed distinct swellings when grown under simulated deep-sea conditions in malt extract broth but grew normally in sediment extract medium (Damare and Raghukumar, 2008). Two deep-sea fungi showed microcyclic conidiation at 10 MPa hydrostatic pressure (Raghukumar and Raghukumar, 1998) wherein germinating conidium formed further conidia without forming vegetative mycelium. This phenomenon is reported to occur under nutrient limitation and helps fungi in early completion of their life cycle.

Spores of a few deep-sea fungi germinated in sediment extracts of different dilutions at 20 MPa pressure at 30º C, but not at 5º C (Damare *et al.*, 2008). At 5º C spores failed to germinate even at 0.1 MPa after incubation period of 20 days. When initiated with mycelia inoculums, all of these fungi grew and produced biomass at elevated hydrostatic pressure and low temperature, but the spores did not germinate under these conditions. Thus low temperature and not elevated hydrostatic pressure appeared to be a limiting factor for spore germination. Thus, metabolically active fungal mycelia fragments are more tolerant to elevated hydrostatic pressure and low temperature than dormant fungal spores. It appears that mycelia fragments have better chances of survival and propagation in deep-sea conditions than spores.

It can be hypothesized that some of the wind- blown spores and mycelial fragments as well as those landing in deep sea with terrestrial runoffs remain active whereas a greater part remain dormant. These dormant propagules during their ascent to the surface undergo depressurization shock and /or availability of nutrients make them metabolically active. On this line, a 4 m long sediment core from a depth of 5900 m of the Chagos Trench in the Indian Ocean yielded several fungi down to 370 cm length of the core. *Aspergillus sydowii* isolate obtained from the depth of 360 m depth showed growth at 50 MPa pressure and 5º C temperature. Germination of spores of *A. sydowii* at 100, 300 and 500 bar pressure and 5º C temperature confirmed its barotolerance and nativity to deep-sea sediments. The sediment at this depth was estimated to be 0.43 million years old by using radiolarian index (Raghukumar *et al*., 2004). Such fungi provide an opportunity to understand cellular adaptations involved in long-term survival under extreme conditions. Discovery of such paleobes will allow us a virtual time travel to the past and pick up organisms for potential biotechnological explorations.

An orange pigmented yeast cells showed filament formation resulting in pseudomycelia under 20 MPa pressure and 5º C temperature (Damare *et al.*, 2006b). Differentially expressed genes in a psychrotolerant deep-sea yeast, *Cryptococcus* sp subjected to 50 MPa (500 bar pressure prevailing at 5000m depth in the Central Indian Basin) and low temperature of 5º C were analysed using suppression substractive hybridization technique (SSH). A total of 17 and 20 upregulated genes were identified at 50 MPa and 50 MPa/5º C, respectively (Singh *et al.*, 2012c). These differentially expressed genes showed homology to the expressed sequence tags (ESTs) listed in the NCBI database. They coded for proteins involved in arachidonic acid metabolism, amino acid transport and unsaturation of membrane fatty acids demonstrated to assist in survival of microbes under stress conditions. About 50% of the upregulated genes matched with hypothetical proteins at a percentage similarity of $\leq$96, suggesting their probability of being novel. Further detailed analysis of such upregulated genes under deep-sea conditions may elucidate mechanisms adapted by microorganisms for survival.

A deep-sea isolate of the psychrotolerant yeast *Cryptococcus* sp. isolated from polymetalic nodule-bearing sediments of the Central Indian Basin showed considerable growth in the presence of $ZnSO_4$, $CuSO_4$, Pb $(CH_3COO)_2$ and $CDCl_2$ at 100 mg/L concentration at 30 and 15º C (Singh *et al.*, 2013). Scanning electron microscopic images revealed altered cell surface morphology of the cells in the presence of heavy metals in comparison with the controls. Fourier transform infrared spectroscopy (FTIR) and Energy-dispersive X-ray analysis (EDAX) demonstrated adsorption of heavy metals to the cells. Atomic absorption spectrometric analysis demonstrated removal of the heavy metals in the range of 30-90% from the culture supernatant after 4 days of growth at 30º C. Being isolated from polymetallic nodule-bearing sediments, this isolate may have evolved a defensive mechanism to detoxify the environment by bioaccumulation of heavy metals and also developed tolerance to them. Thus such microorganisms need to be explored for their ecological role in deep-sea sediments.

DIVERSITY OF CULTURE-DEPENDENT FUNGI IN DEEP-SEA

Several fungi were isolated from water samples collected from surface to 4500 m depth in subtropical Atlantic Ocean (Roth *et al.*, 1964). Deep sea fungi were observed to grow in wooden panels submered at 1615-5315 m depth (Kohlmeyer, 1997). However, these were not cultured. Poulicek *et al.* (1986) documented mycelial fungi inside shells of molluscs at 4830 m depth in the Atlantic. Raghukumar *et al.* (1992) cultured fungi from surface sterilized calcareous fragments collected from 300-860 m depth in the Bay of Bengal. *Aspergillus ustus* and *Graphium* sp. were the most commonly isolated fungi from calcareous sediments from the Arabian sea (Raghukumar and Raghukumar, 1998). Takami (1999) isolated *Penicillium lagena* and *Rhodotorula mucilaginosa* from a depth of 10,500 m sediment samples of the Mariana Trench in the Pacific Ocean. *Aspergillus sydowii* and several non-sporulating fungi were isolated from a 4 m long sediment core taken from Chagos Trench in the Indian Ocean from a depth of 5900 m (Raghukumar *et al.*, 2004). Damare *et al.* (2006b) reported *Aspergillus* sp. as the most dominant form, followed by several non-sporulating cultures from the Central Indian Basin. Most of these reports used classical morphology-based taxonomy to identify culturable fungi **(Table 3).**

Table 3. Deep-sea fungi isolated from various oceanic regions identified by classical morphology-based taxonomy.

Fungi isolated & (substrates)	Oceanic sites and depths	Reference
Filamentous fungi (water samples)	Atlantic Ocean, 0-4500 m	Roth *et. al*. (1964)
Ascomycetous fungi (wooden panels)	1615-5315 m	Kohlmeyer (1997)
Mycelial fungi (molluscan shells)	Atlantic Ocean, 4830 m	Poulicek *et al*. (1986)
Filamentous fungi (calcareous fragments)	Bay of Bengal, 300-860 m	Raghukumar *et. al*. (1992)
Aspergillus ustus and *Graphium* sp (Calcareous sediments)	Arabian Sea, 800 m	Raghukumar and Raghukumar (1998)
Penicillium lagena, Rhodotorula mucilaginosa (sediments)	Marianna Trench Pacific Ocean, 10,500 m	Takami *et. al*. (1999)
Aspergillus sydowii and other non-sporulating mycelia fungi (4 m long sediment core)	Chagos Trench, Indian Ocean, 5900 m	Raghukumar *et. al*. (2004)
Several mycelia fungi (sediments)	Central Indian Basin, 5000 m	Damare *et. al*. (2006b)

With advent of molecular - based taxonomy, several more fungi were reported from deep sea. Using ITS and 18S sequences of SSU rDNA to identify cultured fungi, Singh *et al*. (2010) reported 16 filamentous fungi and 12 yeast species from the Central Indian Basin. These authors noted that most filamentous fungi belonged to *Ascomycetes*, whereas most of the yeast isolates belonged to *Basidiomycetes*. About 62 filamentous fungi, mostly belonging to ascomycetes were isolated from animals in various deep-sea hydrothermal vent sites (Burgaud *et al.*, 2009). Dupont *et al.* (2009) reported a new genus and species of ascomycete, *Alisea longicola* by analysis of 18S and 28S rDNA sequences and morphological characters. This species was found on sunken wood obtained from Pacific Ocean off Vanuatu Islands. Several yeast and yeast-like fungi were isolated from cold hydrothermal environment and basalt rock surfaces from an active deep-sea volcano, Vailulu'u Seamount, Samoa (Connell *et al.*, 2009). Burgaud *et al*. (2010) obtained 32 isolates of yeasts associated with deep-sea fauna at hydrothermal vents.

In the recent past Zhang *et al.* (2014) obtained 20 culturable phylotypes from a depth ~4000 m in the East India Ocean. Of these 30% are new reports from deep-sea sediments. Several reports on occurrence of Malassezia-like organisms in marine environment have appeared. These organisms are associated from dandruff in human beings to pathogens of marine biota. They are associated with healthy corals and sponges. They may be commensals or latent pathogens waiting for loss of immunity in their hosts or some environmental triggers to become pathogens (Amend, 2014). Rédou *et al.* (2015) investigated culturable fungal community from sediment core at a depth of 1928 meters below sea floor (mbsf) sampled from Canterbury Basin (New Zealand). About 200 filamentous fungi and yeasts, belonging to *Ascomycetes* and *Basidiomycetes* were isolated. Wei *et al* (2018) isolated 106 fungal isolates from 10 sediment samples collected at a depth range of 4545-7068 m in the East Pacific Ocean. These fungi were identified based on morphological characteristics and ITS ribosomal DNA sequencing. These belonged to 12 genera including *Aspergillus, Aureobasidium, Candida, Cladosporium, Cystobasidium, Devriesia, Knufia, Nigrospora, Penicillium, Rhodotorula, Sarocladium* and unclassified *Xylariales*.

Ocean drilling programs (ODP) started recently in collaboration with different institutions has enabled examination of microbial abundance and diversity in sediments collected below oceanic seafloor. Biddle *et al.* (2005) recovered ascomycetous fungi belonging to the genera *Cladosporium, Penicillium* and *Acremonium* spp. by direct plating and by enrichment culturing technique from sediment core collected at 200 mbsf from 252 m water depth on the outer shelf edge of the Peru Margin. They were identified by ITS sequencing **(Table 4)**.

Table 4. Deep-sea fungi isolated from various oceanic sites identified by molecular-based taxonomy. Either ITS, 18S and 28S rDNA sequences or a combination of these sequences were used for identification.

Fungi isolated and substrates	Oceanic sites and depths	Reference
16 filamentous fungi and 12 yeasts (sediments)	Central Indian Basin , 5000 m	Singh *et al*. (2010)
62 filamentous fungi (hydrothermal vent animals)	Various hydrothermal vents	Burgaud *et al*. (2009)
Alisea longicola (sunken wood)	Pacific Ocean	Dupont *et al*. (2009)
Yeast and yeast-like fungi	Cold hydrothermal environment, deep-sea volcano,Vailulu'u Seamount, Samoa	Connell *et al*. (2009)
32 yeasts (hydrothermal vent fauna)	Hydrothermal vent	Burgaud *et. al*. (2010)
20 culturable phylotypes	East India Ocean, 4000 m	Zhang *et. al*. (2014)
Malassezia-like organisms	Several marine environment	Amend (2014)
200 filamentous fungi and yeasts (sediments)	Canterbury Basin, New Zealand, 1928 mbsf	Rédou *et. al*. (2015)
106 fungi 9sediments)	East Pacific Ocean, 4545-7068 m	Wei *et. al*. (2018)
Ascomycetous fungi (sediments)	Peru Margin, 200 m below sea floor (mbsf)	Biddle *et. al*. (2005)

DIVERSITY OF CULTURE-INDEPENDENT FUNGI FROM DEEP SEA

With the advent of accurate, fast and clean techniques to isolate DNA samples from natural habitats and availability of high throughput sequencers, assessment of microbial diversity without culturing has been widely reported. Demonstration of fungi as one of the major groups among microbial eukaryotes in deep sea (Lopez-Garcia, 2001; Edgcomb *et al*. ,2002), by methods employing amplification of sediment DNA with fungal specific primers to study culture-independent fungal diversity gained popularity. Using such techniques Le Calvez *et al*. (2009) reported unsuspected diversity from hydrothermal vent samples. They reported new species in three fungal phyla namely, *Chytridiomycota*, *Ascomycota* and *Basidiomycota*. From deep-sea sediments 37 m below seafloor of the Peru Margin and Peru Trench, Edgcomb *et al*. (2011) recovered fungal sequences from DNA and RNA-based clone libraries. Fungal sequences were recovered from deep-sea methane seeps (Takishita, 2007), gas hydrate-bearing sediments (Cao, 2010). Lai *et al.* (2007) reported several fungal sequences from methane hydrate-bearing deep-sea sediments that are not associated to any known fungi or fungal sequences in public data bases **(Table 5)**.

Table 5. Diversity of culture-independent fungi from deep-sea

Fungi phylotypes reported & substrates	Oceanic site and depth	Reference
Fungal signatures among microbial eukaryotes	Deep-sea sediments	Lopez-Garcia (2001); Edgcomb *et. al.* (2002)
New species from Chytridiomycota, Ascomycota & Basidiomycota	Hydrothermal vent	Le Calvez *et. al.* (2009)
Fungal sequences from DNA & RNA-based clone libraries	37 m below sea floor from Peru Margin & Peru Trench	Edgcomb *et. al.* (2011)
Methane seeps	Sagami Bay, Japan	Takishita (2007)
Gas hydrate-bearing sediments	Ocean drilling program sites in the Pacific	Cao (2010)
New fungal sequences from Methane hydrate-bearing sediments	South China Sea	Lai *et. al.* (2007)
32 fungal taxa (8 of them new) from sediments	Central Indian Basin, ~5000 m	Singh *et. al.* (2011)
20 distinct fungal taxa belonging to Ascomycota and Basidiomycota. & 7 totally new	Central Indian Basin, ~5000 m	Singh *et. al.* (2012a)

Culture-independent fungal diversity was intensely studied at different locations and various depths in the Central Indian Basin (CIB) using multiple primer sets. Singh *et al*. (2011) evaluated fungal diversity at 3 locations (station A, B and C) in the CIB by culture-independent approach. Community DNA isolated was amplified using 3 primer sets namely, fungal-specific ITS (primer pair a), universal ITS (primer pair b), and universal 18S rDNA primer pair (primer pair c). A total of 39 fungal operational taxonomic units (OTU), having 32 distinct fungal taxa were recovered from a total of 768 clones generated from 16 environmental clone libraries. Out of these, 8 sequences appeared to be new having less than 97% similarity with known sequences. A majority of sequences belonged to diverse phylotypes of *Ascomycota* and *Basidiomycota* **(Table 5).** Individual primer set appeared to amplify different fungal taxa occasionally. Out of the total 22 OTUs recovered from primer pair a and b, eight OTUs affiliated with sequences reported from marine environment. Out of these 8 OTUs, three were new sequence types. **(Table 5)**. Fungal taxa were unevenly distributed at different stations, some being exclusively in station A, B or C. Distribution of fungal taxa in stations A, B and C was 10, 21 and 16, respectively, indicating richness of diversity in station B.

In a slightly modified approach Singh *et al.*, (2012a) used sediment sample from one station but used four different primer sets. These were fungal-specific primer set and 3 different universal 18S rDNA primers **(Table 5)**. One environmental library was constructed with each of these 4 primer sets and 48 clones per library were sequenced. These sequences yielded 8 fungal OTUs with ITS and 19 OTUs with 18S rDNA primer pairs, respectively. These OTUs belonged to 20 distinct fungal taxa of the phyla *Ascomycota* and *Basidiomycota*. Seven sequences showed divergence by 79-97% from the known sequences in the existing data base and therefore appear to be novel. Phylogenetic affiliation of a few sequences with known environmental sequences from marine and hypersaline habitats suggest their autochthonous nature or adaptation to marine habitat. Amplification of fungal sequences with eukaryotic as well as fungal-specific primers indicates that among eukaryotes, fungi appear to be a dominant group in the sampling site.

None of the above work reported presence of sequences that clustered close to known taxa belonging to *Zygomycota* and *Chytridiomycota*. Cathrine and Raghukumar (2013) pointed out that the taxonomic position of fungal sequences that clustered close to known fungal taxa could be clearly identified. However, many of the environmental sequences clustered away from the known fungal taxa and grouped to form environmental clades. Analysis of these environmental sequences shows that a few distinct environmental clusters can be identified within *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota*.

FUNGAL DIVERSITY BY SIMULTANEOUS CULTURE-DEPENDENT AND CULTURE-INDEPENDENT APPROACH

Singh *et al.* (2012b) further investigated fungal diversity in two deep-sea sediment cores collected at a depth of ~40 cm below sea floor of CIB by adopting culture-dependent and culture-independent approach. Partial region of 18SrDNA of pure fungal isolates was PCR amplified with NS1 and NS4 (White *et al.*, 1990). Sediment DNA samples were amplified using fungal specific ITS primer and universal 18S rDNA primer sets. A total of eight environmental clone libraries from (2 sediment cores x 2 depths each x 2 primer sets each) were constructed and 48 clones were screened from each of these libraries. They recovered 19 culturable fungi and 46 OTUs, respectively **(Table 6)**. Some of the fungi such as *Cerrena, Hortaea* and *Aspergillus* sp. were recovered by culture-dependent as well as culture-independent approaches. Eleven OTUs from environmental libraries showed high divergence (86-97%) from the existing sequences in the Genbank (NCBI database). These two approaches together detected a total of 12 distinct fungal genera and 42 OTUs, respectively from two sediment cores suggesting presence of high fungal diversity in deep-sea sediments.

Table 6. Fungal diversity in deep-sea by simultaneous culture-dependent and culture-independent approaches. Cultured fungi were identified on the basis of 18S rDNA gene sequences. Phylogenetic affiliations of the OTUs obtained using primer set NS1/NS2 (of 18S rDNA gene sequences) were compared.

Cultured fungi (closest identified relative)	Fungal operational taxonomic units (OTUs)
Nigrospora oryzae	Uncultured soil ascomycete (3)
Cladosporium sp	Uncultured fungus clone (2)
Trametes versicolor	Uncultured *Aspergillus* clone
Chaetomium elatum	*Phialosimplex caninus*
Aspergillus versicolor (2)	Uncultured marine non-fungal eukaryote
Ascotricha lusitanica	*Saccharomyces* sp.
Pleaospora herbarum	Uncultured marine fungus clone
Cladosporium sp.	*Pycnosporus* sp.
Eurotium herbariorum	Uncultured *Malassezia* clone
Cerrena sp. (4)	*Sterigmatomyces halophilus*
Penicillium griseofulvum (2)	*Dothideomycete* sp
Sagenomella sp	*Cerrena unicolor*
Hortaea werneckii (2)	

Numbers within brackets denote number of isolates/OTUs obtained.

ROLE OF FUNGI IN THE DEEP-SEA HABITAT

Besides bacteria, major role of fungi is in biotransformation of organic matter in terrestrial environment. However, the role of fungi in deep-sea is mostly hypothesized without concrete evidence. Damare and Raghukumar (2008) demonstrated that besides biodegradation fungi are important in forming aggregates in deep sea. These aggregates help in binding the soil, prevent leaching of enzymes and may serve as food to the detritus-feeding animals in deep sea. Fungi produce a large amount of exopolysaccharides which help in binding the sediment and also offer a major source of food to detritivores. Melanin and chitin, the polymers of fungal cell walls are not easily degradable and thus, fungus-mediated C storage is more persistent than that sequestered by bacteria (Bailey *et al.*, 2002). Several of the fungal sequences and Malassezia-like organisms are known to be animal parasites (Nagano *et al*., 2010) and may impact host population in deep-sea and hydrothermal vents. Some of them may be symbionts and have stimulating effects on host defence responses (Domart-Coulon *et al*., 2004). Nagano *et al*. (2010) reported 14 OTUs belonging to DSF-1 group dominating in oxygen-depleted environment like methane cold seeps. These might be anaerobic fungi or facultative anaerobes. Siderophore-producing and heavy metals tolerant yeasts in deep sea may play an active role in biomineralization processes (Connell *et al*., 2009, Singh *et al*., 2013). Methylotrophic yeasts reported in methane seeps (Lai *et al.*, 2007) may be used as biomarker for tracing methane seeps in deep-sea. Several extracellular enzymes produced by fungi may be used as indicators of nutrient cycling processes. Alkaline phosphatase activity of fungi play an important role in regeneration of inorganic phosphate through its catalysis to organic esters to inorganic P (Chróst, 1991; Raghukumar *et al.*, 2010). Damare *et al*. (2006a) demonstrated that 11% of the total of 221 fungi isolated from deep-sea sediments of the CIB produced low-temperature active alkaline protease. Miura *et al*. (2001) purified two novel endopolygalacturonases active at 0-10º C from a deep-sea yeast isolated from the Japan Trench at a depth of 4500-6500 m. Deep-sea fungi have added new dimension to the discovery of natural products too (Petit, 2011).

CONCLUSION

As is evident from this discussion, studies on deep-sea fungi from cold has become a hot topic. A vast biodiversity of fungi from cultured and culture-independent approaches is being reported from different oceanic regions. Understanding their role in ecological processes and potential biotechnological applications are pursued in great detail.

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