

# 15. Marine Fungi and their Enzymes for Decolorization of Colored Effluents

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## Introduction

Fungi play an important role in degradation and mineralization of lignocellulosic substrates in the marine environment. The importance of lignicolous fungi occurring in wood submerged in the sea was reported by Barghoorn and Linder (1944). These lignicolous fungi comprising Ascomycetes, Basidiomycetes and Deuteromycetes have distinct spore structures that set them apart from their terrestrial counterparts. Such fungi have been defined as obligate marine fungi "which grow and sporulate exclusively under marine conditions" (Kohlmeyer and Kohlmeyer 1979). In order to accommodate the possibility that terrestrial species might also be active in the sea, Kohlmeyer and Kohlmeyer (1979) offered a definition for "facultative marine fungi" as those originating from freshwater or terrestrial environment that are capable of growth and sporulation in the sea. Marine filamentous fungi, obligate and facultative are known to occur in algae, corals and detritus of marine macrophytes (Kohlmeyer and Kohlmeyer, 1979).

## Lignocellulosic substrates and associated fungi in the marine environment

Mangrove plants that are found along estuaries in tropical and subtropical belts, contain about 50% lignocellulosic structural polymers and about 50% soluble organics which include tannins and phenolics (Benner and Hodson, 1985). Degrading mangrove leaves, wood, seagrasses and algae are the primary contributors to the detrital biomass which is an important source of food in the estuarine and near shore food web (Pomeroy, 1980; Hodson and Moran, 1989). Mangrove leaves, twigs and seagrasses fallen into water are colonized and decomposed by bacteria and fungi. The fine particles of such detritus further colonized by microorganisms are utilized by detritus-feeding zooplankton and small animals such as shrimps, crabs and numerous larvae (Teal, 1962; Odum *et al.*, 1979).

Bacteria colonize the maximum surface area and form the initial biofilm on submerged plant parts, have high substrate affinities and produce enzymes diffusible into solid particles to release dissolved organic carbon (DOC). On the other hand, fungal hyphae can grow on the surface (epibiotic) and also penetrate (endobionts) dead plant surfaces (Fig. 1) and cause active decomposition (Raghukumar, 1990). In marine ecosystem (Sathe *et al.*, 1991; Raghukumar *et al.*, 1995) and in fresh water vascular plant-decay (Gessner and Chauvet, 1994; Baldy *et al.*, 1995), it has been shown that fungi contribute to a substantial biomass (75-100% of its nitrogen as fungal biomass).

Enumeration of fungi, their succession and decomposition of mangrove wood is reported from various sites in the tropical and subtropical parts of the world by numerous workers (Borse, 1984; Vrijmoed and Tam, 1990; Chinnaraj and Untawale, 1992; Vishwakiran *et al.*, 2001). Several researchers have reported active loss in weight of various timber blocks and presence of soft rot and white-rot cavities in these blocks (Mouzuras, 1986; Eaton, 1986; Nilsson *et al.*, 1989; Pointing *et al.*, 1998). Similarly, fungi have been isolated, enumerated from mangrove leaves, seagrasses and saltmarsh grass (Sathe *et al.*, 1991, Newell, 1993, Raghukumar *et al.*, 1995).

## Lignin-degrading enzymes

Next to cellulose, lignin is the most abundant and widely distributed renewable aromatic polymer (Boominathan and Reddy, 1992). Lignin, cellulose and hemicellulose are the major structural components of woody plants. About 70% of the dry weight of woody plants consists of lignocellulose of which 20-30% is contributed by lignin. Lignin occurs mainly in the primary and secondary plant cell wall layers, interspersed with hemicellulose and forms a matrix around cellulose microfibrils.

There is a wealth of information available on the extracellular lignin-degrading enzymes of terrestrial wood-rot fungi. These fungi are classified into three major categories based on the type of wood decay caused; white-rot, brown-rot and soft-rot fungi. White-

rot fungi are the only kind of wood-rotting fungi that efficiently attack all the wood components i.e., lignin, cellulose and hemicellulose. White-rot fungi belong to Basidiomycotina and have extensively been studied for their suite of lignin-degrading enzymes. Lignin degradation in these fungi is a cometabolic process, in that lignin is degraded coincidental to their metabolism of energy/carbon substrates such as cellulose, hemicellulose, as well as simpler carbohydrates and glycerol. It has been suggested that degradation of cellulose or hemicellulose provides carbohydrate for the growth of the fungus and when this carbon source is exhausted, the fungus starves and transfers from a primary metabolism to a secondary metabolism mode which in turn triggers the production of enzymes involved in lignin degradation. During utilization of sugars from polysaccharides of wood,  $H_2O_2$  is produced by the action of glucose oxidase and glyoxal oxidase (Reddy and Kelley, 1986) and this is a prerequisite for white-rot fungi such as *Phanerochaete chrysosporium* and *Trametes versicolor* to degrade lignin. When cultivated under optimal ligninolytic conditions of limiting nitrogen

(2.4 mM) and unlimiting carbon (56 mM) conditions, the white-rot fungi produce two families of extracellular glycosylated heme proteins, the lignin peroxidase (LIP) and manganese-dependent peroxidase (MNP).

The LIP (EC 1.11.1.7) family contains multiple, up to 15 isozymes with  $pI$  values ranging from 3.3 to 4.7 and molecular mass ranging from 38 to 43 kDa. LIPs oxidize a spectrum of substrates and the reactions can be classified into five categories such as 1) benzylic alcohol oxidation 2) carbon-carbon bond cleavage 3) hydroxylation 4) phenol dimerization or polymerization and 5) demethylation (Boominathan and Reddy, 1992). Fungus-degraded lignin isolated from decayed wood suggested the presence of an aromatic cleavage system. Whether each isozyme of LIP has a specific role in lignin-degradation is not clearly known. However, all the isozymes catalyze the oxidation of veratryl alcohol to veratraldehyde which is the standard assay procedure for this enzyme. However, the LIP isozymes exhibit differing specific activities against veratryl alcohol (Boominathan *et al.*,

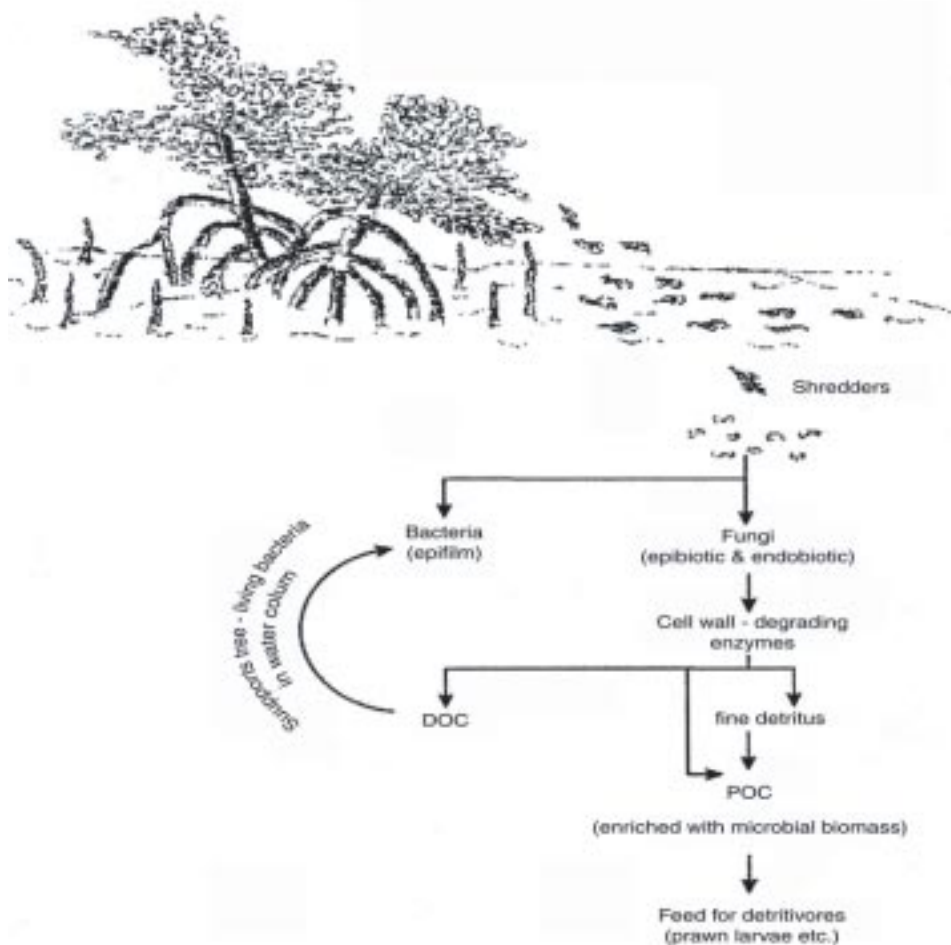


Fig. 1. Schematic diagram showing role of fungi in the degradation of mangrove leaves and production of particulate organic carbon (POC) and dissolved organic carbon (DOC) in the marine ecosystem.

1990). It was further suggested that expression of different LIP gene was regulated by various nutritional and environmental triggers (Reddy, 1993).

The second family of extracellular heme proteins produced by white-rot fungi includes 4-6 or more manganese-dependent peroxidases (EC 1.11.1.7) with  $pI$  values ranging from 4.2-4.9. MNPs, similar to LIPs are glycosylated heme proteins and have molecular mass of approximately 46 KDa. MNPs oxidize Mn (II) to Mn (III) and can be activated by lactate which probably acts by chelating Mn (II) to form stable complexes with a high redox potential. The Mn (III) in turn oxidizes phenols to phenoxy radicals (Glenn and Gold, 1985). The MNPs apparently utilize freely diffusible Mn II/ Mn III as an obligatory redox couple to oxidize terminal phenolic substrates such as lignin and various model compounds. About 10-15% of the phenolic moiety in lignin is free and it is likely that Mn (III) can diffuse into polymeric matrix and oxidize the polymeric phenolic substrates.

Laccases (EC 1.10.3.2) are copper-containing glycoproteins and have been implicated in lignin degradation because they are capable of oxidizing and depolymerizing different lignin preparations and lignin model compounds. They bring about solubilization of lignin from lignocellulose without the presence of any peroxidase (Galliano *et al.*, 1988). They catalyze oxygen-dependent oxidation of phenolic compounds and are found in both plants and fungi. Both inducible and constitutive laccases have been reported from fungi. The current evidence indicates that this enzyme is part of the lignolytic system of some of the most efficient and selective lignin degraders (Roy and Archibald, 1991; Ruttiman *et al.*, 1992).

A major requirement for production of these lignin-degrading enzymes by most of the white-rot fungi is oxygen-saturated culture conditions and therefore extremely shallow cultures are most suitable for this purpose. The direct evidence of lignin-degradation by white-rot fungi is provided by mineralization of  $^{14}\text{C}$ -labeled lignin to  $^{14}\text{CO}_2$  (Freer and Detroy, 1982).

## Lignin-degrading enzymes of marine fungi

The first report on mineralization of  $^{14}\text{C}$ -labeled lignin to  $^{14}\text{CO}_2$  was published by Sutherland *et al.* (1982) wherein it was demonstrated that a number of marine fungi decomposed  $^{14}\text{C}$ -labeled maple and spruce lignins. However, only 5-6% of the  $^{14}\text{C}$ -labeled lignin was mineralized at the end of 30 days. An ascomycete *Phaeospheria spartinicola* was demonstrated to degrade lignocellulose and contributed to dissolved organic carbon (DOC) formation from a salt marsh cord grass *Spartina alternifolia* (Bergbauer & Newell,

1992). *Phaeospheria spartinicola* is the predominant fungus on decaying standing leaves of *Spartina alternifolia* in salt marsh ecosystem. After 45 days of incubation at 20°C, 3.3 % of lignin moiety was mineralised to  $^{14}\text{CO}_2$  and 2.7% solubilized to  $\text{DO}^{14}\text{C}$  by this fungus. Investigation of cupric oxide oxidation products of whole and degraded lignocellulose revealed a preferential utilization of the syringyl and cinamyl phenols compared with vanillyl phenols (Bergbauer and Newell, 1992). We demonstrated the mineralization of U-ring  $^{14}\text{C}$ -labeled lignin by one obligate marine fungus, *Halosarpheia ratnagiriensis* (strain # NIOCC 321) and two facultative marine fungi, namely *Sordaria finicola* (strain # NIOCC 298) and *Flavodon flavus* (strain # NIOCC 312) to  $^{14}\text{CO}_2$  (Raghukumar *et al.*, 1996). One of them, *Flavodon flavus*, a white-rot fungus effectively mineralized about 24 % of the labeled lignin (Raghukumar *et al.* 1999) within 24 days. The fungus was efficient in mineralizing labeled lignin when grown in sea water medium (Fig.2).

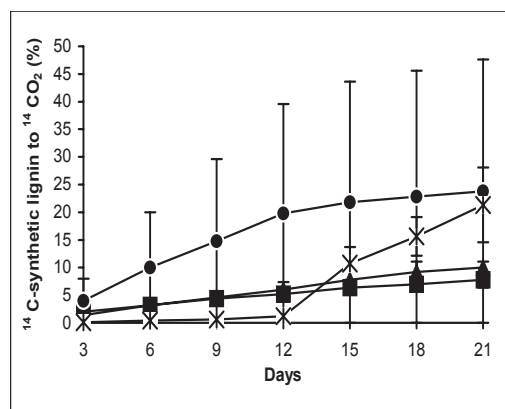


Fig. 2. Percentage mineralization of side chain  $^{14}\text{C}$ -labeled lignin to  $^{14}\text{CO}_2$  by ■NIOCC #298, ▲NIOCC #321, ●NIOCC #312, ×NIOCC #312 (at alkaline pH) in sea water. Low nitrogen medium (LNM) was used for culturing these fungi.

Qualitative screening of degradative enzymes such as cellulase, xylanase, laccase and tyrosinase in 16 marine fungi was reported by Rohrmann and Molitoris (1992). These authors reported laccase activity in a higher percentage of marine basidiomycetes and ascomycetes grown in seawater media than in fresh water media. Ligno-cellulosic enzymes such as cellulases, hemicellulases and pectinases of a marine fungus *Corrollospora maritima* play an active role in the degradation of leaves of an angiosperm *Posidonia oceanica* (Cuomo *et al.*, 1987). We have demonstrated presence of laccase, cellulase and xylanase activities in several, obligate as well as facultative marine fungi isolated from mangrove and seagrass leaves and sediment of mangrove stands (Table 1). These fungi were isolated using selective media incorporating

phenol red or Poly-R 481 (Sigma Chemicals, St. Louis, MO, USA) and prepared with sea water. About 70% of the fungi tested showed laccase activity and about 80% of fungi showed cellulase activity (Raghukumar *et al*, 1994).

Three of these fungi possessed MNP and laccase activity and, one LIP activity (Raghukumar *et al*. 1996; 1999) (Table 2). Subsequently, Pointing *et al* (1998) showed peroxidase-like activity in a few and laccase activity in several marine fungi. Vrijmoed *et al*. (1999)

Table 1. Lignocellulolytic activities of marine fungi

Fungus	Strain No.	Source	Lignin-olytic (1)	Laccase (1 & 2)	Xylanase (2)	Cellulase (1)
<i>Gliocladium</i> sp	NIOCC 145	B	+	+	+	+
Ascomycete (unidentified)	289	A	-	-	+	-
<i>Sordaria fimicola</i>	298	C	+	+	+	+
<i>Flavodon flavus</i>	312	D	+	+	+	+
<i>Gongronella</i> sp.	313	A	+	+	+	-
<i>Aigialis grandis</i>	318	E	+	+	+	+
<i>Aigialis mangrovei</i>	319	E	+	-	+	+
<i>Lophiostoma mangrovei</i>	320	E	+	-	+	+
<i>Halosarpheia ratnagiriensis</i>	321	E	+	+	+	+
<i>Zalerion varium</i>	326	E	+	+	+	+
<i>Hypoxylon oceanicum</i>	328	E	+	+	+	+

A = Decaying mangrove leaves, Andaman Islands, India; B = Mangrove soil, Andaman islands; C = Decaying mangrove leaves, Chorao island, Goa, India; D = Seagrass leaves, Lakshadweep Islands, India; E = Decaying mangrove wood, Chorao Island, Goa. Strains numbers marked in bold and underlined were studied in detail. (1), qualitative assay, (2), quantitative assay. Qualitative and quantitative assay were carried out as described by Raghukumar *et al*. (1994).

Table 2 Lignin-degrading enzymes by fungi isolated from marine habitats grown in low nitrogen medium.

Enzyme assayed	Fungus	Days						
		2	4	6	8	10	12	14
<b>MNP (U L-1)</b>	# 298 <i>Sordaria finicola</i>	11	19	31	24	17	17	10
	# 312 <i>Flavodon flavus</i>	13	20	265	360	244	5	0
	# 321 <i>Halosarpheia ratnagiriensis</i> *	0	10	12	15	21	20	28
<b>LIP (U L-1)</b>	# 298	-	-	-	-	-	-	-
	# 312	7.6	12.4	29.3	52.3	15.1	0.1	0
	# 321	-	-	-	-	-	-	-
<b>Laccase (U L-1)</b>	# 298	85	178	177	90	85	67	111
	# 312	33	26	11	6	4	0	0
	# 321	235	227	205	213	167	409	411

\* obligate marine fungus isolated from mangrove wood. The enzymes were assayed spectrophotometrically (Raghukumar *et al*, 1999).

demonstrated laccase and peroxidase activity, albeit qualitatively based on plate assay using Poly-R and 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) in a mangrove fungus *Julella avicerniae*.

A basidiomycetous fungus *Flavodon flavus* isolated from decaying sea grass from a coral lagoon off the west coast of India, produced all the three major classes of extracellular lignin-degrading enzymes namely LIP, MNP and laccase (Fig. 3a,b,c) when grown in low-nitrogen medium containing 2.4 mM nitrogen prepared with distilled water or synthetic sea water using Instant Ocean salts (IO). Relatively high MNP and moderate levels of laccase were produced in media containing natural lignocellulosic substrates such as pine, poplar or sugarcane bagasse when used as sole carbon and nitrogen source suspended in distilled water but LIP production either was not seen or was minimal (Figs. 4a, b).

### Decolorization of colored pollutants by lignin-degrading marine fungi

As the three fungi showed mineralization of <sup>14</sup>C-labeled lignin, they were also used for decolorization of bleach plant effluent (BPE) from paper and pulp industry.

### Decolorization of bleach plant effluent:

Waste lignin derived from pulp and paper industries is of the order of 30 to 50x10<sup>6</sup> tons per year (Kuhad & Singh, 1993). Large volumes of waste waters are generated for each metric ton of paper produced. These effluents are dark colored containing high molecular weight, modified and chlorinated lignins, phenols, dioxins and furans. Several investigators demonstrated that BPEs can be decolorized by cultures of lignin-degrading white-rot fungi. Among these, *Phanerochaete chrysosporium* (Michel *et al.*, 1991), *Tintoporia borbonica* (Fukuzumi *et al.*, 1977) and *Trametes versicolor* (Bajpai *et al.*, 1993) have been shown to be the best.

There is a total paucity of data in the field of obligate marine fungi or marine-related fungi on these aspects. We demonstrated decolorization of bleach plant effluent (BPE) by 3 marine fungi (Fig. 5). One of the cultures, a basidiomycetous fungus, *Flavodon flavus* was effective in decolorizing BPE at acidic (pH 4.5) as well as at alkaline pH (Fig. 5). BPE in fact stimulated production of lignin-modifying enzymes, MNP and laccase in these fungi (Table 3). Increased production of these enzymes in the presence of BPE indicates that modified lignin or degradation products of lignin, in the form of small molecular weight aromatic

compounds act as inducers. Presnel *et al.*, (1992) reported similar induction of the lignin-degrading enzyme system in *Phanerochaete chrysosporium* in the presence of pulp mill effluent. Decolorization of paper mill effluent by a non white-rot obligate marine fungus, *Halosarpheia ratnagiriensis* calls for a thorough screening program of marine fungi for biotechnological application.

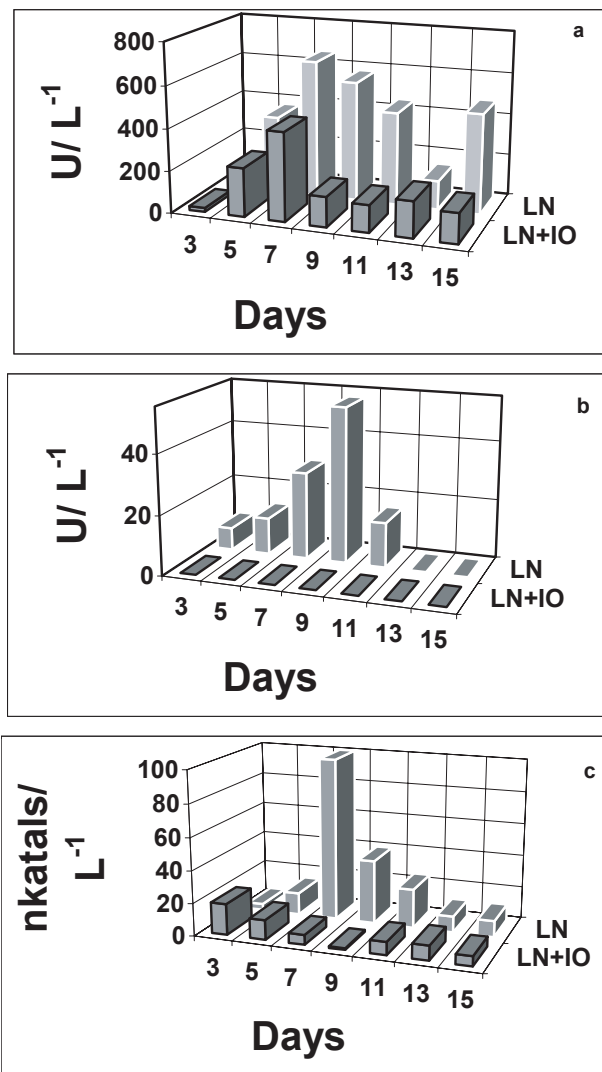


Fig. 3. Comparison of a) MNP b) laccase and c) LIP production by NIOCC # 312 grown in low nitrogen medium (LNM) and LNM prepared with synthetic Instant Ocean salts (LN+IO).

### Decolorization of synthetic dyes

About 50% of industrial colorants produced in the world are azo dyes (Rodriguez *et al.*, 1999). These effluents are from dye-making and textile industries. It is estimated that between 10 and 15% of the total dye used in the dyeing process may be found in wastewater. These can be transformed to carcinogenic compounds under anaerobic conditions (Chung and Stevens 1992). Although azo dyes are generally



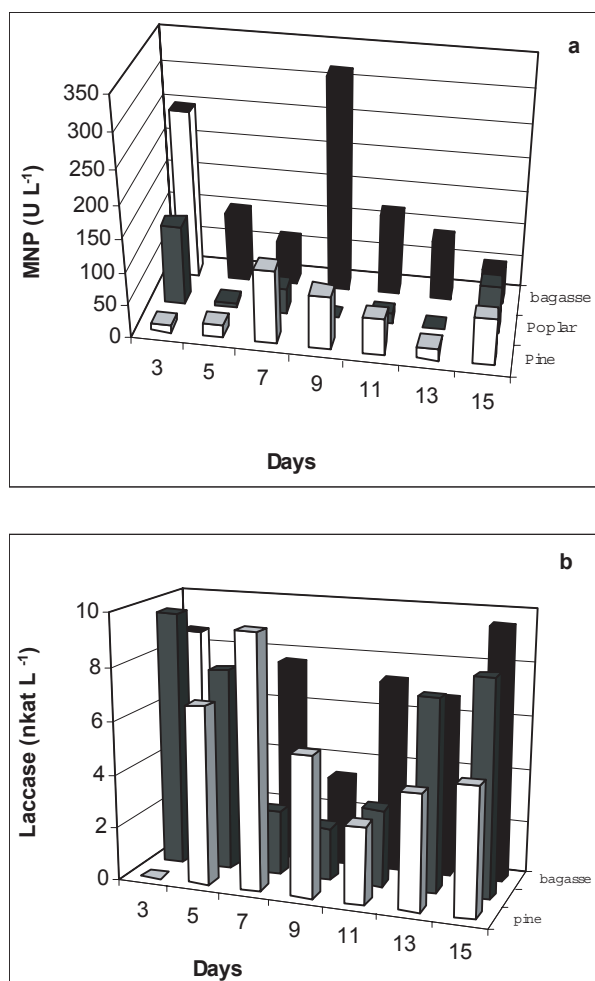


Fig. 4. Comparison of a) MNP and b) laccase production by NIOCC # 312 grown in natural wood substrates using sugarcane bagasse, poplar and pine wood powder (at 1% concentrations) suspended in distilled water. There was no production of LIP in these substrates.

considered to be non-biodegradable under aerobic conditions, the nonspecific nature of the lignin-degrading enzyme system could be expected to be effective in degrading these dyes.

Ligninolytic cultures of *P. chrysosporium* were demonstrated to degrade several azo dyes including orange II, azure B, tropaeolin O, congo red, amaranth and orange G (Cripps *et al.*, 1990). Cultures grown under nitrogen limitation were more effective in degradation of these dyes than those grown under nitrogen sufficient conditions. Crude lignin peroxidase from nitrogen-limited cultures were able to partially decolorize azure B, tropaeolin O and orange II within 20 min of incubation in the presence of H<sub>2</sub>O<sub>2</sub>. Similar results were obtained upon incubation of the dyes with isolated isozymes of LIP (Cripps *et al.*, 1990). Other complex dyes such as crystal violet, cresol red, bromophenol blue, malachite green and brilliant green were also degraded by nitrogen-limited cultures of *P. chrysosporium* (Bumpus and Brock, 1988). Crude lignin peroxidase preparation was effective in

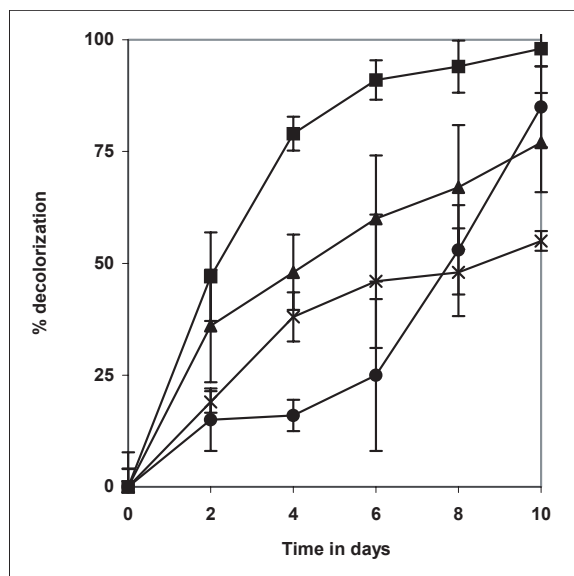


Fig. 5. Decolorization of bleach plant effluent (BPE) from paper mill by ■ NIOCC #312, ▲ NIOCC # 312 in alkaline pH, × NIOCC # 298 and ● NIOCC # 321. Decolorization was measured as platinum cobalt units and expressed in percentage.

Table 3. Effect of bleach plant effluent (BPE final concentration of 10%) on the production of lignin-degrading enzymes in marine fungi.

MNP(U L <sup>-1</sup> )	Days					
	2	4	6	8	10	12
# 298 (-)	11	19	31	24	17	17
# 298 (+)	12	14	21	23	47	16
# 312 (-)	16	20	265	360	21	5
# 312 (+)	16	255	331	483	66	13
# 321 (-)	0	10	12	15	21	20
# 321 (+)	0	12	26	25	18	12
Laccase nkat L <sup>-1</sup> )						
# 298 (-)	85	178	177	90	167	113
# 298 (+)	53	124	80	56	46	0
# 312 (-)	33	26	11	6	4	0
# 312 (+)	17	8	7	6	4	0
# 321 (-)	235	227	205	213	167	409
# 321 (+)	201	217	296	315	267	227

degradation of these dyes too. Sulfonated azo dyes and sulfanilic acid were also demonstrated to be mineralized by *P. chrysosporium* (Paszczynski *et al.*, 1992).

The basidiomycete *F. flavus* isolated from marine environment decolorized several synthetic dyes to various degrees in nitrogen-deficient and nitrogen-sufficient media as well as in sea water media (Fig. 6). The visible absorption spectra of congo red, Poly-B, Poly-R and remazol brilliant blue R decayed totally whereas that of Azure B, Brilliant green and crystal violet remained unchanged qualitatively (Raghukumar et al., 2002). Decolorization was not due to pH

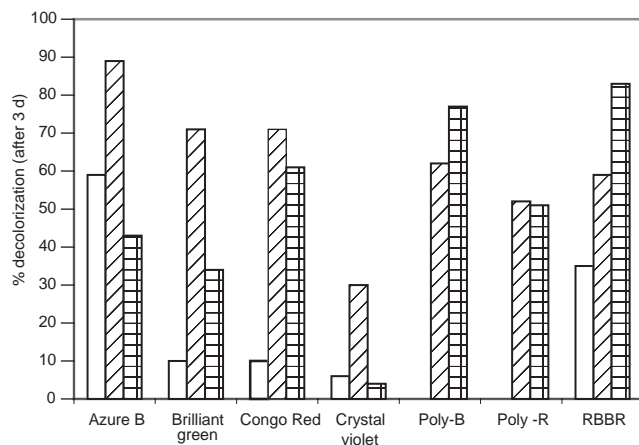


Fig. 6. Percentage of decolorization of various synthetic dyes on day 3 by *Flavodon flavus* when grown in low nitrogen medium (LNM; □), LNM with sea water (▨) or malt extract (▤) media

changes since the pH of the culture media remained essentially unchanged throughout the experiment (Raghukumar et al., 1999). Activity of MNP was found to be directly correlated with decolorization of Poly R and remazol brilliant blue R (Figs 7a & 7b), whereas it was not so with other dyes (Raghukumar 2000). Laccase or LIP activities were not detected in cultures with dyes, whereas MNP was detected in all such cultures. It appears that MNPs play an important role in degradation of these dyes.

## Decolorization of molasses spent wash

Distilleries in India use sugarcane molasses as the raw material for ethanol production. There are about 285 distilleries in India producing about 2.7 billion litres of alcohol each year and generating 40 billion litres of wastewater annually (Joshi, 2001). The effluent if disposed untreated can cause considerable stress on the water courses leading to widespread damage to aquatic life. The effluents from such distilleries called molasses spent wash (MSW) contain large amounts of melanoidin pigments. Melanoidin pigments are the products of the "Maillard reaction" between sugars and amino compounds produced on heating (Fitzgibbon et al., 1995). MSW pollutes aquatic ecosystems due to its intense brown color which cuts off light, prevents photosynthesis and causes anaerobic conditions. When disposed in soil, it acidifies the soil and thereby

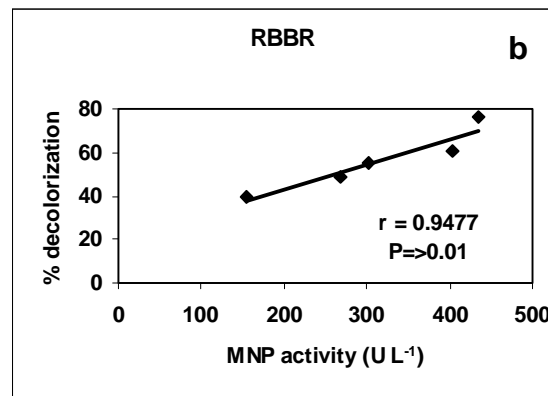
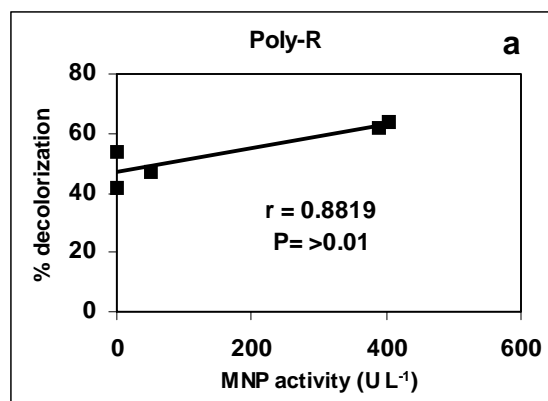


Fig. 7. Positive correlation between MNP activity and decolorization of a) Poly-R dye b) and remazol brilliant blue R (RBBR) by *Flavodon flavus* grown in low nitrogen medium.

affects agricultural crops. Anaerobic digestion of MSW-containing effluents is one of the treatments followed by distilleries and the resulting dark brown sludge is used as a fertilizer. Chemical oxygen demand (COD) and biological oxygen demand (BOD) are reduced in treated effluents but the effluents are still dark brown in color and is a major problem for the distilleries as far as safe disposal is concerned. Color removal in such effluents using terrestrial fungi has been reported (Sirianuntapiboon et al., 1988). The possibility of using obligate and facultative marine fungi occurring in coastal habitats has not been explored for the bioremediation of saline soil and waste waters. We reported decolorization of MSW by *Flavodon flavus*, isolated from marine habitat (Raghukumar and Rivonkar 2001).

*Flavodon flavus* decolorized pigments in the MSW by 80% after 8 days of incubation, when used at 10% and 50% concentration (Table 4) under various culture conditions such as low-nitrogen, nutrient rich medium (eg. malt extract broth) and natural medium containing sugarcane bagasse suspended in distilled water or sea water (Table 4). Highest decolorization was seen when the fungus was grown in the presence of glucose or sucrose (Raghukumar and Rivonkar, 2001). MSW had negative influence on the production of lignin-

degrading enzymes as was evident by negative correlation between MNP activity and decolorization percentage obtained (Fig. 8a). The production of both MNP and laccase decreased in the presence of MSW (Fig. 8b).

Besides color, total phenolics and COD were reduced by 50% in MSW treated with *F. flavus*. Toxicity of the fungus-treated-MSW was totally removed in contrast to the untreated MSW as demonstrated by toxicity assay using an estuarine fish, *Oreochromis mossambicus* (Raghukumar and Shailaja 2003). Increased level of serum sorbitol dehydrogenase (SSDH) is a specific indicator of chemically-induced liver damage in fish. In the estuarine fish, *O. mosambicus* exposed to 10% diluted, untreated MSW for 4 days, the SSDH activity was  $121.7 \pm 5.3$  U ml<sup>-1</sup> serum. On the other hand, with fungus-treated MSW at the same dilution, the activity was  $2.6 \pm 2.6$  U ml<sup>-1</sup> serum. Thus, treatment of MSW with fungus has affected a near total (ca.98%) removal of the damage-causing factor (Raghukumar et al., 2004). Toxicity test was also done using Comet assay, a technique to detect DNA damage in individual cells (Singh et al., 1988). In the presence of untreated MSW, 85% of the hepatic cells of *O. mosambicus* showed damaged nuclei as comets whereas in the presence of fungus-treated MSW only 9% of the cells showed presence of comets indicating removal of toxic factor after the fungal treatment. Benzo(a)pyrene, a known environmental carcinogen, showed ~30% reduction in MSW treated with the fungus (Raghukumar et al., 2004).

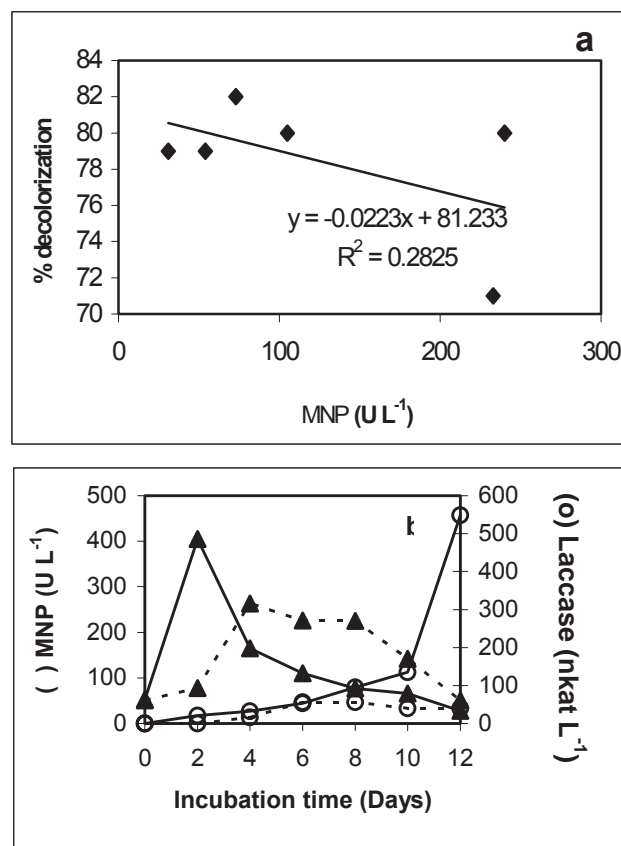


Fig. 8. a) Negative correlation between MNP production and decolorization of molasses spent wash (MSW) and b) production of lignin-degrading enzymes in the presence (---) and absence (-) of MSW.

### Degradation of polycyclic aromatic hydrocarbons

White-rot fungi of terrestrial origin are known to metabolize phenanthrene besides several other PAHs (Reddy 1993). Most of the research concerning bioremediation by these fungi is focused on a single

Table 4. Decolorization of molasses spent wash (MSW) by *Flavodon flavus* under various culture conditions.

Culture medium	Concentration of MSW used (%)	% color removal	Time (days)
Malt extract broth	10	70	8
Low nitrogen medium (LNM)	10	90	8
LNM prepared with half-strength sea water	10	90	8
Sugarcane bagasse powder medium	10	85	8
Sugarcane bagasse powder medium prepared with half-strength sea water	10	40	8
LNM	50	70	8
LNM with sea water	50	70	8



species, the wood-degrading white-rot fungus *Phanerochaete chrysosporium* (Paszczynski and Crawford, 1995).

*Flavodon flavus* isolated from sea grass degraded 60-70% of phenanthrene and chrysene used at a concentration of 12 mg L<sup>-1</sup> (12 ppm) in low nitrogen medium by day 6 (Raghukumar et al., 2003). Nuclear magnetic resonance (NMR) spectra of the ethyl acetate extracts of the fungal biomass revealed that in the heat-killed fungal biomass, the phenanthrene peak remained unchanged throughout the observation period of 6 days. On the other hand, in the live fungal biomass, no phenanthrene peak was detected on day six suggesting that it was metabolized or transformed into non-aromatic fragments (Fig. 9). Fluorescence spectroscopy of these extracts from fungal biomass

showed that only 28% of phenanthrene was removed by the heat-killed fungal biomass (may be due to irreversible adsorption) while the live fungal biomass degraded 63% of the phenanthrene by day 6.

### Degradation of pollutants by immobilized *F. flavus*

The use of immobilized cells and enzymes has been considered as an alternate technology for environmental applications. Fungi have been encapsulated for biocontrol (Axtell and Guzman, 1987) and biodegradation (Anselmo et al, 1985).

The white-rot fungus *F. flavus* was immobilized on 1 cm<sup>3</sup> cubes polyurethane foam (PUF) and these were used for decolorization of molasses spent wash. About 60% of the color from MSW used at 10%

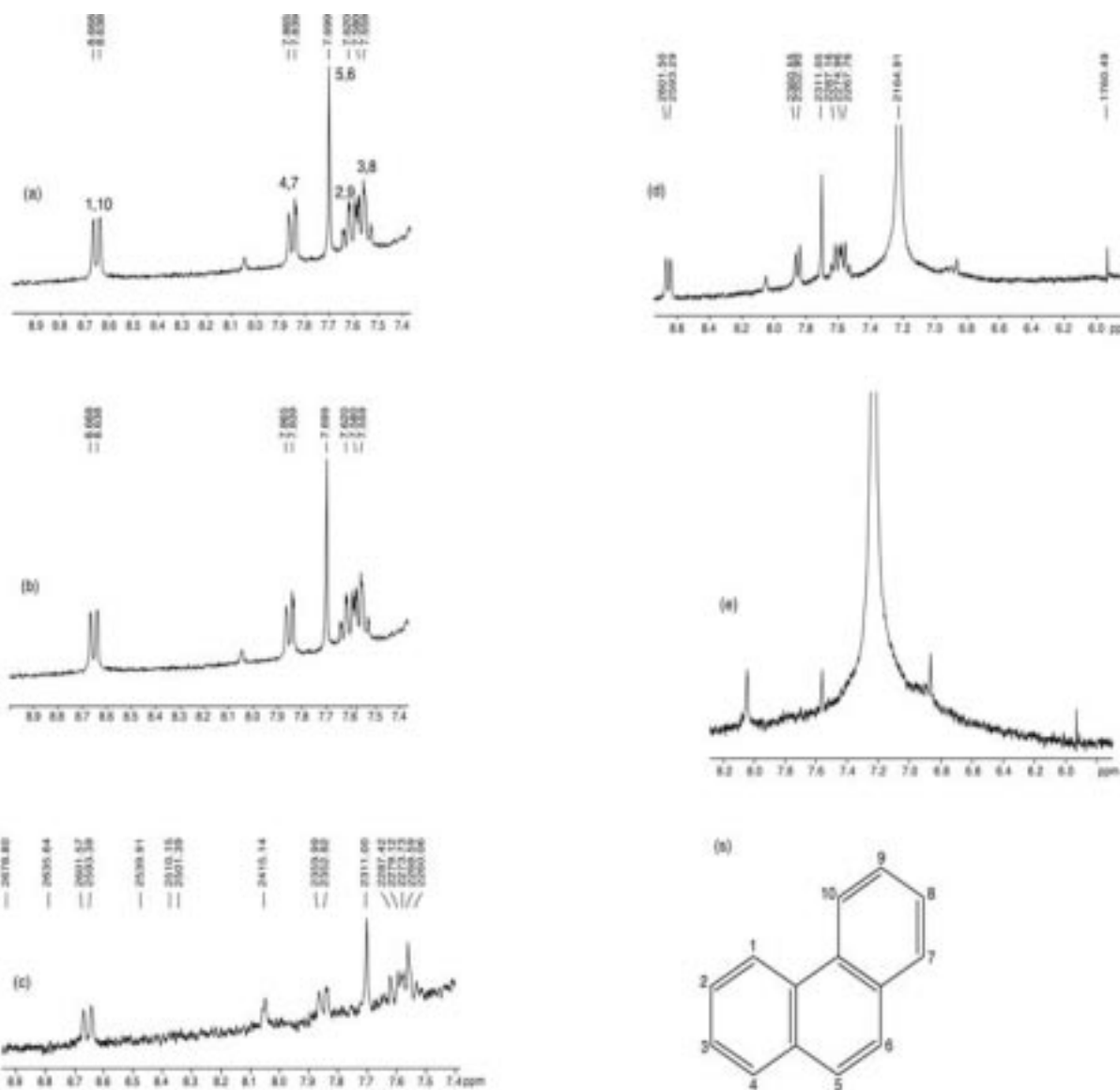


Fig. 9. (a) 300-MHz Proton NMR spectra of the standard phenanthrene , (b) phenanthrene extracted from the heat-killed fungal biomass on day 0 and (c) day, 6 (d) phenanthrene extracted from the live fungal biomass on day 0 and (e) day 6. (f) The chemical structure of phenanthrene ; the peaks corresponding to numbers in the structure are shown in (a).

concentration was removed within 5 days and further about 73% of the color was removed by day 7 (Fig. 10a). No decolorization of MSW was observed in PUF cubes used without the fungal inoculum, thereby ruling out color removal by adsorption (Raghukumar et al, 2004). The same batch of immobilized fungus could be used effectively for three consecutive cycles of decolorization of fresh MSW used at 10% concentration (Fig. 10b). Fungus-treated MSW with 50% of the color removed, when further treated with

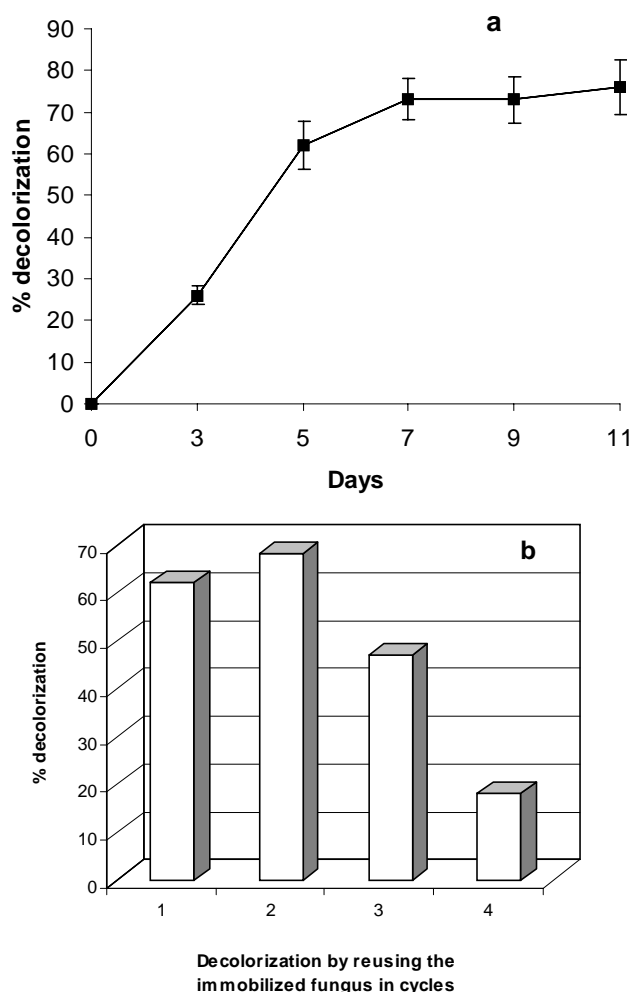


Fig. 10. a) Progress of decolorization of molasses spent wash used at 10% concentration using the immobilized fungus *Flavodon flavus*, b) successive decolorization of several batches of fresh molasses spent wash used at 10% concentration by reusing the same batch of the immobilized fungus. Color was measured on day 5 after each treatment.

a new batch of the immobilized fungus, an additional 30% of the color was removed. Therefore, in effect 78-80% of the color could be removed by employing immobilized fungus. In MSW treated with immobilized fungus reduction in color (after 5 days) of high molecular weight fractions was proportionately greater than the low molecular weight fractions (Fig. 11).

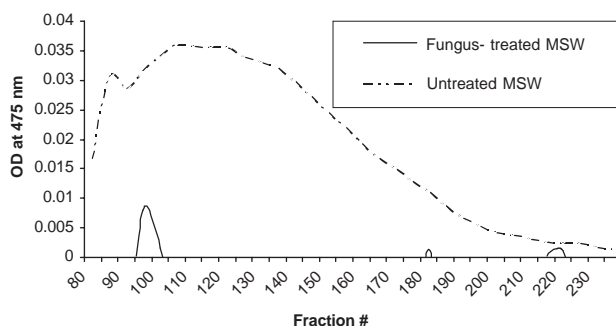


Fig. 11. Gel filtration chromatography of molasses spent wash (MSW) on a Sephadex G-50 column. Solid line represent 10% diluted MSW decolorized by the live immobilized fungus and broken line represents fractionation of untreated, 10% diluted MSW. The color absorbance of the fractions was read at 475 nm. MSW treated with the fungus for 5 days was used for gel filtration.

## Possible mechanisms of pollutants degradation by *Flavodon flavus*

The results obtained with *F. flavus* indicate that MNP and laccase are actively involved in decolorization of BPE as the production of these enzymes increased in the presence of BPE (Table 3). On the other hand, only MNP appeared to be involved in decolorization of synthetic dyes (Figs. 7 a & b). None of these enzymes appeared to be involved in decolorization of MSW by *F. flavus* as evidenced by a lack of correlation between percentage decolorization and MNP activity the inhibitory effect of MSW on these enzymes (Fig. 8a,b). However, addition of starch to the medium improved the efficiency of decolorization of MSW by *F. flavus*. Although amylase activity did not show much difference, there was increased glucose oxidase activity in the medium with MSW which also correlated well with decolorization of MSW (Table 5). Therefore, it is possible that  $H_2O_2$  produced as a result of glucose oxidase activity might act as a bleaching agent. This is further confirmed by the fact that decolorization is lesser when the fungus is grown in malt extract broth (in the absence of glucose). On addition of starch to this medium, amylase activity is stimulated releasing reducing sugars and these are oxidized further by glucose oxidase producing  $H_2O_2$  as a byproduct. This results in decolorization of MSW (Table 5). Production of  $H_2O_2$  is reported in several white-rot fungi and the amount produced varies depending on the substrates used and other culture conditions (Zhao and Janse, 1996). Hydrogen peroxide is implicated in lignin and cellulose degradation by white and brown-rot fungi (Ander, 1994). The lignin degrading enzymes, manganese peroxidase and lignin peroxidase require  $H_2O_2$  as co-substrate.

It is pertinent at this point to suggest that purified enzymes of this fungus be tested for their efficiency in decolorization of various colored pollutants. The heat-killed mycelia or the culture filtrate (without the

fungal biomass) did not decolorize BPE, dyes or MSW (even in the presence of exogenous H<sub>2</sub>O<sub>2</sub>) showing that the color removal was not due to adsorption and that direct contact with live mycelium is necessary for color removal. Swamy and Ramsay (1999) have suggested involvement of mycelial-bound lignin-degrading enzymes or a H<sub>2</sub>O<sub>2</sub>-generating mechanism in the cell wall to be responsible for decolorizing capacity of *Trametes versicolor*.

In case of degradation/removal of PAHs by *F. flavus*, the possible mechanism of action may be as follows. The loss of up to 70% phenanthrene from the culture medium containing live or heat-killed fungus was due to its instant adsorption to the fungal biomass owing to the presence of the exopolysaccharide envelope around the fungal hyphae (Fig. 12). It has been shown that extracellular lignin-degrading enzymes are readily available in this matrix of the exopolymer in some of the white-rot fungi (Bes et al., 1987). Therefore, it is hypothesized that phenanthrene thus adsorbed is subsequently degraded by the lignin-degrading enzymes present in the exopolysaccharide envelope (Fig. 13). Thus, fungal biomass alone could be used for adsorption and ultimate degradation of PAHs from

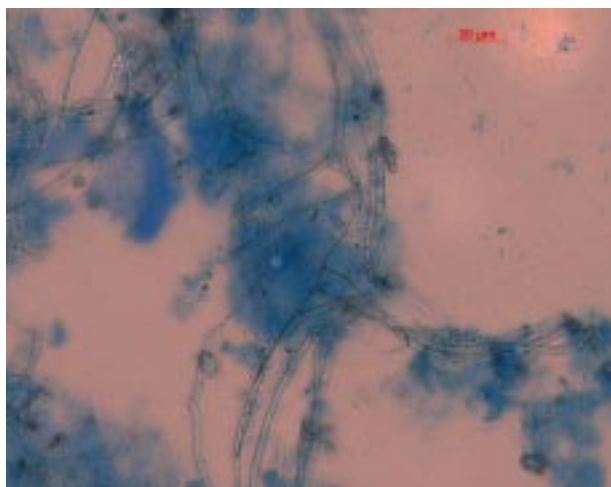


Fig. 12. Exopolysaccharide (arrow) around the fungal mycelium stained blue with acidic Alcian blue (1% in 3% acetic acid, W/V).

contaminated sites (Raghukumar et al., 2004). The possible mechanism of decolorization/degradation of various effluents by *F. flavus* is summarized in Table 6.

A continuous process for color removal from BPE with the white-rot fungus *P. chrysosporium* was developed at the pilot scale (Chang et al. 1983). The process, mycelial color reduction (MyCoR) utilizes a rotating biological contactor on the surface of which the fungus

Table 6. Possible mechanism of decolorization/degradation

Effluent	Enzymes/active principles involved in decolorization/degradation	Reference
Bleach plant effluent	MNP and laccase	Raghukumar 2000
Dyes	MNP	Raghukumar 2002
Molasses spent wash*	Glucose oxidase	Raghukumar et al. 2004
Polyaromatic hydrocarbons*	Lignin-degrading enzymes and extracellular polymeric substance (EPS)	Raghukumar et al. 2004

\*Immobilized fungus also effective in decolorization/degradation

is immobilized. In the MyCoR process, chlorolignins are somewhat degraded and low molecular mass chlorinated derivatives are metabolized.

Use of free enzymes may face some problems due to thermal instability, susceptibility to protease attack, inhibition of activity and the difficulty of separating and reusing them after completion of the reaction. The phenol-oxidases have been immobilized on natural and synthetic supports such as soil, clay and porous glass beads to overcome some of these hurdles (Leonowicz et al., 1988; Sarkar et al., 1989).

A concerted effort from workers in different disciplines is required to use effectively the lignin-degrading fungi for application in bioremediation of colored effluents.

## Future Directions

The dye stuff market share by the Asian countries is about 42% and India consumes some 600 thousand tons of dyes per annum (Wesenberg et al., 2003). With such demands which keep growing, the remediation of effluents from dye-making industries and dye-using industries is going to be a challenging task. Decolorization of such effluents using lignin-degrading fungi and their enzymes has generated a large amount of information on basic aspects. One of these aspects has directed the current research into a search for mediators which are cheap and affordable to be used on a large scale (Wesenberg et al., 2003). These low-molecular weight mediators may be able to reach and contact large polymer of lignin much easier than the high molecular weight lignin-degrading enzymes. Several natural and synthetic mediators are already

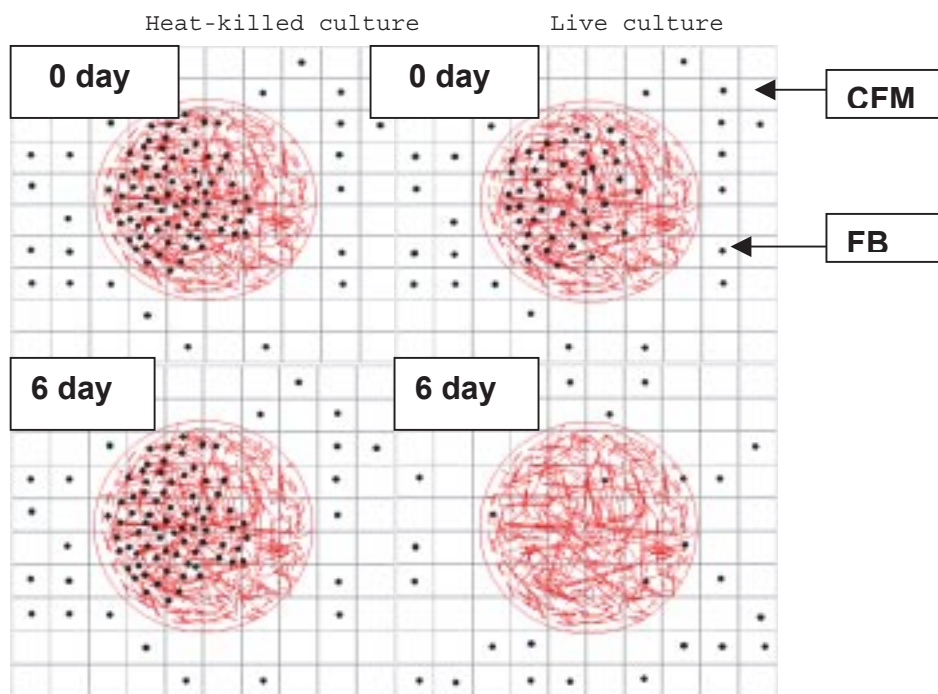


Fig. 13. Model showing the fate of phenanthrene in the live and heat-killed fungal biomass (FB) and the cell-free medium (CFM). The black dots represent phenanthrene, the large round disc with red strands represent the fungal mycelial mat. The square grid represent the culture medium. The phenanthrene dots correspond to its distribution in the culture medium and the fungal biomass, amounting to a total of 100%.

known which are involved in the formation of reactive oxygen species which could degrade lignin and other similar xenobiotic compounds (Wesenberg et al., 2003). A search for mediator-producing organisms or genetically engineering them will certainly be rewarding.

Decolorization of colored effluents need not always solve the problems as they can still be toxic and therefore detoxification and mechanisms involved in it need to be studied simultaneously. As the colored effluents also contain salts at high ionic strength marine fungi or marine-related fungi are effective for treatment of such effluents. More attention should be paid to decolorization of complex colored effluents from industries than individual dyes. Effect of environmental parameters such as temperature, pH, ionic concentrations besides molecular structure of chromophores also needs to be considered.

Future research should be targeted towards search for or creating fungal strains with over-producing lignin-degrading enzymes. Such organisms could be used in bioreactors for treatment of wastewaters or scaling up of enzyme productions. Biofilms for immobilization of lignin-degrading fungi or their enzymes for continuous use in wastewater treatment is yet another challenge for the future.

## Conclusion

A high throughput screening strategy for lignin-degrading marine fungi is required to tap the vast resources in marine ecosystem. Most of the pollutants have high load of inorganic salts of chlorides and sulphides and are of alkaline nature and therefore, obligate and facultative marine fungi that live under such conditions are better suited for bioremediation purposes. Finally, proper coordination or liaison between marine mycologists and industries can realize bench-scale results to final, working processes.

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