

Marine fungal biotechnology: an ecological perspective

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This paper reviews the potential of marine fungi in biotechnology. The unique physico-chemical properties of the marine environment are likely to have conferred marine fungi with special physiological adaptations that could be exploited in biotechnology. The emphasis of this review is on marine fungi from a few unique ecological habitats and their potential in biotechnological applications. These habitats are endophytic or fungi associated with marine algae, seagrass and mangroves, fungi cohabiting with marine invertebrates, especially corals and sponges, fungi in marine detritus and in marine extreme environments. It is likely that microorganisms, including fungi may be the actual producers of many bioactive compounds reported in marine plants and animals. Fungi occurring in decomposing plant organic material or detritus in the sea have been shown to be source of several wood-degrading enzymes of importance in paper and pulp industries and bioremediation. One of the major applications of the thraustochytrids occurring in marine detritus and sediments is the production of docosahexaenoic acid (DHA), an omega-3 fatty acid used as nutraceutical. The deep-sea, an extreme environment of high hydrostatic pressure and low temperatures, hydrothermal vents with high hydrostatic pressure, high temperatures and metal concentrations and anoxic marine sediments are some of the unexplored sources of biotechnologically useful fungi. An understanding of the adaptations of extremotolerant fungi in such habitats is likely to provide us a greater insight into the adaptations of eukaryotes and an avenue from which to discover novel genes.

Key words: deep sea, detritus, endophytes, enzymes, fungi, hypoxic sediments, bioremediation, biotechnology

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Introduction

Fungal biotechnology or ‘mycotechnology’, has advanced considerably in the last five decades. Terrestrial fungi are used in the production of various extracellular enzymes, organic acids, antibiotics and anti-cholesterolemic statins (Pointing and Hyde, 2001). They have been used as expression hosts as well as a source of new genes. With modern molecular genetic tools, fungi have been used as “cell factories” for heterologous protein production (Punt *et al.*, 2002) and human proteins (Bretthauer, 2003). What prospects do marine fungi offer in such a scenario? How do marine fungi compare with terrestrial fungi that have become an important target group for pharmaceuticals and enzymes (Bull *et al.*, 2000)? In this review uniqueness of marine fungi and their potential in the area of

marine mycotechnology is discussed. The focus of this review is oriented towards fungi in special ecological niches, as a basic understanding of the ecology would help to reveal the novelty of an organism and its properties. The emphasis is on i) fungi associated with or endophytic fungi in marine algae, seagrasses and mangroves and their implications ii) fungi associated with marine invertebrates especially corals and sponges and their potential towards production of bioactive molecules iii) fungi from extreme environments such as the deep sea with elevated hydrostatic pressure and low temperatures, hypersaline waters of the Dead Sea and anoxic or hypoxic (oxygen deficient) sediments from the marine environment.

What are marine fungi?

Marine fungi form an ecological, and not

a taxonomic group (Hyde *et al.*, 2000). Among these, the obligate marine fungi grow and sporulate exclusively in sea water, and their spores are capable of germinating in sea water. On the other hand, facultative marine fungi are those from fresh water or a terrestrial *milieu* that have undergone physiological adaptations that allow them to grow and possibly also sporulate in the marine environment (Kohlmeyer and Kohlmeyer, 1979). About 800 species of obligate marine fungi belonging to the Fungi have been reported so far (Hyde *et al.*, 2000). These belong mostly to ascomycetes (Fig. 1B), the anamorphs (Fig. 1A) and a few basidiomycetes. Among the straminipilan fungi, those belonging to *Labyrinthulomycetes*, comprising the thraustochytrids, aplanochytrids, and labyrinthulids (Fig. 2A, B, C) are obligately marine (Raghukumar, 2002) and those belonging to the oomycetes are also fairly widespread in the marine environment.

Marine fungi are most common in decomposing wood (Fig. 3A) and plant detritus in coastal waters (Kohlmeyer and Kohlmeyer 1979; Newell, 1996; Hyde *et al.*, 2000; Raghukumar, 2004; Sridhar, 2005), are also common in calcareous animal (Fig. 3B) shells (Raghukumar *et al.*, 1992), algae (Raghukumar, 2006) and corals (Golubic *et al.*, 2005). They have been isolated from deep-sea sediments (Fig. 3C. Damare *et al.*, 2006) and detected in anoxic marine sediments (Stoeck *et al.*, 2003).

Unique features of marine environment and their relevance to marine fungi

A consideration of the unique properties of the marine environment is important for marine biotechnology for several reasons: 1) A good understanding of the ecosystem will help prospect for novel genes and 2) biotechnological production processes are influenced by the special adaptations of organisms to their environment. The physical factors that influence the marine fungi most are a) salinity and pH, b) low water potential, c) high concentrations of sodium ions, d) low temperature, e) oligotrophic nutrient conditions and f) high hydrostatic pressure, the last three parameters being unique to the deep-sea environment.

(a) Sea water on an average has a salinity of 33-35 ppt. Fresh water in compa-

ison contains less than 0.05% salts (0.5 ppt). Far more saline than the sea are hypersaline waters such as those in the Dead Sea, containing 5-10% salts or salinities of 50 to 100 ppt.

- (b) Low water potential is one of the problems posed by seawater. Organisms living in it therefore, need to maintain water potentials lower than that of seawater in their cells to enable water uptake. Marine fungi maintain this gradient by accumulating osmolytes such as glycerol, mannitol, polyol and trehalose (Blomberg and Adler, 1992). Marine yeasts produce glycerol to maintain their internal osmotic potential in response to increased salinity (Hernández-Saavedra *et al.*, 1995).
- (c) The presence of high levels of sodium ions in seawater also confers some unique properties to the cells of organisms living in the sea. Sodium, even in small concentrations is toxic to most of the living cells in the terrestrial and freshwater environments. Many marine fungi are known to reduce the toxicity of sodium ions by sequestering them in vacuoles (Jennings, 1983) or have a very efficient sodium efflux ATPase (Benito *et al.*, 2002). The straminipilan fungi, the thraustochytrids and labyrinthulids on the other hand have an absolute requirement for sodium for their growth and sporulation (Jennings, 1986). Thus, these two groups are excellent tools for understanding the physiology of growth and enzyme production in the presence of sodium.
- (d) Terrestrial fungi generally grow best at pH 4.5-6.0, whereas facultative marine fungi were demonstrated to grow and produce various extracellular enzymes at pH 7-8 (Raghukumar *et al.*, 1994; 1999; 2004, Damare *et al.*, 2006b). Lorenz and Molitoris (1992) demonstrated that salinity optimum for growth in some marine fungi show upward shift with increasing incubation temperature. This interaction of salinity and temperature is termed *Phoma* pattern since it was first described in the marine species of *Phoma*.

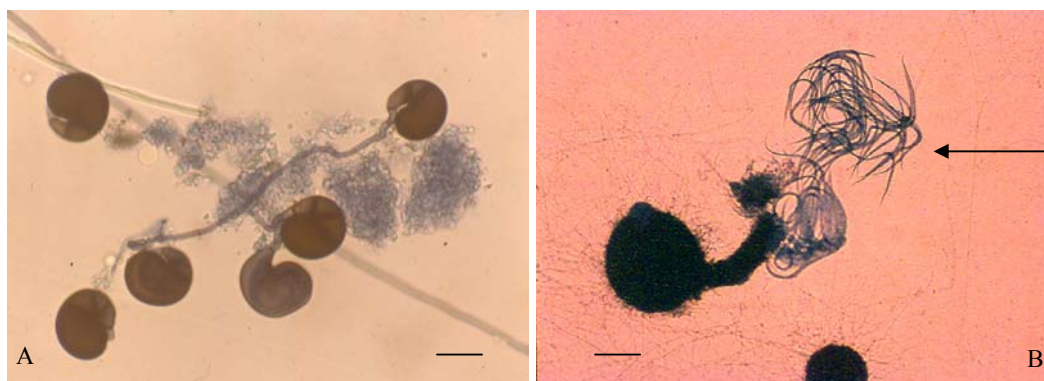


Fig. 1. **A.** *Cirrenalia pygmae*, an anamorphic taxon isolated from decaying mangrove wood. **B.** *Lindra thalassiae*, an ascomycetous fungal pathogen on the brown alga *Sargassum cinerea*. Note the thin filamentous ascospores emerging from the fruit body (arrow). Bars = 10 μ m.

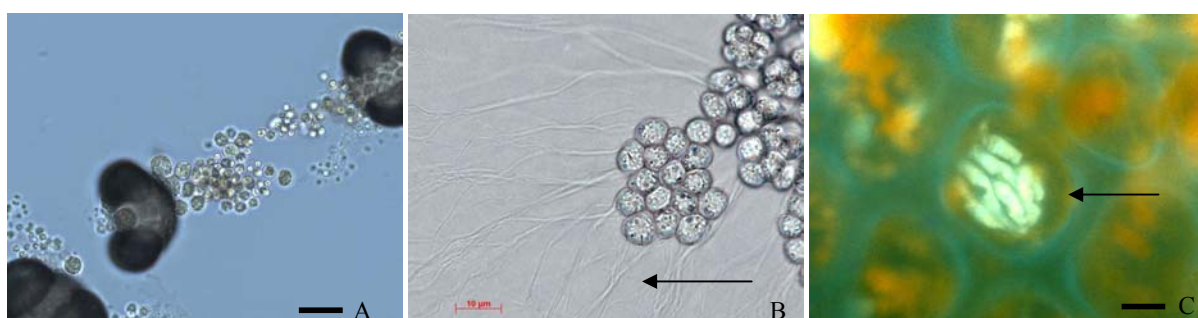


Fig. 2. **A.** *Thraustochytrium aggregatum* growing on pine pollen which is normally used as bait to isolate these thraustochytrids fungi from marine habitats; bar = 20 μ m. **B.** *Aplanochytrium* sp. in culture showing a network of ectoplasmic net elements which are extension of plasma membrane and serve to increase the surface area. **C.** Epifluorescence picture showing cells of *Labyrinthula* sp. (arrow) in the necrotic lesion of the sea grass *Thalassia hemprichii*. The chlorophyll appears red due to autofluorescence; Bars: A = 20 μ m; B-C = 10 μ m.

- (e) Growth of fungi on microscope lenses, contact lenses and glass slides in terrestrial environments is indicative of oligotrophic existence of terrestrial fungi. In the water column, the organic nutrient levels are comparatively low and are mostly in steady state. Fungi have not been observed to grow freely in water columns. However, fungi probably form microcolonies in marine sediments.

Marine endophytic fungi

Endophytic organisms are mostly fungi, bacteria and actinomycetes that live in the intercellular spaces of plant tissue and cause no apparent damage to their host (Strobel, 2002; Sánchez Márquez *et al.*, 2007; Wei *et al.*, 2007a). Endophytic fungi have been isolated and successfully cultured from several terrestrial plants (Hu *et al.*, 2007; Wei *et al.*, 2007b)

and have even been isolated from lichens (Li *et al.*, 2007). Some of these endophytes produce bioactive substances that are products of the host-microbial interactions (Tejesvi *et al.*, 2007). The most famous example being that of taxol, a multibillion dollar anti-cancer compound produced in yew plant *Taxus brevifolia* by the terrestrial endophytic fungus *Taxomyces andreanae* (Strobel, 2002).

These exciting discoveries with terrestrial plants make one wonder if marine algae, sea grasses (the only angiospermic plants that grow submerged in the sea) harbor endophytic fungi that produce secondary metabolites. Kobayashi and Ishibashi (1993) are of the opinion that marine endophytic fungi may have potential to produce different types of compounds from those of terrestrial sources. Several fungi isolated as endophytes from marine algae and plants have been found to produce interesting secondary metabolites (Table 1). To cite one example, a novel polyketide ascosalipyrro-

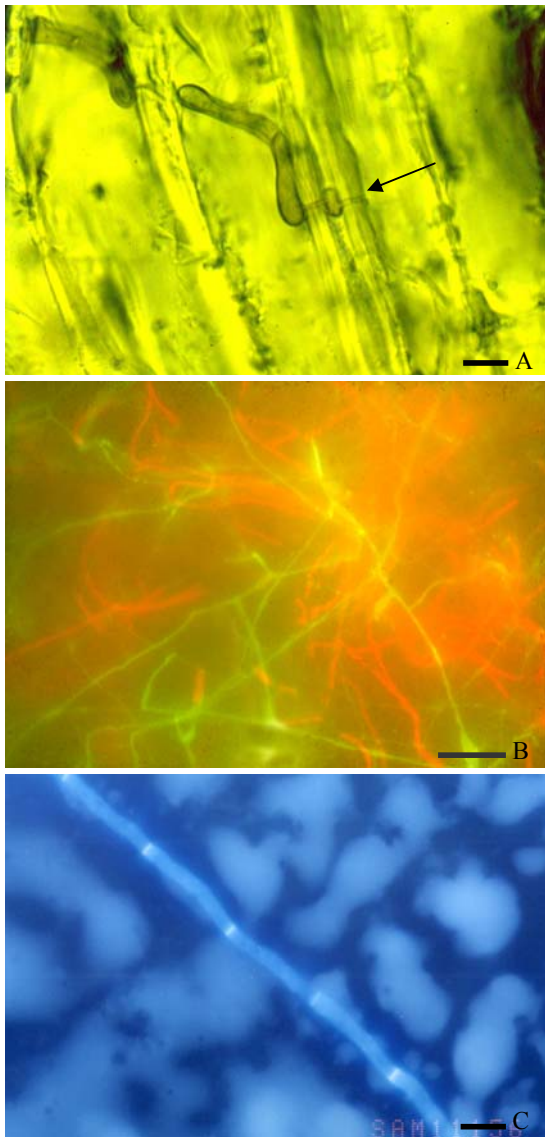


Fig. 3. **A.** A fungal hypha penetrating the cell walls in mangrove wood. Note the thinning of hypha while passing through the cell wall (arrow) and broader hyphae in the lumen. **B.** Endolithic fungi (green colored) and cyanobacterial filaments (red colored due to autofluorescence of chlorophyll pigment) growing inside a window pane oyster shell. They were observed under an epifluorescence microscope. **C.** A fungal filament seen after staining the deep-sea sediments with the optical brightener Calcofluor. Bars = 10 μ m.

lidinone-A was isolated from an obligate marine fungus *Aschochyta salicorniae* associated with the marine green alga *Ulva* species (Osterhage *et al.*, 2000). This compound showed anti-plasmodial activity toward *Plasmodium falciparum* strain K1, a strain resistant to chloroquinone, and strain NF 54, susceptible to standard anti-malarials.

Several marine plants produce interesting secondary metabolites. This has led to the discovery of several antibacterial, antifungal, antimalarial, antitumor, antiviral and several such pharmacologically useful compounds from them (Mayer and Hamann, 2004). However, there is no information whether these plants have endophytic fungi associated with them and if these are responsible for the production of such novel secondary metabolites.

There are several interesting algal-fungal associations which have not been investigated for bioactive metabolites. Several marine algae from the Indian coast harbor endophytic fungi (Raghukumar, 1996). The green filamentous algae *Cladophora* and *Rhizoclonium* species from various localities of the Indian coast always harbor the polycentric chytrid *Coenomyces* sp. The algae do not show any external symptoms of fungal infection. Only on incubation in sterile sea water, do fungal sporangia emerge from the algal filaments (Raghukumar, 1986). Attempts to culture this fungus in artificial media were unsuccessful. It is not known whether this fungus resides as an endophyte in these algal species. Several filamentous fungi and thraustochytrids were isolated from surface-sterilized green algae *Ulva fasciata* and *Valoniopsis pachynema*, the brown algae *Sargassum cinereum* and *Padina tetrastomatica* and the red algae, *Centroceras clavulatum* and *Gelidium pusillum* (Table 2, Raghukumar *et al.*, 1992). Growth of fungi always arose from the cut edges of algal discs and never on the surface indicating that actively growing hyphae of fungi were present within the healthy algae. It is possible that these fungi are endophytes in algae. In order to prove this, routine temporal and spatial isolation of such fungi from marine algae should be carried out. Thraustochytrids and labyrinthulids are often isolated from marine algae. A labyrinthulid, *Aplanochytrium minutum* was isolated from the brown algae *Sargassum cinereum* and *Padina tetrastomatica*, where the algae did not display any disease symptom (Sathe-Pathak *et al.*, 1993). The labyrinthulid could also be detected within the tissues of the host plant. *Labyrinthula* sp was also reported from *Cladophora*, several other green algae and in seagrass (Schmoller and Wieggershausen, 1969; Raghukumar, 1987).

Table 1. Some examples of secondary metabolites produced in fermentation broth from marine endophytic fungi.

Host	Fungus	Secondary metabolite	Activity/application	Reference
Marine algae				
Red alga (<i>Actinotrichia fragilis</i>)	<i>Penicillium citrinum</i>	Alkaloid	Anti-cancer compound	Tsuda <i>et al.</i> , 2004
Green alga (<i>Codium fragile</i>)	<i>Fusarium</i> sp.	Cyclic tetrapeptide	Anti-cancer	Ebel, 2006
Red alga (<i>Polysiphonia violacea</i>)	<i>Apiospora montagnei</i>	Diterpene	Activity against human cancer cell lines	Klemke <i>et al.</i> , 2004
Seagrasses				
<i>Halodule wrightii</i>	<i>Scytalidium</i> sp.	Hexa peptide	Inhibitor of Herpes simplex virus	Rowley <i>et al.</i> , 2003
Mangrove plants <i>Kandelia candel</i>	Unidentified endophytic fungus	Lactones	Active against bollworm and parasitic copepods	Chen <i>et al.</i> , 2003

Table 2. Number of fungi isolated from 10 mm² area of surface-sterilized (SS) and non-surface-sterilized (NS) algae.

Algae	Treatment	Mycelial fungi	Yeasts	Thraustochytrids
Ulva fasciata	NS	3	171	0
	SS	4	0	0
<i>Valoniopsis pachynema</i>	NS	4	0	0
	SS	7	0	0
<i>Centroceras clavulatum</i>	NS	36	106	0
	SS	14	4	0
<i>Gelidium pusillum</i>	NS	8	0	14
	SS	4	0	39
<i>Sargassum cinereum</i>	NS	4	0	7
	SS	1	0	43
<i>Padina tetrastomatica</i>	NS	2	0	20
	SS	2	0	16

Source: Raghukumar *et al.*, 1992

Do such host algae produce special compounds that act as defence chemicals against grazing animals such as limpets and periwinkles (Bakus *et al.*, 1986)? This raises the question whether fungi, labyrinthulids and thraustochytrids play a mutualistic role in algae. Kohlmeyer and Kohlmeyer (1979) described such mutualistic association between fungi and algae as mycophycobiosis.

Several endophytic fungi have been isolated and cultured from the mangrove plants *Rhizophora apiculata* and *Dendrophthoe falcate* (Kumaresan *et al.*, 2002). Do these fungi induce the host plants to produce novel secondary metabolites? Similarly, about 26 fungi are reported as endophytes in the seagrasses *Thalassia testudinum*, *Zostera marina*

and *Z. japonica* (Alva *et al.*, 2002). Are any antiforaging compounds produced in seagrasses and mangrove plants harbouring the endophytic fungi and if so, they need to be investigated in detail.

Animal-fungal associations

Several examples of insect-fungal associations in terrestrial ecosystem are known. These include the mound-building termites and the mushroom *Termitomyces*, the leaf-cutting ants (the Attine ants) and their fungal gardens and ambrosia beetles and their fungal symbionts (Breznek, 2004). Enormous amount of research on biological control of insect pests using entomogenous fungi and their secondary

metabolites has been reported (Gillespie and Claydon, 1989 and the references therein).

Association of fungi with marine animals ranges from saprotrophic to symbiotic to parasitic. Saprotrophic fungi, some of them being common terrestrial fungi have been isolated from the surface, guts and coelomic fluids of holothurians or the sea cucumbers (Pivkin, 2000).

Several fungi isolated from invertebrates have been found to produce interesting secondary metabolites. A novel group of platelet-activating factor (PAF) antagonists, phomactins A, B, B1 and B2 were isolated from culture broth of *Phoma* isolated from the shell of a crab *Chionoectes opilio* collected off the coast of Fukui, Japan (Sugano *et al.*, 1991). Two lipophilic tripeptides from *Penicillium fellutanum* living inside the gastrointestinal tract of a marine fish and three quinazoline derivatives from *Aspergillus fumigatus* from gastrointestinal tract of the fish, *Pseudolabrus japonicus* from the coast of Japan were described (Kobayashi and Ishibashi, 1993). An endolithic fungus *Ostracoblabe implexa* living inside the shells of rock oyster *Crassostrea cucullata* from the coast of Goa was reported (Raghukumar and Lande, 1988). It will be interesting to examine if this fungus, which can be easily cultured in organic media produces such novel bioactive molecules. Fungal metabolites effective against arthropods and insects should be tested against mites, that cause havoc in fungal culture collections. This will be a great service to mycological culture collection centres. Strong miticides of well known brands are available for agriculture and horticultural crops but these are too toxic to be used in a closed laboratory atmosphere.

Several invertebrates produce novel secondary metabolites. Holothurians or sea cucumbers produce triterpene glycosides which show fungitoxic, hemotoxic and cytotoxic activities. Sponges are also known for production of several bioactive molecules with antifungal, antibacterial, antimalarial and antiviral activities (Mayer and Hamann, 2004). Marine sponges, the natural biofermentors of microorganisms harbour a variety of microorganisms such as bacteria, fungi (Namikoshi *et al.*, 2002) and thraustochytrids (Rinkevich, 1999). Several bioactive compounds in sponges

are assumed to be produced mostly by symbiotic bacteria or actinomycetes (Kobayashi and Ishibashi, 1993; Piel *et al.*, 2004). Jensen and Fenical (2000) have described 10 compounds from sponge-derived fungi. Several mycelial fungi isolated from sponges and other marine animals yielded not only new natural products, but also compounds identical or related to those formerly attributed to their hosts (Proksch *et al.*, 2003). For any endosymbiotic association, it is necessary to reconfirm by direct visualization of the alleged symbionts inside their hosts. A recent report on the detection of an endosymbiotic yeast in a sponge by transmission electron microscopy and immunocytochemical labelling of β -1,4-N-acetyl-D-glucosamine residues of chitin walls, a fungal signature confirmed its presence (Maldonado *et al.*, 2005). Interestingly these yeasts are reported to be maternally transmitted from the soma through the oocytes to the fertilized eggs. Bioactive molecules from such sponge-fungal endosymbiosis will be worth investigating.

There are several interesting associations of fungi with marine animals which have not been investigated. Fungi belonging to the class of Trichomycetes are found in the guts of marine arthropods, isopods, decapods and amphipods in a symbiotic association (Misra and Lichtwardt, 2000). Endolithic fungi in coral skeleton are common (Le-Campion-Alsumard *et al.*, 1995; Ravindran *et al.*, 2001; Golubic *et al.*, 2005). They often intermingle with endolithic algae, frequently parasitizing the latter (Le-Campion-Alsumard *et al.*, 1995). A fungal species taxonomically similar to the terrestrial aspergilli coexists with live polyp tissues of the coral *Porites lobata* on Moorea island near Tahiti, French Polynesia (Bentis *et al.*, 2000). A basidiomycetous yeast *Cryptococcus* sp associated with *Pocillopora damicornis* coral skeleton was shown to produce a transient cryoprotective effect, selectively enhancing the survival of skeletogenic cell types (Domart-Coulon *et al.*, 2004). A *Scolecobasidium* species was isolated from necrotic patches in several massive corals in the Andamans (Raghukumar and Raghukumar, 1991). Do these endolithic fungi produce bioactive molecules to combat defence reactions of coral polyps and against other endolithic

microorganisms? As many of these fungi are culturable, they can be easily tested for bioactive molecules and examined if similar molecules are also produced inside their hosts.

Detritus-associated fungi

Processes involved in the detritus formation are crucial to the remineralization processes in the marine ecosystem and detritus, the dead organic matter with its associated microbes is an important link of marine food web to the detritivores feeding on them (Mann, 1988). Detritus from coastal marine macrophytes, particularly mangroves, contribute an enormous amount of organic matter to the adjacent waters (Wafar *et al.*, 1997). The major role of microorganisms in detrital processes is the biochemical transformation of the detritus by production of extracellular degradative enzymes and these properties can be harnessed for biotechnological applications.

Extracellular degradative enzymes such as cellulases, xylanases and ligninases of several terrestrial fungi have found biotechnological applications in paper and pulp industries (Eriksson, 1993). In the production of paper, residual lignin from wood pulp is chemically liberated by using chlorine bleaching. Elemental chlorine reacts with lignin and other organic matter in the pulp, forming chlorinated lignin derivatives such as chloro-lignols. These are toxic, carcinogenic and recalcitrant to degradation. Biobleaching is an important alternative to reduce the use of chlorine. This is carried out using ligninolytic enzymes manganese peroxidase and laccase or by using hemicellulolytic enzyme xylanase (Eriksson, 1993). Microbial xylanases that are thermostable, active at alkaline pH and cellulase-free are generally preferred for biobleaching of paper pulp.

Gessner (1980) demonstrated that filamentous fungi isolated from the salt marsh grass *Spartina alterniflora* and other salt marsh substrata were capable of degrading cellulose, cellobiose, lipids, pectin, starch, xylan and tannic acid. Wainright (1980) and Schaumann and Weide (1990) have demonstrated alginate-degrading enzyme production in several marine fungi. A great diversity of fungi were isolated from decaying mangrove and seagrass leaves

(Fell and Newell, 1981; Coumo *et al.* 1985). Marine fungi isolated from various stages of decomposing brown alga *Sargassum cinereum* from a sampling site in Goa were shown to grow on detritus of the same alga suspended in sea water (Sharma *et al.*, 1994). These fungi produced cellulase, xylanase, polygalacturonase, amylase, alginate hydrolase and laminarinase in the detritus medium. Raghukumar *et al.* (1994) investigated degradative enzyme production by fungi isolated from detritus of the leaves of the mangrove *Rhizophora apiculata*. Cellulases were produced by all the fungi isolated, while some produced xylanase, pectin lyase, amylase and protease when grown in detritus of *R. apiculata*. A marine-derived fungus NIOCC #3 isolated from mangrove detritus produced xylanase that was thermostable at 55°C, cellulase-free, and active at pH 8.5 and was found to be effective in biobleaching. The crude culture filtrate containing 58 U l⁻¹ of xylanase could bring about bleaching of sugarcane bagasse pulp by a 60 minutes treatment at 55°C, resulting in a reduction of 30% chlorine consumption during bleaching (Raghukumar *et al.*, 2004).

An enormous amount of work is reported from lignin degrading enzymes from terrestrial white-rot fungi (Basidiomycetes). These fungi are well known for their lignin-degrading enzymes, the most common being lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Reddy, 1995). These fungi and their enzymes have been used for decolorization of bleach plant effluent from pulp and paper mill, effluent from textile and dye-making industries and molasses spent wash from alcohol distilleries (Pointing, 2001). Colour removal from these effluents is a major challenge and therefore most of the mills dilute the effluent several times before disposal. A large quantity of freshwater is thus wasted for this purpose and in its absence effluents are clandestinely disposed off without treatment. Several of these effluents have alkaline pH and high salt content and therefore, marine fungi may be ideally suited for the bioremediation of such effluents (Raghukumar, 2002).

A white-rot fungus NIOCC #312 (*Flavodon flavus*) isolated from detritus of the sea grass *Thalassia hemprichii* and two ascomycetous fungi *Sordaria fimicola*, (NIOCC#298

Table 3. Detoxification tests carried out with fungus-treated molasses spent wash from alcohol distillery. The fungus used was the white-rot basidiomycete *Flavodon flavus* (NIOCC # 312).

Toxicity tests used	Untreated MSW	Fungus-treated MSW
Serum sorbitol dehydrogenase (SSDH)	122 U L ⁻¹	2.6 U L ⁻¹
Comet assay (to assess damaged nuclei)	85 %	9 %
Benzo (o) pyrene (a PAH) concentration	3.8 µg mL ⁻¹	1.2 µg mL ⁻¹

PAH= polycyclic aromatic hydrocarbon

Table 4. Decolorization of colored effluents by the fungal biomass, culture supernatant and EPS of the white-rot basidiomycetous fungus NIOCC # 2a isolated from decaying mangrove wood.

Percentage decolorization obtained when added to the culture			
Days	2	4	6
Molasses spent wash	22	80	100
Black liquor	40	50	50
Textile effluent A	30	32	34
Textile effluent B	60	62	62
Percentage decolorization obtained with the culture supernatant containing 18 U mL ⁻¹ of laccase			
Hours	6	12	
Molasses spent wash	34	33	
Black liquor	71	59	
Textile effluent A	9	11	
Textile effluent B	14	22	
Percentage decolorization obtained when treated with 10 mg of exopolymeric substance (EPS) of the fungus			
Hours	12	24	
Molasses spent wash	12	100	
Black liquor	35	100	
Textile effluent A	12	100	
Textile effluent B	41	100	

Table 5. Comparing dye-decolorization activities of NIOCC # 312, isolated from decaying seagrass with NIOCC # 2a, isolated from decaying mangrove wood.

Dyes/Pollutant	(NIOCC#312) % Decolorization on day 3	fungus (NIOCC # 2a)
Azure B (0.02 %)	55	Nt
Brilliant Green (0.02 %)	45	100
Congo Red (0.02 %)	60	90
Crystal Violet (0.02 %)	10	15
Poly B (0.02 %)	74	Nt
Poly R (0.02 %)	50	30
Remazol Brilliant Blue R (0.02 %)	80	60*
Black liquor (10 %)	75	40
Molasses spent wash (10 %)	75	80

Cultures were grown in low nitrogen medium prepared with half-strength sea water.

from mangrove sediment) and *Halosarpheia ratnagiriensis* (NIOCC #321 from mangrove wood) decolorized bleach plant effluent (BPE) from paper and pulp mill industries to a great extent (Fig. 4). The culture NIOCC # 312 was able to decolorize the bleach plant effluent at pH 4.5 as well as at 8.5. It produced all the three lignin degrading enzymes namely LiP, MnP and laccase whereas, the other 2 fungi

produced laccase as the major lignin-degrading enzyme and MnP to a lesser extent (Raghukumar *et al.*, 1994). These authors showed laccase production in the presence of sea water by several marine fungi. Pointing *et al.* (1998) showed laccase activity in several marine fungi. Such salt-tolerant lignin-degrading enzymes have not been sufficiently explored for their biotechnological applications.

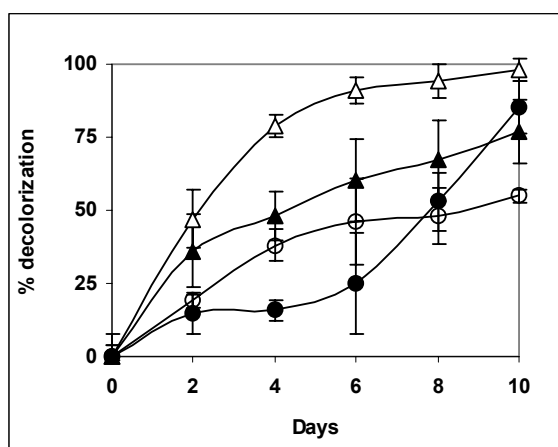


Fig. 4. Percent decolorization of bleach plant effluent (BPE) by \triangle NIOCC # 312 (a white-rot fungus grown in the presence of bleach plant effluent at pH 4); \blacktriangle NIOCC # 312 grown in the presence of bleach plant effluent at pH 8.5; \circ NIOCC # 298 (*Sordaria fimicola*, an ascomycetous fungus); \bullet NIOCC # 321 (*Halosarpheia ratnagiriensis*, an ascomycetous fungus) grown in the presence of bleach plant effluent at pH 4.

Besides paper mill effluent and molasses spent wash from the alcohol distillery and several synthetic dyes were also effectively decolorized by marine fungi (Raghukumar, 2002). The fungus NIOCC #312 not only decolorized molasses spent wash (MSW) from alcohol distillery but also detoxified it (Raghukumar *et al.*, 2004). Toxicity testing using the comet assay of the fungus-treated molasses spent wash using an estuarine fish *Oreochromis mossambicus* showed no liver damage (Table 3) in contrast to untreated effluent, which showed significant liver damage. Increased levels of serum sorbitol dehydrogenase (SSDH) is a specific indicator of chemically-induced liver damage in fish. The levels of SSDH in the estuarine fish liver, exposed to fungus-treated MSW was reduced by 98% (Table 3). The concentration of benzo(a)pyrene, a polycyclic aromatic hydrocarbon in fungus-treated MSW decreased by 68% (Table 3). In many cases decolorized dye waste waters are more toxic than the original untreated ones and therefore toxicity assays of fungal-treated effluents are equally important to report.

Another white-rot fungal isolate (NIOCC # 2a) obtained from decaying mangrove wood decolorized textile mill effluents, molasses spent wash from alcohol distillery and black liquor from paper and pulp mills (Table 4). It

produced laccase as the major lignin-degrading enzyme (D'Souza *et al.*, 2004). The cell-free culture supernatant containing laccase as the only lignin-degrading enzyme and the exopolymeric substance (EPS) produced by the fungus also decolorized these effluents to various degrees (Table 4). Thus, the white-rot fungus (NIOCC # 312) obtained from seagrass detritus producing laccase > lignin peroxidase and > manganese peroxidase, and the fungus obtained from the decaying mangrove wood (NIOCC # 2a) producing laccase as the only major lignin-degrading enzyme were equally efficient in decolorization of synthetic dyes and colored effluents (Table 5). It will be interesting to further investigate and compare degradation of other xenobiotics by more such isolates to understand the role of different lignin-degrading enzymes in the process.

Mineralization of labelled lignin to labelled CO_2 is the ultimate test of lignin-degrading abilities of fungi. A marine ascomycete *Phaeosporia spartanicola* isolated from the salt marsh grass mineralised radiolabeled lignin moiety to CO_2 and solubilized a part of it to dissolved organic carbon (DOC) after 45 days of incubation at 20°C (Bergbauer and Newell, 1992). The mangrove fungi *Halosarpheia ratnagiriensis* (NIOCC # 321) and *Sordaria fimicola* (NIOCC # 298) and *Flavodon flavus* (NIOCC #312) isolated from the seagrass detritus mineralised ^{14}C -labeled synthetic lignin into $^{14}\text{CO}_2$ (Raghukumar *et al.*, 1996). The culture NIOCC # 312 when grown in the medium prepared with freshwater as well as artificial sea water mineralised about 24 % of the side chain-labelled lignin to $^{14}\text{CO}_2$ within 21 days, comparable to the terrestrial white-rot fungus *Phanerochaete chrysosporium*, the benchmark fungus used for lignin-degradation experiments (Fig. 5).

Fungi, besides being directly utilized as feed and contributing to changing C:N ratio in detritus also help in removing antigustatory compounds present in just-dead plant parts (Raghukumar, 2004). The fibrous lignocellulose tissues may be softened by the lignocellulose degrading enzymes of the fungi colonizing vascular plant detritus. Fungi might also supply essential nutrients to detritivores (Philips, 1984). One such essential nutrient is the polyunsaturated fatty acids which play an

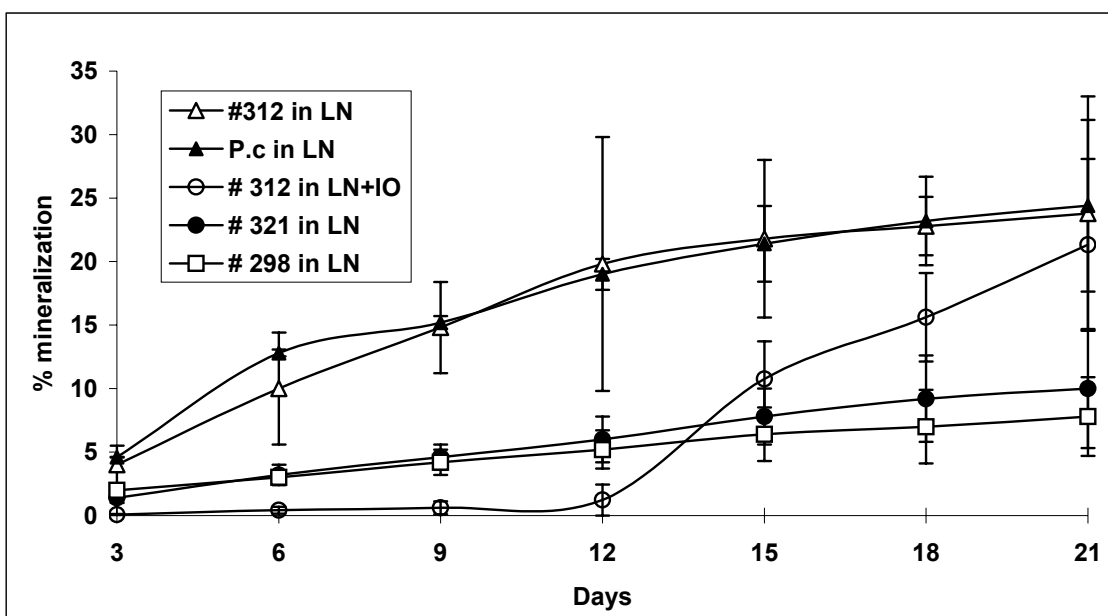


Fig. 5. Comparison of mineralization of ¹⁴C-labeled lignin by NIOCC # 312 (△) grown in low nitrogen medium (LNM) and in LNM prepared with Instant Ocean salt (IO), dehydrated synthetic sea salt (○). ¹⁴C-labeled lignin mineralization by the well-known terrestrial lignin-degrading basidiomycetous fungus *Phanerochaete chrysosporium* (▲) used as the positive control. The obligate marine fungus *Halosarpheia ratnagiriensis* (●) and the facultative marine fungus *Sordaria fimicola* (□) also mineralised ¹⁴C-labeled lignin to a limited extent.

important role in feeds for detritivores. Some of them, such as omega-3 fatty acids, the docosahexaenoic acid (DHA) is essential for growth and maturation of crustaceans (Harrison, 1990). Thraustochytrids have a high DHA content. Does the high biomass of these straminipiles found on the algal and mangrove detritus serve to function in the detrital food web (Raghukumar, 2004)? DHA is now commercially manufactured from thraustochytrids and a vast amount of literature and patents describe the current status of this technology (Lewis *et al.*, 1999; Ward and Singh, 2005).

Several secondary metabolites are reported from fungi isolated from marine detritus. A polyketide metabolite, obionin-A was isolated from the liquid culture of the marine fungus *Leptosphaeria obiones*, a halotolerant ascomycete obtained from the salt marsh grass *Spartina alterniflora* (Poch and Gloer, 1989). These workers also reported two novel compounds helicascolides A and B from the Hawaiian mangrove ascomycetous fungus *Helicascus kanaloanus*

Fungal succession on fallen mangrove litter may to a limited extent give insight in to the type of r- or k-strategists. The oomycetous fungus *Phytophthora vesicula* colonizes the

fallen mangrove leaves within 2 hours of leaf submergence and disappears after 2 weeks (Newell *et al.*, 1987). This fungus thus may be r-strategist. The slow growing lignin-degrading fungi colonizing woody substrates in the marine environment are probably k-strategists and may be a good source of antibiotics (Strongman *et al.*, 1987). In fact a number of antimicrobial compounds have been reported from fungi colonizing woody substrates in the marine environment (Bugni and Ireland, 2004). Such ecological information is important in drug discovery and screening programs.

Fungi in extreme marine environments

Fungi have been reported from extreme terrestrial environments such as Arctic glaciers (Skidmore *et al.*, 2000), glacial ice (Ma *et al.*, 2000), soils of polar deserts with less than 5% moisture content, and carbon content of 0.03% and temperature of -20°C (Fell *et al.*, 2006) and in stratospheric air samples, at an altitude of 41 km (Wainwright *et al.*, 2003). In marine environments extreme conditions are encountered in the form of elevated hydrostatic pressure and low temperature in the deep-sea, low temperatures in sea-ice, high temperature

and elevated hydrostatic pressure with high concentrations of metals in hydrothermal vents, hypersaline water bodies and hypoxic (oxygen deficient) conditions in coastal as well as off-shore waters.

Several pharmaceutical companies are engaged in bioprospecting marine extreme environments. They are focussing on extremophilic bacteria for their biodiversity, thermostable and cold-tolerant enzymes, novel secondary metabolites, metal-tolerant enzymes, stress proteins and bioremediation potentials to make profit. The famous thermostable enzyme Taq polymerase, vent polymerase, detergents supplemented with enzyme, surfactants, antioxidants, industrial enzymes and colorants are some of the examples of the commercial products obtained from extremophilic microorganisms (Bull *et al.*, 2000). Highly solvent-tolerant bacteria for their application in degradation of xenobiotic compounds, have been isolated from deep-sea sediments and waters (Inoue and Horikoshi, 1989). Fungi from extreme marine environment have not been tapped for any of these commercial applications.

Elevated hydrostatic pressure is one of the extreme conditions encountered in the deep sea. Hydrostatic pressure increases by 1 bar with every 10 m depth in the sea. Thus the deep-sea with elevated hydrostatic pressure, is home to barotolerant and barophilic microorganisms. Deep-sea sampling while maintaining the *in situ* pressure and temperature, culturing the deep-sea microorganisms under similar conditions require special equipment and techniques. Several new bacterial and archaeal species have been described and an exhaustive literature is available on their physiology, enzymes and molecular biology (Kato *et al.*, 1998). Awareness regarding presence of fungi in deep-sea is very meagre. Roth *et al.* (1964) isolated marine fungi from oceanic waters of the Atlantic Ocean from a depth of 4450 m. Lorenz and Molitoris (1997) demonstrated growth of yeasts at 400 bar pressure. Barotolerant fungi were isolated from calcareous sediments obtained from 965 m depth in the Arabian Sea (Raghukumar and Raghukumar 1998). Takami (1999) isolated *Penicillium lagena* and *Rhodotorula mucilagenosa* from Mariana Trench at about 11,500 m depth in the

Pacific Ocean. However, isolation of these fungi was carried out at 1 bar pressure from sediments or water samples collected from deep sea. Several culturable fungi have been recovered recently from deep-sea sediments from 5000 m depth of the Central Indian Basin by incubating the sediments under hydrostatic pressure of 200-300 bar (Damare *et al.*, 2006a). The biotechnological potentials of such barotolerant fungi for application in commercial fermentors of large capacities (above 10,000-50,000 L) needs to be assessed.

Deep sea is characterised by constant low temperature of 2-4°C. One of the biotechnological applications of deep-sea microbes will be low temperature-active enzymes. Cold-active enzymes are sought for waste digestion in cold, food processing, detergents for cold-wash, preservation and industrial processing where use of low temperature-active enzymes helps in conservation of heat energy (Gerday *et al.*, 1999). Several of the deep-sea fungi were shown to produce alkaline protease and one of the fungi, *Aspergillus terreus* produced low-temperature active serine protease (Damare *et al.*, 2006b). The half-life of alkaline protease produced by the deep-sea fungus at 5°C and pH 4.5 was 196 h (Damare *et al.*, 2006b). About 26% of its maximum activity was present at 15°C and about 10% at 2°C. In contrast, the protease of the mesophilic *Penicillium* species 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 (Agarwal *et al.*, 2004) Fungi from such extreme habitats need to be screened for novel enzymes and bioactive molecules.

Ancient microorganisms or paleobes form an another example of extremophiles. Viable microorganisms preserved for millions of years have been reported from terrestrial sources of ancient origin. Some of these are intestinal contents of 12,000 year old mastodon remains (Rhodes *et al.*, 1998), 400,000 year old deep ice cores in the Antarctic ice sheets (Castello *et al.*, 1999) and 50 m deep core samples from 3 million year old Siberian permafrost (Shi *et al.*, 1997). Lake Vostok in Antarctica, isolated by ~ 4000 m in depth from overlying ice and for nearly 1 million years (Karl *et al.*, 1999; Priscu *et al.*, 1999) is

suspected to have a rich diversity of viable, actively growing microorganisms. Ma *et al.* (2000) reported the presence of culturable and unculturable fungi from a ~2000 m long glacial ice core. Studies on such culturable ancient microorganisms are important in understanding long-term biological survival strategies as well as in paleoclimatology (Abyzov, 1993). Deep-sea sediments are a source of ancient microorganisms wherein low but constant sedimentation rates bury microorganisms in deeper layers. In one instance, culturable fungi were obtained from subsections of a 460 cm long core obtained from 5900 m depth in the Chagos Trench in the Indian Ocean (Raghukumar *et al.*, 2004). These sediments were dated using a radiolarian biostratigraphy index as they are the index organisms used for estimating sediment age by paleoceanographers (Gupta, 2002). Based on the radiolarian assemblage, the age of the sediments from which fungi were obtained was estimated to range from > 0.18 to 0.43 million years. This is the oldest recorded age for recovery of culturable fungi. A collection of fungi, both culturable and unculturable from deep-sea sediments of other oceans is worth attempting to study their biodiversity and biotechnological applications.

Recent reports on presence of filamentous fungi in the hypersaline waters of the Dead Sea (340 g L⁻¹ total dissolved salts), survival of their spores and mycelia in this hostile environment have invoked great interest (Buchalo *et al.*, 1998; Kis-Papo *et al.*, 2003). Further, a gene responsible for High Osmolarity Glycerol (HOG) response pathway from one such Dead Sea-fungus *Eurotium herbariorum* has been identified for stress tolerance to freezing and thawing (Jin *et al.*, 2005). This has further led to produce a recombinant yeast *Saccharomyces cerevisiae* containing the gene HOG. The genetically transformed yeast proved to withstand high salinity and also extreme heat and cold. However, high salt tolerance is governed by several genes and this might be the first step towards genetic engineering of salt-tolerant agriculture crops.

Studies conducted using cultivation-independent, small-subunit (SSU) rRNA-based survey in suboxic waters and anoxic sediments in Cape Cod, Massachusetts and from permanently anoxic basin off Venezuela have shown

large microeukaryotic diversity and bulk of their sequences represent deep novel branches within green algae, fungi and unclassified flagellates (Stoeck and Epstein, 2003; Stoeck *et al.*, 2003). This suggests that oxygen-depleted environments harbor diverse communities of novel organisms, each of which might have an interesting role in the ecosystem.

In such hypoxic environment the process of denitrification plays a major role. Bacteria are known to be the major denitrifiers in terrestrial soils. Recent work has shown that some of the terrestrial fungi exhibit denitrifying activity where nitrate or nitrite is converted to nitrous oxide under anaerobic conditions (Shoun *et al.*, 1992). Therefore, fungi might also play an important role in denitrification in coastal waters of the Arabian Sea where intermittent hypoxic conditions prevail (Naqvi *et al.*, 2000).

Oil-contaminated sites in the marine habitats are examples of artificial extreme environments. Fungi have been isolated from oil-polluted water bodies in the terrestrial environment. Surface films and tar-balls in the marine environment may contain populations of fungi. Degradation of crude oil by filamentous fungi in the coastal waters has been demonstrated (Leahy and Colwell, 1990). Raikar *et al.* (2001) have shown that thraustochytrids isolated from several sites in Goa, chronically polluted by oil spills were capable of degrading tar-balls. Up to 30% of tar-balls added to peptone broth was degraded by thraustochytrids in 7 days, as estimated by gravimetry and gas chromatography. Fractions above the retention time for C-20 saturates were preferentially degraded, suggesting possible affinity of thraustochytrids to long chain aliphatics. Such fungi and protists occurring in marine environment might play an important role natural degradation of oil spills in the coastal sediments. However, this has not received much attention.

Discovery of enigmatic microorganisms from marine environment such as the protist *Corallochytrium limacisporum*, described as a common ancestor of animals and fungi (Sumathi *et al.*, 2006) may provide new tools for studies in evolutionary biology and hopefully newer biotechnological applications.

Future prospects

From the foregoing discussion, the following areas are recommended to be strengthened in future.

- We need to investigate whether endophytic fungi living in algae and other marine plants and endosymbionts in marine invertebrates induce the host to produce structurally as well as activity-wise novel bioactive molecules.
- A systematic search should be initiated for fungi from special extreme environment such as deep-sea, hypoxic zones (with low oxygen levels) and hydrothermal vents for enzymes, degradation of xenobiotics and bioremediation applications.
- Bioremediation of pollutants using salt-tolerant fungi and their salt-tolerant enzymes on a pilot scale and industrial scale should be carried out.
- Genomic and proteomic studies with novel organisms such as *Corallochytrium limacisporum* as a model of animal-fungal allies will hopefully help in basic research on evolutionary biology.

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