

Treatment of colored effluents with lignin-degrading enzymes: An emerging role of marine-derived fungi

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Running title: Decolorization of colored effluents with marine-derived fungi

Abstract

Some of the industries that discharge highly colored effluents are paper and pulp mills, textiles and dye-making industries, alcohol distilleries and leather industries. Terrestrial white-rot basidiomycetous fungi and their lignin-degrading enzymes laccase, manganese-peroxidase and lignin peroxidases are useful in the treatment of colored industrial effluents and other xenobiotics. Free mycelia, mycelial pellets, immobilized fungi or their lignin-degrading enzymes from terrestrial fungi have been reported in treatment of several effluents. Marine obligate or facultative (marine-derived) fungi may have unique properties but have not been explored sufficiently for this purpose. This article presents a critical review of bioremediation potential of such fungi and their lignin-degrading enzymes in comparison with the state-of-the-art in terrestrial white-rot fungi.

Keywords: Industrial effluents, decolorization, detoxification, laccase, peroxidases, marine fungi.

1. Introduction

Paper and pulp mills, molasses based-alcohol distilleries, tanneries, dye-making units and textiles are some of the major industries that produce and discharge highly colored effluents. Each of these industrial effluents creates some specific problem besides producing aesthetically unacceptable intense coloring of soil and water bodies. They block the passage of light to the lower depths of the aquatic system resulting in cessation of photosynthesis, leading to anaerobic conditions, which in turn result in the death of aquatic life causing foul smelling toxic waters.

Colored industrial wastewater is usually treated by physico-chemical processes. These processes include flocculation, flotation, electro flotation, membrane-filtration, ion exchange, irradiation, precipitation, ozonation and adsorption using activated carbon or biological adsorption using bacteria, fungi, algae or plant biomass (Robinson et al. 2001; Husain 2006; Whiteley and Lee 2006 and references therein). Both living and dead cells have been used for bio-adsorption (Fu and Viraraghavan 2001).

Bioremediation is also possible with naturally occurring (Reddy 1995) or genetically modified organisms (Chen et al. 1999) for treatment of soil, ground or surface water for general protection of human health and the environment. This has to be primarily safe and comparatively less expensive than conventional treatments. Bacteria and fungi along with their products such as enzymes (Whiteley and Lee 2006) and exopolymeric substances (Liao et al. 2001) aid in bioremediation. The application of bioremediation has remained limited due to incomplete understanding of the degradation processes performed by organisms in natural systems and engineering of suitable systems for the optimum utilization of the organism is required (Pritchard et al. 1996). Maintaining the optimum catalytic activity of an organism for a long period of time under controlled conditions for treatment of massive volumes of effluents is a rather difficult task. Therefore bioremediation has not been as successful as physical and chemical methods (Pritchard et al. 1996).

A number of biotechnological approaches have been tried for the treatment of colored effluents and one of the most successful groups of organisms in this context has been the white-rot basidiomycetous fungi that are capable of extensive degradation of lignin under aerobic conditions. White-rot fungi are known to play a major role in mineralization of the lignin polymer to CO₂ and H₂O in the terrestrial environment. These fungi produce a wide range of

lignin-degrading enzymes (LDEs), which in turn act on lignin and lignin-analogous compounds. The LDEs share common features such as broad substrate specificity, high redox potential and are mostly extracellular in nature. The high redox potential and broad substrate specificity, increases the range of pollutants the enzyme is capable of degrading. These features combined with the fact that LDEs are mostly expressed under nutrient deficient conditions (which is usually the case in the nature) and their ability to oxidize substrates with low solubility have made them the preferred candidates for bioremediation along with the fungi, responsible for their production (Reddy 1995).

The process of bioremediation can be monitored by measuring any of the following factors: (i) by measuring the redox potential, together with pH, temperature, oxygen content and concentrations of electron acceptor (s) / donor(s) and the breakdown products such as carbon dioxide or (ii) by measuring chemical oxygen demand (COD) and biological oxygen demand. Biological oxygen demand (BOD) represents only the organic matter which is capable of being degraded / oxidized by microbes whereas COD represents all the oxidizable matter, including organic matter in any particular effluent (Marmagne and Coste 1996). For colored effluents, bioremediation is measured by estimating the reduction in color units of effluents and percentage of detoxification achieved besides measuring a few of the above-mentioned parameters.

2. Lignin-degrading enzymes

Lignin-degrading enzymes (LDEs) belong to two classes *viz* the heme-containing peroxidases and the copper-containing laccases. A series of redox reactions are initiated by the LDEs, which degrade the lignin (or lignin-derived pollutants). The LDEs oxidize the aromatic compounds until the aromatic ring structure is cleaved, which is followed by further degradation with other enzymes.

Peroxidases are heme-containing enzymes that comprise manganese-dependant peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP). Lignin peroxidase (EC 1.11.1.14) requires H_2O_2 as the co-substrate as well as the presence of a mediator like veratryl alcohol to degrade lignin and other phenolic compounds (Fig. 1). Here H_2O_2 gets reduced to H_2O by gaining an electron from LiP (which itself gets oxidized). The oxidized LiP then returns to its native reduced state by gaining an electron from veratryl alcohol and oxidizing it to veratryl aldehyde. Veratryl aldehyde then gets reduced back to veratryl alcohol by gaining an electron

from lignin or analogous structures such as xenobiotic pollutants. This results in the oxidation of lignin or the aromatic pollutant (ten Have and Teunissen 2001). MnP (EC 1.11.1.13). This too, requires H_2O_2 as its co-substrate and the presence of Mn^{2+} (naturally present in wood). It catalyses oxidation of Mn^{2+} to Mn^{3+} , resulting in an Mn^{3+} chelate-oxalate, which in turn oxidizes the phenolic substrates. The resulting Mn^{3+} chelate-oxalate is small enough to diffuse into areas inaccessible even to the enzyme, as in the case of lignin or analogous structures such as xenobiotic pollutants buried deep within the soil, which are not necessarily available to the enzymes (Tortella et al. 2005).

A novel enzyme which can utilize both veratryl alcohol and Mn^{2+} , versatile peroxidase (EC 1.11.1.16) has been recently described as a new family of ligninolytic peroxidases (Marti'nez 2002; Marti'nez et al. 2004). The most noteworthy aspect of versatile peroxidase (VP) is that it combines the substrate-specificity characteristics of LiP, MnP as well as cytochrome *c* peroxidase (Du et al. 1992). In this way, it is able to oxidize a variety of (high and low redox potential) substrates including Mn^{+2} , phenolic and non-phenolic lignin dimers, veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols and hydroquinones (Heinfling et al. 1998). It has an Mn-binding site similar to MnP and an exposed tryptophan residue homologous to that involved in veratryl alcohol oxidation by LiP. It is suggested that the catalytic properties of the new peroxidase is due to a hybrid molecular architecture combining different substrate-binding and oxidation sites (Camarero et al. 2000).

Laccase (EC 1.10.3.2) is a benzenediol:oxygen oxidoreductase (a multi-copper enzyme), present across the kingdoms from bacteria, eg. *Azospirillum lipoferum* and actinomycetes like *Streptomyces*, to fungi to plants and even in insects (Baldrian 2006). This multi-copper oxidase has the ability to oxidize phenolic compounds. Unlike peroxidases, it does not contain heme as the cofactor but copper. Neither does it require H_2O_2 as the co-substrate but rather molecular oxygen. Laccase often sports a high degree of glycosylation, which confers a degree of self-resistance to attack by proteases (Yoshitake et al. 1993). The downside however, is that the redox potential although varying between different laccase isozymes, cannot be compared with that of the peroxidases, especially LiP. Although laccase does not have the exclusive requirement for the presence of mediators like veratryl alcohol and Mn^{+2} , their presence increases the effective range of substrates, which can be degraded by laccase. Laccase, can not only catalyze

depolymerizing reactions but polymerizing reactions as well. Whilst depolymerization is obviously useful for the breakdown of pollutants, polymerization can also be useful, even though larger compounds are created. This is because sequestration is acceptable as a method for bioremediation. While forming a larger compound does not remove it from the environment, it can be rendered non-toxic thus negating the need for its removal (Ali and Sreekrishnan 2001).

Based on the enzyme production patterns of white-rot fungi, Hatakka (1994) suggested three categories of fungi: 1) lignin peroxidase-manganese peroxidase group, 2) manganese peroxidase-laccase group and 3) lignin peroxidase-laccase group. The most efficient lignin degraders are able to mineralize lignin to CO₂ and belong to the first category of fungi. Only moderate and very poor mineralization of lignin occurs in the second and third category of fungi respectively. The terrestrial white-rot fungus *Phanerochaete chrysosporium* which produces multiple isozymes of MnP and LiP but mostly no laccase, has been the laboratory model for physiological and molecular biological studies of LDEs (Fu and Viraraghavan, 2001). *Trametes versicolor* producing laccase as the major LDE has been studied widely for industrial application in biobleaching of paper pulp, treatment of effluents and various other industrial applications (Wesenberg et al. 2003). However, production of MnP in strains of *T. versicolor* has also been demonstrated recently (Snajdr and Baldrian, 2007; Diorio et al. 2008).

3. What and why marine fungi for lignin degradation?

Mangrove plants and seagrasses contain 50 % lignocellulosic material as structural polymers and are the major contributors of lignocellulose substrate in coastal marine environment (Benner and Hodson 1985). Mangrove leaves, twigs, wood pieces and seagrasses fallen into the intertidal zone are colonized by epibiotic bacteria and epi- and endobiotic fungi (Fig. 2). The term 'marine-derived' fungi, is used here since the marine ecosystem comprises of obligately marine as well as facultative marine fungi. The facultative forms although having counterparts in the terrestrial ecosystem, have adapted to the marine environment. Obligate and facultative marine fungi colonizing these substrates produce cell wall-degrading enzymes and are responsible for the production of dissolved organic carbon (DOC) and particulate organic carbon (POC) in water (Newell 1996). The resulting DOC is utilized by bacteria for biomass build up and the microbially colonized POC is utilized as feed by detritus-feeding larvae and other macroorganisms such as crabs and shrimps (Odum et al. 1979).

Fungi have been isolated and enumerated from mangrove leaves, seagrasses and salt marsh grass (Sathe and Raghukumar 1991; Newell 1993; Raghkumar et al. 1995). Several reports have demonstrated active loss in weight of various timber blocks colonized by marine wood-degrading fungi (Nilsson et al. 1989; Pointing et al. 1998; Pointing and Hyde 2000; Bucher et al. 2004). Interestingly, most of these reported fungi belong to ascomycetes and a very few to basidiomycetes or white-rot fungi. Enumeration of fungi, their succession and decomposition of mangrove wood is reported from various tropical and subtropical parts of the world by numerous workers (Vrijmoed and Tan, 1990; Chinnaraj and Untawale 1992; Vishwakiran et al. 2001). Therefore, fungi growing under such marine conditions are expected to have adapted to grow under saline (ranging from 10-34 ppt) and alkaline conditions since the pH of seawater ranges from 7.5 to 8.2. Such LDE-producing fungi should find application in bioremediation of lignin-based derivatives in colored industrial pollutants such as paper and pulp mills, tanneries, molasses-based distilleries and textile mills. These effluents are mostly alkaline and have high salt content (Bartlett 1971) and therefore, marine fungi, facultative or obligate that grows in the presence of saline and alkaline conditions perhaps are well suited for treatment of such effluents.

3.1. Lignin-degrading ability of marine fungi

Mineralization of ^{14}C (ring)-labeled synthetic lignin to $^{14}\text{CO}_2$ is considered the acid test for the lignin-degrading ability of any fungus (Kirk and Farrell 1987). Sutherland et al. (1982) demonstrated limited mineralization of ^{14}C -labeled maple and spruce lignin to $^{14}\text{CO}_2$ by a number of marine fungi. Only 5-6% of the labeled lignin was mineralized at the end of 30 days by these fungi. *Phaeospheria spartinicola*, an ascomycetous fungus growing on the decaying leaves of the salt marsh cord grass *Spartina alternifolia* was shown to degrade lignocellulose and contribute to dissolved DOC formation (Bergbauer and Newell 1992). After 45 days of incubation, only 3.3 % of the lignin moiety was mineralized to $^{14}\text{CO}_2$ and 2.7 % solubilized to DO^{14}C by this fungus. An obligate marine fungus *Halosarpheia ratnagiriensis* (strain NIOCC #321) and one facultative marine fungus *Sordaria finicola* (NIOCC #298) mineralized about 9-10 % of the U-ring ^{14}C -labeled lignin to $^{14}\text{CO}_2$ within 21 days (Raghukumar et al. 1996). A basidiomycete, NIOCC #312 isolated from decaying leaves of the sea grass *Thalassia hemprichii* on the other hand, mineralized 21 % of the U-ring ^{14}C -labeled lignin to $^{14}\text{CO}_2$ within 21 days

(Raghukumar et al. 1999). In the same experiment, the lignin-degrading terrestrial fungus *Phanerochaete chrysosporium* generally used as a benchmark for lignin-degradation was shown to mineralize about 21% of the ^{14}C -labeled lignin (DHP) to $^{14}\text{CO}_2$ within 21 days (Raghukumar et al. 1999). Thus, this marine-derived fungus, NIOCC #312 has been the only one reported among the marine fungi to match the efficiency of terrestrial white-rot fungi in lignin mineralization.

3.2. Lignocellulose-degrading enzymes in marine fungi

Lignocellulose-degrading enzymes; cellulase and xylanase have been detected in marine fungi isolated from salt marsh grass (Gessner 1980). Rohrmann and Molitoris (1992) have also reported the presence of laccase in addition to the above enzymes in marine fungi isolated from algae. Marine basidiomycetes and ascomycetes grown in seawater media showed higher laccase activity than those grown in fresh water media (Rohrmann and Molitoris, 1992). Schaumann et al. (1986) demonstrated laccase activity in 65 % of marine ascomycete *Lulworthia* sp. in substrates like guaiacol, naphthol and benzidine. Subsequently, presence of laccase, cellulase and xylanase activities in several facultative and obligate marine fungi isolated from mangrove and seagrass leaves and sediments from mangrove stands were reported (Raghukumar et al. 1994). About 70% (12 out of 17 fungi screened) of these fungi showed laccase activity and ~ 80 % of the fungi showed cellulase activity when grown in media prepared with half strength sea water. Among these, two of the marine ascomycetous fungi *Halosarpheia ratnagiriensis* (NIOCC #321) and *Sordaria fimicola* (NIOCC #298) secreted MnP and laccase in seawater media. Thus, these two fungi belong to the second category of lignin-degrading fungi, which are classified to produce MnP and laccase (Hatakka 1994). Pointing et al. (1998; 1999) reported presence of laccase, cellulase and xylanase in several marine fungi from tropics. Although a thorough list of marine fungi in tropical America and Africa is available (Kohlmeyer and Kohlmeyer 1979) and other tropical countries (Kohlmeyer 1984), lignin-degrading activity of these fungi have not been investigated. On the other hand, large amount of information is available on biologically active natural product chemistry from marine and marine-derived fungi (Liberra and Lindequist 1995; Bugni and Ireland 2004) but not on lignin-degrading enzymes. Recently a number of filamentous fungi have been isolated from hypersaline environment of the Dead Sea (Molitoris et

al. 2000). They were demonstrated to decolorize several synthetic dyes at various salinities but no information is available regarding presence of LDE system in these.

Lignin-degrading marine-derived fungi that do not fall into any of the categories described by Hatakka (1994) have also been reported. The basidiomycetous fungus NIOCC #312, isolated from decaying seagrass (*Thalassia hemprichii*) of the Lakshadweep island, India, produced all three LDEs, also does not conform to any of the above categories (Raghukumar et al. 1999). However, the major LDEs in this fungus were MnP and LiP whereas laccase was minimal (Table 1).

Another basidiomycetous fungus NIOCC #2a, isolated from decaying mangrove wood in Chorao island, Goa, showed laccase positive reaction when grown in LNM prepared with seawater containing 4 mM guaiacol or 2 mM ABTS (2,2'-azino-bis-(3-ethyl benzothiazoline-6-sulphonic acid). Production of an intense brown color under and around the fungal colony in guaiacol-supplemented agar and a deep green color in ABTS-supplemented agar was considered a positive reaction for the presence of laccase activity (D'Souza et al. 2006)(Table 1). It produced MnP and LiP in LNM at negligible levels and thus too, does not fall into any of the reported categories of the lignin-degrading fungi. Several obligate marine fungi have been reported to produce only laccase (Pointing et al. 1998; Luo et al. 2005). Thus, it appears that lignin-degrading marine fungi may not be strictly classified in to groups as described by Hatakka (1994). However, it would be interesting to screen for the presence of all the LDE genes in marine fungi in general.

The test of adaptation of marine-derived fungi to their environment is growth and production of degradative enzymes in seawater media. Although, marine fungi showed growth and LDE activities in agar media (Raghukumar et al. 1994; Pointing et al. 1998) or in liquid media containing sea water (Raghukumar et al. 1999; Li et al. 2002a; 2002b; D'Souza et al. 2006) laccase and MnP activity of NIOCC #312 and NIOCC #2a, decreased drastically when seawater was added during enzyme assay (in vitro) studies (unpublished results). Purified laccase from NIOCC #2a was not inhibited in the presence of NaCl roughly upto 0.3 M, above which it was reversibly inhibited (unpublished results). Luo et al. (2005) reported that inhibitory effect of seawater on the laccase activity of two marine fungi tested was reversible. A basidiomycetous fungus *Phlebia* sp (strain #MG-60) isolated from mangrove stands was identified as a

hypersaline-tolerant lignin-degrading fungus (Li et al. 2002a; 2003a) which participated in biodegradation of sugarcane bagasse, biobleaching of paper pulp and decolorization of dyes (Li et al. 2002b) in the presence of different concentrations of sea salts. Subsequently these authors showed production of hypersaline-tolerant MnP in #MG-60, in the presence of sea salt and NaCl (Li et al. 2003b). Raghukumar et al. (1999) and D'Souza et al. (2006) demonstrated growth and LDEs production in NIOCC #312 and NIOCC #2a respectively, in media prepared with 50 % diluted seawater. These two cultures also decolorized several synthetic dyes and industrial effluents when grown in seawater medium.

Since ascomycetes are more dominant than basidiomycetes in the marine environment (Kohlmeyer and Kohlmeyer 1979), it is to be expected that ascomycetes would play a major role as biomass degraders in marine habitats. *In vitro* production of cellulase and xylanase was reported among 47 ascomycetes obtained from mangrove stands of tropics (Bucher et al. 2004). Lignin-degrading enzyme production among these was comparatively less common. Most isolates were able to cause loss in birch wood mass, when used as substrate during a 24-week period. Five of these ascomycetous fungi solubilized lignin, with indices of lignin-solubilization comparable to terrestrial white-rot basidiomycetes. The authors conclude that to a certain extent, marine ascomycetes in the marine realm play a similar ecological role as that of terrestrial white-rot fungi (Bucher et al. 2004). Ascomyceteous species were shown to participate in the decay of dead plant biomass in salt marshes (Lyons et al. 2003).

Recent approach has been to screen for the laccase gene in marine environmental samples to assess their role in lignin degradation. Analysis of the fungal community in the salt marsh ecosystem using the diversity of the functional laccase gene indicated high diversity of laccase sequences in clones from environmental DNA and ascomyceteous fungi isolated from the decaying blades of *Spartina alterniflora* (Lyons et al. 2003).

3.3 Effect of nutritional parameters on production of lignin-degrading enzymes

Production of LDEs is affected by several culture conditions such as medium composition, carbon and nitrogen ratio, pH, temperature and aeration. Production of LiP and MnP in several terrestrial white-rot fungi was reported in the presence of high carbon and low nitrogen medium, a condition found in plants. This resulted in the development of a special culture medium (Tien and Kirk 1988) termed 'low nitrogen medium' (LNM). In contrast,

several white-rot fungi were reported to produce LDEs in the presence of high nitrogen (Kuhad et al. 1997). Production of LDEs was reported to take place only in shallow undisturbed stationary cultures (Boominathan and Reddy 1992). Addition of surfactants such as Tween 20 or Tween 80 to the culture media helped in overcoming this problem and production of these enzymes in bioreactors and agitated cultures was reported (Gomez-Alarcon et al. 1989). Addition of manganese to culture medium induced MnP but suppressed LiP production in *Phanerochaete chrysosporium* (Boominathan and Reddy 1992). Addition of veratryl alcohol induced both, LiP and MnP production in several white-rot fungi (Gill and Arora 2003; Boominathan and Reddy 1992). Several natural substrates like wood chips and shavings from soft and hard wood have been used to induce production of both peroxidases (Niku-Paavola et al. 1990).

With increasing interest in laccase from fungi for bioremediation applications, efforts have been made to enhance the laccase titre. Addition of various aromatic compounds analogous to lignin or lignin derivatives, have induced laccase production (Gianfreda et al. 1999). Nitrogen source and concentration in the culture medium are known to influence laccase production (Gianfreda et al. 1999). Addition of copper has been reported to increase laccase production by several folds (Galhaup and Haltrich, 2001; Galhaup et al. 2002). Recently, novel approaches to increase laccase production in white-rot fungi by addition of ethidium bromide and a range of vitamins, amino acids and antibiotics to the culture medium have been reported (Dhawan et al. 2003; Dhawan and Kuhad 2002; Dhawan et al. 2005). To make the fermentation process cost-effective, optimizing the culture conditions is a prerequisite for large-scale production of these enzymes. In recent years, several statistical designs collectively under response surface methodology have been introduced into the fermentation field to replace the “one-factor-at-a-time” method (Levin et al. 2005). Using these methodologies, production of laccase has been optimized in several species of white-rot fungi (Levin et al. 2005).

In light of this scenario in terrestrial fungi, marine fungi have received a scant attention. The marine-derived fungus NIOCC #312 produced both, MnP and laccase in low as well as high nitrogen medium containing 2.4 mM and 24 mM ammonium tartrate respectively (Raghukumar et al. 1999). It produced all the three LDEs to varying extents when grown in sea water medium containing sugarcane bagasse fibres, pine and poplar wood shavings as carbon and nitrogen source (Table 2). Another marine-derived fungus, NIOCC #2a, produced laccase in low as well

as high nitrogen medium prepared with seawater of 25 ppt (Table 2). Laccase production was enhanced by several folds on addition of copper or a combination of copper and guaiacol to 6 day-old culture growing in high nitrogen medium (peptone as the nitrogen source). Several phenolics and lignin-derivatives also enhanced laccase production in this culture (D'Souza et al. 2006). Glutamic acid and glycine were good sources of nitrogen in the presence of fructose as carbon source (Table 2) for enhancing laccase production in this fungus (D'Souza-Ticlo et al. 2006).

4. Application of lignin-degrading enzymes

Growing public awareness of the environment is forcing several industrial units to practice stringent pollution treatment on a top priority. Several strategies including biological approaches besides physical and chemical methods are devised to restore polluted environments. Oxidoreductive enzymes play an important role in degradation and transformation of polymeric substances. The partially degraded or oxidized products can easily be taken up by microbial cells where they are completely mineralized. Lignin-degrading enzymes are one such group of oxidoreductive enzymes, which have practical application in bioremediation of polluted environment (Husain 2006).

4.1. Decolorization of black liquor

The paper and pulp industry release large volumes of intensely colored black-liquors that contain toxic chlorinated lignin-degradation products. These products include chlorolignins, chlorophenols and chloroaliphatics (Ali and Sreekrishnan 2001). Besides, these paper mill effluents are highly alkaline and alter the pH of the soil and water bodies into which they are discharged. A vast literature is available on the involvement of lignin-degrading fungi from terrestrial sources in treatment of such effluents (Garg and Modi, 1999). Several white-rot fungi producing lignin peroxidases are shown to be involved in decolorization of black liquor (Thompson et al. 2001; Sahoo and Gupta, 2005; Wu et al. 2005) but in a few instances non-ligninolytic fungi have also been used for this purpose (Nagarathnamma and Bajpai, 1999; Sumathi and Phatak, 1999). The relative important role of the peroxidases in decolorization of black liquor has been debated. Frederick et al. (1991) demonstrated negligible decolorization of bleach plant effluent from paper and pulp mills by a mutant of *Phanerochaete chrysosporium* that lacked the ability to produce the peroxidases. A mutant of the same fungus which produced

only MnPs but not LiPs showed about 80% of the decolorizing activity exhibited by the wild type, indicating the relatively major role of MnPs in decolorization of this bleach plant effluent.

Very few attempts have been made to tap the potential of marine-derived fungi in treatment of colored effluents from paper and pulp mills. The marine-derived fungus NIOCC #312 which produced the peroxidases was better than NIOCC #2a, which produced laccase as the major LDE, in decolorizing black liquor from paper and pulp mills (Fig. 3). Although the production of MnP was induced in the presence of black liquor in NIOCC #2a, it did not decolorize the effluent (D'Souza et al. 2006). Earlier studies demonstrated that marine fungi *Sordaria fimicola* (NIOCC #298) and *Halosarpheia ratnagiriensis* (NIOCC #321), which produced MnP and laccase, brought about 65-75 % decolorization of bleach plant effluent within 8 days (Raghukumar et al. 1996). These results may indicate that MnP and not laccase plays an important role in decolorization of black liquor.

The pH of black liquor is always alkaline ranging from 8-11 and most of the lignin-degrading enzymes show optimum activity between pH 3-6. Therefore, black liquor is usually acidified prior treatment with these fungi (see references in Wu et al. 2005). These authors have reported optimum decolorization of black liquor at pH 8-11 using three terrestrial fungi. Raghukumar et al. (1996) reported decolorization of bleach plant effluent at pH 11 by the isolate NIOCC #312. These are the only reports demonstrating effective decolorization of black liquor at alkaline pH.

4.2. Decolorization of molasses spent wash

Molasses spent wash (MSW) is a by-product of sugar mills and alcohol distilleries, where the starting material is molasses. It contains mostly dark brown colored recalcitrant compounds, collectively termed as melanoidin, which are formed by the Maillard amino-carbonyl reaction (Wedzicha and Kaputo 1992). These compounds are toxic to many microorganisms including those generally involved in wastewater treatment processes (Kitts et al. 1993). Anaerobic digestion of MSW-containing effluents is one of the treatments followed by distilleries and the resulting dark brown sludge is used as fertilizer. Dark brown color of these effluents remains a major problem for the distilleries.

Color removal from MSW using terrestrial white-rot fungi was shown to be MnP-dependent in *Phanerochaete chrysosporium* (Dehorter and Blondeau 1993) and laccase-dependent in *Trametes versicolor* (González et al. 2007). The process was sorbose oxidase- and glucose oxidase-dependent in mitosporic fungi *Aspergillus fumigatus* (Ohmomo et al. 1987) and *A. oryzae* (Ohmomo et al. 1988) and in the basidiomycete *Coriolus* sp. No. 20 (Watanabe et al. 1982). Raghukumar and Rivonkar (2001) demonstrated MnP-independent decolorization of MSW by the marine-derived fungus NIOCC #312. About 60% decolorization of MSW was brought about when added at 50% concentration in seawater medium. Subsequently it was demonstrated that decolorization was dependent on glucose oxidase levels in the culture medium (Raghukumar et al. 2004). The activity of MnP and percentage decolorization of MSW by the isolate NIOCC #312 did not correlate (Fig. 4A) but there was a direct co-relation between concentration of glucose oxidase and decolorization of MSW (Fig. 4B). It was suggested that H₂O₂ produced by glucose oxidase may act as a bleaching agent. In another marine-derived fungus NIOCC #2a, 80% of color removal was obtained by day 3 (Fig. 3). Glucose oxidase production in this fungus ranged from 8769 to 23,253 U L⁻¹ but did not correlate with decolorization of MSW. It was further demonstrated that marine fungi are capable of decolorizing MSW effectively in the presence of seawater of 15-34 ppt salinity (Raghukumar et al. 2000; D'Souza et al. 2006). Studies to understand the mechanism of decolorization of MSW in marine fungi merit further attention.

4.3. Decolorization of synthetic dyes and textile effluents

Textile industries release highly colored effluents containing large amounts of a mixture of dyes, many of which are resistant to degradation leading to deleterious effects on the aquatic life (Rodriguez et al. 1999). Exhaustive reviews on decolorization of synthetic dyes (Wong and Yu 1999; Peralta-Zamora et al. 2003) and dye wastewaters using white-rot fungi and their lignin-degrading enzymes have appeared (Fu and Viraraghavan 2001; Wesenberg et al. 2003). Textile effluents besides containing dyes, also have extreme pH values and contains salts, often at very high ionic strength. Thus, in spite of the highly efficient terrestrial strains reported, marine fungi may find use in decolorization of industrial effluents with these added factors of salt content and extreme pH values. The marine-derived culture NIOCC #2a with laccase proved to be more efficient in the decolorization of textile effluents and synthetic dyes than the culture NIOCC

#312 having MnP and LiP activity (Fig. 3). Two textile effluents, textile effluent A (TEA) with pH of 8.9 and the textile effluent B (TEB) with pH 2.5 were added separately at 10% final concentration in the fungal cultures grown in seawater medium. Among the synthetic dyes, Brilliant Green and Congo Red were almost totally decolorized by NIOCC #2a, whereas Remazol Brilliant Blue R and Poly R-478 were better decolorized by NIOCC #312 than NIOCC #2a (Fig. 3, Raghukumar et al. 1999; D'Souza et al. 2006).

The role of laccase in terrestrial fungi in decolorization of dyes and dye wastewaters is undisputed (Wong and Yu 1999; Fu and Viraraghavan 2001; Wesenberg et al. 2003). In the marine-derived fungus NIOCC #312, decolorization of the dye RBBR directly correlated with MnP concentration (Fig. 4C). On the other hand, decolorization of the effluents or dyes did not correlate with laccase concentration in the marine-derived fungus NIOCC #2a (D'Souza et al. 2006). A number of filamentous fungi including a halophilic new species of *Gymnoscella marismortui* isolated from the Dead Sea decolorized synthetic dyes belonging to 4 different groups (Molitoris et al. 2000). However, it is not known whether these fungi produced any of the lignin-degrading enzymes.

4.4. Degradation of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are formed during incomplete combustion of fossil fuels. They consist of analogs of benzene having two or more aromatic rings in various alignments. Most of the low molecular weight PAHs are very toxic and adversely affect aquatic life. Degradation of PAHs by MnP-producing terrestrial white-rot fungi has been demonstrated in *Irpex lacteus* (Baborova et al. 2006), *Nematoloma frowardii* (Sack et al. 1997), *Phanerochaete chrysosporium* (Moen and Hammel 1994) and several other fungi. Laccase-producing white-rot fungi such as *Trametes versicolor* (Collins et al. 1996), *Pleurotus ostreatus* D1 (Pozdnyakova et al. 2006) and *Coriolopsis gallica* (Picard et al. 1999) have been implicated in PAHs degradation.

The marine-derived fungi, NIOCC #312 and NIOCC #2a facilitated removal of phenanthrene, a PAH from the medium by adsorption on the fungal mycelium. Phenanthrene was completely metabolized or transformed into more polar derivatives by NIOCC #312 by day 6 (Raghukumar et al. 2006). Phenanthrene added to the culture NIOCC #2a at 20 ppm concentration was metabolized to an intermediate compound (Fig. 5A & B). The NMR spectrum obtained was consistent with earlier data obtained with *Phanerochaete chrysosporium*, a

terrestrial basidiomycete (Sutherland et al. 1991). The mass spectrum of this metabolite showed a molecular mass of 212 (data not shown). The comparison of the fragmentation pattern of this peak with the library established its identity to be phenanthrene (*trans*)-9,10-dihydrodiol (unpublished results). The fragment ions at m/z 194,181,166,165 and 152 were consistent with mass spectrum of phenanthrene 9,10-dihydrodiol reported previously (Sutherland et al. 1991). In both the fungi, majority of phenanthrene removal and further metabolism was by the fungal mycelia. It was suggested that EPS that form a sheath around the fungal mycelium plays an active role in the adsorption and degradation of xenobiotic pollutants (Bes et al. 1987). We have shown the presence of EPS in the isolate NIOCC #312 (Raghukumar et al. 2006) and in NIOCC #2a (D'Souza et al. 2006). A part of the extracellular LDEs are likely to get trapped in the EPS surrounding the mycelium, which may further help in degradation of PAHs (Barassa et al. 1998).

5. Effect of pollutants on lignin-degrading enzymes

Several workers have demonstrated the production of LDEs especially laccase, in terrestrial fungi induced in the presence of aromatic compounds (Mai et al. 2000; Carbajo et al. 2002; Marques et al. 2004). This induction was associated with induced expression of laccase gene *cglcc1* in the presence of tannic acid in the white-rot fungus *Coriolopsis gallica* and *lcc1* and *lcc2* in *Trametes* sp. I-62 (Carbajo et al. 2002; González et al. 2007).

We found that marine fungi were no exception to this. Raghukumar (2000) demonstrated increased MnP production in three marine fungi, *Sordaria fimicola* (NIOCC # 298), *Halosarpheia ratnagiriensis* (NIOCC #321) and marine-derived basidiomycete (NIOCC #312) in the presence of bleach plant effluent from a paper and pulp mill. Laccase production in these fungi was reduced in the presence of bleach plant effluent. In the culture medium containing either MSW or black liquor, production of MnP was generally enhanced, whereas laccase production was inhibited in NIOCC #312 (Table 3). In the isolate NIOCC #2a, black liquor and both the textile effluents in the culture medium induced laccase production whereas, MSW resulted in decreased laccase production (Table 3). D' Souza et al. (2006) demonstrated that textile dye effluent, black liquor and MSW enhanced laccase production in the same fungus. However, the media used in these two different studies differed in their nitrogen source. Subsequently, it was proven that laccase production in the presence of effluents was strongly influenced by the nitrogen source (D' Souza et al. 2006a).

Addition of phenanthrene (12 ppm) increased fungal biomass production in both NIOCC #312 (Raghukumar et al. 2006) and in NIOCC #2a. However in the presence of phenanthrene, maximum biomass was attained earlier in NIOCC #2a than in NIOCC # 312 (Fig. 6A & 6B).

6. Methods of treatment of effluents

Several methods of treatment of industrial effluents with fungi for decolorization have been reported (Thompson et al. 2001). Whole cultures, mycelial pellets, immobilized fungi or their enzymes, free enzymes and biofilm (Wu et al. 2005) are some examples of such treatments.

6.1. Treatment of effluents with whole versus immobilized cultures

Most of the experiments for decolorization of various effluents and xenobiotics with terrestrial basidiomycetes are carried out by addition of effluents to pre-grown, shallow, static cultures having substantial fungal biomass (Reddy 1995). Care should be taken not to disturb the fungal mat, as this affects LDE production. The efficiency of the fungal mat decreases on repeated usage. In nature, most of these fungi grow in solid-state conditions, in the near absence of free water. Such cultures when immobilized on a variety of solid supports have been shown to decolorize various effluents (Ohmomo et al. 1987; Shin et al. 2002; Šušla et al. 2007). Zhang et al. (1999) used immobilized a white-rot fungus strain F29 to decolorize Orange II, a toxic dye. Wu et al. (2005) used biofilm of white-rot fungi grown on a porous plastic rings for decolorization of paper mill effluent.

The marine-derived fungus NIOCC #312 when immobilized in polyurethane foam (PUF) cubes, decolorized MSW by 70% by day five and most of the high molecular weight compounds were removed by this treatment. The same batch of immobilized fungus could be reused up to four cycles for treatment of MSW. The immobilized fungus remained viable for a minimum of 75 days (Raghukumar et al. 2004). When NIOCC # 2a, was immobilized in PUF, it was able to decolorize MSW to varying degrees and also reduce COD levels by 50% within 72 h (unpublished results). The porous nature of PUF increases the diffusion of oxygen, resulting in increased production of LDEs as well as enhanced activity. Since many of the white-rot fungi produce LDEs under oxygen-saturation conditions (Boominathan and Reddy, 1992). Thus immobilized marine fungi offer an alternative technology for an effective environmental bioremediation.

6.2. Treatment of effluents with crude/purified enzyme and immobilized enzymes

Several studies with terrestrial white-rot fungi showed involvement of either mycelial-bound lignin-degrading enzymes or H₂O₂ generating mechanism for degradation of lignin and decolorization of several effluents (Wesenberg et al. 2003; Svabodová et al. 2008). It has been shown by immunochemical methods that sites of H₂O₂ production are located in the periplasmic space of the fungal mycelium in *P. chrysosporium* (Forney et al. 1982). By using an immunocytochemical technique Garcia et al. (1987) demonstrated that LiPs are located very close to the cell membrane in *Phanerochaete chrysosporium* and other white-rot fungi. Further, washed pellets were shown to retain a part of the lignin-degrading enzyme activity (Kurek and Odier 1990). Earlier studies on lignin-degradation therefore were concentrated using whole cultures of white-rot fungi showing lignin-peroxidases activity. As reports about laccase-producing white-rot fungi increased, lignin-degradation work and decolorization applications with partially purified or crude laccase gained importance (Wong and Yu 1999; Rodriguez et al. 1999; Kokol et al. 2007; Šušla et al. 2007). Laccase-mediated degradation of PAHs has also been widely reported (Pozdnyakova et al. 2006 and references therein).

Decolorization of various effluents by culture supernatant collected from liquid culture was possible with the marine-derived fungus NIOCC #2a (Table 4) whereas, the same by NIOCC #312 was not effective. It is possible that the mycelial-bound lignin-degrading enzymes or the H₂O₂-generating mechanism in NIOCC #312 is responsible for decolorization of various effluents. This isolate produces more peroxidases than laccase and the former require constant supply of H₂O₂, which is not possible practically while using mycelia-free culture supernatants.

Immobilized laccases have been extensively used for decolorization of textile effluents and synthetic dyes (Abadulla et al. 2000; Peralta-Zamora et al. 2003). Various supports such as alumina particles, chemically modified silica, amberlite and glass-ceramic have been used for this purpose (Abadulla et al. 2000; Peralta-Zamora et al. 2003). It has been demonstrated that treatment of azo dyes with free laccase enzyme results in darkening of the solution due to coupling of the degraded products with the unreacted dyes (Zille et al. 2005). Using immobilized enzyme would avoid these coupling reactions. This knowledge gained with terrestrial fungus, *Trametes villosa* should be considered while using laccases from marine-derived fungi for any bioremediation processes.

6.3. Treatment of effluents with exopolymeric substances (EPS) produced by white-rot fungi

Color removal by biosorption is an alternative to the economically disadvantageous physical and chemical methods of treatment (Namasivayam et al. 1996). Color removal by adsorption is an alternative option. Biological adsorbents include plant, fungal and bacterial biomass, either live or dead (Robinson et al. 2001). Bioadsorption potential of microbial exopolymeric substance is well known (Wingender et al. 1999). Basidiomycetous fungi are reported to produce large amount of EPS (Smith et al. 2002; Maziero et al. 1999). These polymeric substances form a sheath around the fungal hyphae and may be water soluble or insoluble forms. The ligninolytic fungus *Phanerochaete chrysosporium* also produces polysaccharide sheath and dissolution of this sheath by addition of glucanase inhibited lignin degradation (Bes et al. 1987). This suggests an active involvement of EPS in lignin degradation process.

The participation of fungal EPS in degradation of xenobiotics has been assumed but not yet investigated. D'Souza et al. (2006) reported for the first time its use in decolorization of colored industrial effluents. Extracellular polymeric substance was produced by the marine-derived fungi NIOCC #2a and NIOCC #312. Freeze-dried EPS of NIOCC #2a was effective in decolorizing various industrial effluents (Table 4) but that of NIOCC #312 was not effective for this purpose. The EPS produced by a marine cyanobacterium *Cyanothece* sp. was reported to remove dyes from textile effluents by gelation under alkaline conditions (Shah et al. 1999). EPS are highly charged and thus absorb water and become gel-like (Whiteley and Lee, 2006). EPS produced by basidiomycetes function as a supporting network in which some of the excreted enzymes get trapped (Ruel and Joseleau, 1991). Biofilm prepared with EPS for treatment of effluent needs to be considered for the future bioremediation processes. Lignin-degrading enzymes immobilized in fungal EPS is another possible strategy for bioremediation purpose. As EPS production is reported to be NaCl-dependent in cyanobacteria (Philippis & Vincenzini, 1998), marine fungi should be screened for EPS production.

7. Detoxification of effluents

It has been reported that effluents from paper and pulp mills and textile dye waste waters are toxic and mutagenic (Reddy 1995). Laccases are shown to render phenolic compounds in

effluents, less toxic via degradation or polymerization reactions or by cross-coupling of pollutant phenols with naturally occurring phenols (Abadulla et al. 2000). Toxicity of several textile dyes, including azo compounds, was reduced by treatment with laccase from *Trametes hirsuta* (Abadulla et al. 2000). Eight white-rot fungi grown in green olives reduced phenolic content by nearly 70-75 % but phytotoxicity was not reduced (Aggelis et al. 2002). All of these fungi produced laccase and some of them produced MnP. *Rhizomucor pusillus* strain RM7, a mucoralean fungus and a white-rot fungus *Coriolus versicolor* were shown to detoxify bleach plant effluent (Driessel and Christov 2001).

Molasses spent wash treated with the marine-derived fungus NIOCC #312 was detoxified as measured by serum sorbitol dehydrogenase (SSDH) assay (Raghukumar et al. 2004). Increased levels of SSDH are indicative of chemically induced liver damage in fish (Dixon et al. 1987). Accordingly, there was a drastic reduction in SSDH activity in fungus-treated MSW (Table 5). Toxicity test was also carried out by 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 an estuarine fish *Oreochromis mosambicus* showed damaged nuclei as comets, whereas, in the presence of fungus-treated MSW only 9 % of the cells showed presence of comets indicating a near total removal of toxic components after the fungal treatment (Table 5). There was a 30% reduction in benzo(α)pyrene, one of the toxic polycyclic aromatic hydrocarbons in MSW on treatment with the fungus (Table 5), which might be one of the toxic components present in MSW. A decrease in phenolics and PAHs might be responsible for cumulative reduction in toxicity of the fungus-treated MSW (Raghukumar et al. 2004). Reduction in COD as an indicator of detoxification of the effluents has been shown in several white-rot fungi (Table 6). Molasses-based pharmaceutical effluent in the presence of 1 % glucose when added to NIOCC #2a and NIOCC #312 cultures, showed a 57 and 67 % reduction in COD after 72 h respectively. The total phenolics were reduced by 20 and 35% respectively in the same treatment (unpublished results). Thus, besides decolorization, detoxification of wastewaters is an important parameter to be monitored.

8. Role of mediators in treatment of effluents

Lignin-degradation by white-rot fungi that produce only laccase led to the discovery of low molecular weight enzyme mediators. These laccase-mediator systems (LMS) involves use of low

molecular weight compounds that are oxidized by the enzyme to stable radicals which in turn act as redox mediators and oxidize other compounds that are not substrates of laccase. Lack of correlation between laccase activity and degradation of xenobiotic compounds further supports the role of LMS (Johannes and Majcherczyk 2000). These mediators can either be natural compounds produced by fungi or plants or synthetic compounds such as, ABTS, 1-hydroxybenzothiazole (HBT), violuric acid (VIO) and N-hydroxyacetanilide (NHA) (Camarero et al. 2005). Degradation products of lignocellulose such as acetosyringone, *p*-coumaric acid, syringaldehyde, vanillin, can also act as mediators. A metabolite, 3-hydroxyanthranilic acid produced by the white-rot fungus *Pycnoporus cinnabarinus* was shown to act as a mediator in degradation of lignin (Eggert et al. 1996). Natural mediators have been extracted from black liquor of eucalyptus-based kraft pulping (Camarero et al. 2007). The redox potential of laccase alone is not high enough to break C-H aliphatic bonds. In the presence of a redox mediator, oxidation of such bonds becomes feasible. The enzyme oxidizes the mediator, which can diffuse away from the enzyme and oxidize a substrate. The reduced mediator is ready for the next cycle (Wells et al. 2006). Laccase-mediator system has found applications in paper pulp delignification (Camarero et al. 2007), degradation of PAHs (Johannes and Majcherczyk 2000) and industrial dyes (Camarero et al. 2005).

Preliminary results with the marine-derived fungus NIOCC #2a showed that treatment of sugarcane baggase with laccase brought about reduction in lignin content, as determined by kappa number. Lignin content was further reduced in the presence of ABTS, a mediator (unpublished results). Role of mediators in laccases from marine fungi has remained a totally ignored subject. As marine-derived fungi are a vast source of biological compounds, search for the presence of natural mediator compounds in them is an exciting new field.

9. Conclusion and future perspectives

Microbial decolorization and degradation of colored effluents is a cost-effective and promising green technology for treatment of such effluents. Reports of white-rot fungi that show lignin-degrading ability in saline conditions are very few. Industrial effluents are mostly alkaline and rich in carbonates, chlorides and sulfates. In light of this, the marine fungi discussed here hold good promise for the application of bioremediation of colored effluents under saline conditions. The marine-derived fungi NIOCC #312 with MnP and LiP and NIOCC #2a with

laccase as the major lignin-degrading enzyme were equally effective in decolorization of various effluents and degradation of PAHs under marine conditions. Marine-derived fungi are often more effective than terrestrial fungi in treatment of various colored effluents since they are better adapted to perform under such extreme conditions (Table 6).

Isolation of fungi from marine environment using media prepared with sea water of different salinities incorporated with lignin model compounds (Poly R 478) or laccase substrates, guaiacol or ABTS is recommended for obtaining selective lignin-degrading fungi. Growth and enzyme production of such cultures under saline conditions is to be maintained by growing them in seawater. Media with low nitrogen and high carbon content is preferable for culturing these fungi.

Development of EPS-based bioremediation with marine fungi needs concerted efforts of basic and applied research. Dependency of EPS production on the concentration of NaCl in cyanobacteria is well known (Philippis and Vincenzini 1998). Similarly, production of EPS in obligate and facultative marine fungi in the presence of NaCl needs to be studied in detail to increase the production of EPS in such fungi to bring out a biotechnologically-developed product in the field of bioremediation. Search for hyper-production of EPS in marine fungi is a challenging field.

Screening of marine-derived fungi as a source of new naturally occurring mediators for enhancing laccase production require to be carried out systematically on a large scale. Understanding the mechanism of degradation and detoxification of industrial effluents by marine lignin-degrading fungi are some of the key areas for future research.

Most of the LDEs show optimum activity around 30-35°C and at pH of 3-4. Industrial effluents generally have alkaline pH and temperatures above ambient. Therefore, fungi from the marine environment with LDE activity at alkaline pH preferably around 40°C should be selectively isolated for bioremediation purposes. Recombinant enzymes with these properties or protein engineering of the enzymes should be considered to achieve this goal. Increasing efficiency of these enzymes for electron transfer should be aimed at, by producing hybrid enzymes. Effect of carbon and nitrogen sources and pH of the effluent on the fungus and / or enzyme used for bioremediation should be studied in detail. The efficacy of the isolates in treating effluents from common effluent treatment (CET) plant which contain mixtures of

effluents from various small-sized mills should also be assessed. Laccase can act alone or in the presence of low molecular weight mediators as well as in the absence of the fungal biomass. On the other hand, LiP and MnP require an H₂O₂-generating system from the fungal mycelium and thus they require presence of the live fungal biomass for lignin-degradation. Immobilized peroxidase-producing fungi may be tested for this purpose. A laccase-hyperproducing strain from marine environment using inexpensive growth medium would be a suitable alternative. Co-immobilization of all the three LDEs or fungal isolates that produce them could be a promising technology for treatment of colored effluents.

Acknowledgements

Ms D'Souza-Ticlo acknowledges the senior research fellowship from Council for Scientific and Industrial Research (CSIR), New Delhi. Mr.Verma is grateful to Department of Biotechnology, New Delhi for a junior research fellowship. The senior author thanks CSIR for financial support. This is NIO's contribution No.

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Table 1. Comparison of the lignin-degrading enzyme production in low nitrogen medium (LNM) by the two marine-derived fungi, NIOCC #312 and NIOCC #2a.

NIOCC #312	Days						
	3	5	7	9	11	13	15
MnP (U L⁻¹)	0	47	112	298	338	276	118
LiP (U L⁻¹)	8	12	29	52	15	0.1	0
Laccase (U L⁻¹)	0.8	0.24	30.5	1.4	2.3	0.6	0.6
Days							
NIOCC #2a	3	5	7	9	11	13	15
MnP (U L⁻¹)	1.5	80	82	79	84	105	93
LiP (U L⁻¹)	0.3	0.5	0	0	0	0.4	0.6
Laccase (U L⁻¹)	571	8425	5097	6125	8946	12613	7415

Table 2. Production of lignin-degrading enzymes (U L^{-1}) by the two marine-derived fungi grown in seawater media. Maximum activity recorded in each media is shown.

Culture	Media	MnP	LiP	Laccase
NIOCC #312	Low nitrogen medium (LNM)*	654	52	30
	High nitrogen medium (HNM)*	50	0	20
	Sugarcane bagasse*	450	20	15
	Pine shavings*	110	4	9
	Poplar shavings*	110	0	8
NIOCC #2a	LNM with Ammonium tartrate as N source	40	0	3428
	HNM (peptone as N source)**	13	1	921
	HNM + Cu**	37	0	42,933
	LNM with KNO_3 as N source***	0	26	16,687
	LNM with glycine as N source***	12	2	34,659
	LNM with glutamic acid as N source***	0	17	47,567
	LNM with beef extract as N source***	25	25	10,346
	LNM with corn steep liquor as N source***	46	26	12,973

*Source data: Raghukumar et al. 1999; ** source data: D'Souza et al. 2006a;

*** source data: D'Souza et al. 2006b

Table 3. Effect of effluents on lignin-degrading enzyme production in NIOCC #312 and NIOCC #2a. All the effluents were added to 10% final concentration in the culture medium.

LDE	Treatment	Enzyme Activity (U L ⁻¹) in NIOCC #312					
		Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
MnP	Without effluent	47	112	298	338	276	118
	With MSW	51	78	263	226	225	142
	With BL	16	255	331	483	66	13
	With TEA	104	0	0	54	0	0
	With TEB	52	0	0	0	0	0
Laccase	Without effluent	0	0	0	0	0	0
	With MSW	0	0	16	56	56	40
	With BL	1	1	0	1	0	0
	With TEA	104	0	0	54	0	0
	With TEB	52	0	0	0	0	0
		Enzyme Activity (U L ⁻¹) in NIOCC #2a*					
MnP	Without effluent	80	82	79	84	105	93
	With MSW	0	22	69	78	193	79
	With BL	197	513	516	683	530	367
	With TEA	86	53	94	0	0	0
	With TEB	8	14	8	48	13	0
Laccase	Without effluent	8425	5097	6125	8946	12613	7415
	With MSW	566	629	644	1266	720	704
	With BL	197	513	516	683	530	367
	With TEA	5603	3348	3278	3195	4279	2900
	With TEB	1728	799	412	676	1549	848

* Source data: D'Souza et al. 2006a. MSW=molasses spent wash; BL=black liquor from paper and pulp mill; TEA= textile effluent A; TEB=textile effluent B.

Table 4. Decolorization of effluents by fungus-free extracellular culture supernatant and extracellular polymeric substance (EPS) produced by the fungus NIOCC #2a (D'Souza et al. 2006a)

Effluents (10%)	(% Decolorization by culture supernatant)		(% Decolorization by EPS)	
	6 h	12 h	12 h	24 h
Textile Effluent A	9	11	11	100
Textile Effluent B	14	22	35	100
Molasses Spent Wash	34	33	12	100
Black Liquor	71	59	41	100

Culture supernatant (500 μ l) with 9 U ml⁻¹ laccase activity was incubated with an equal volume of diluted effluent at pH 6.0 and 60°C. Freeze-dried EPS (10 mg) of the fungus was incubated with effluents at 60°C. Decolorization of both was measured at the absorbance maxima specific to the effluents. The percentage decolorization was calculated based on the initial readings. All the values are mean of 2 replicates.

Table 5. Detoxification of molasses spent wash by NIOCC #312 tested by various toxicity assays (Raghukumar et al. 2004).

Toxicity Test	Untreated MSW	Treated MSW
Serum sorbitol dehydrogenase	122 U ml ⁻¹	2.6 U ml ⁻¹
Comet assay (damaged nuclei)	85 %	9 %
Benzo (α)pyrene	3.8 μg ml ⁻¹	1.2 μg ml ⁻¹

Table 6. Comparison of bioremediation potential of terrestrial white-rot fungi with that of marine-derived fungi

NIOCC #312 and NIOCC #2a.

Effluent	Fungus	% Decolorization	% Phenol Reduction	% COD Reduction	Enzyme (s) involved	Technique	Reference
Alcohol Distillery Waste (3%)	<i>Pycnoporus coccineus</i>	50 – 55 (9 days)	70 – 80 (9 days)	193 (9 days)	MnP	Immobilised on Polyurethane foam	Charattanamankorn et al. 2005
Pulp and Paper Mill Effluent	Consortium containing: 1) <i>Merulius (Syn. Phlebia) aureus</i> . 2) An unidentified basidiomycete 3) <i>Fusarium Sambucinum</i>	79 (4 days)	---	89 (4 days)	---	Static Liquid Culture	Malaviya & Rathore 2006
Textile Effluent	<i>Phanerochaete sordida</i>	90 (48 h)	---	---	MnP	Static Liquid Culture	Harazono & Nakamura 2005
Olive Mill Wastewater (20% & 50%)	<i>Phanerochaete chrysosporium</i>	93 (15 days)	90 (15 days)	74 (15 days)	MnP, LiP & Laccase	Static Liquid Culture	Kissi et al. 2001

Pulp Mill Effluent	<i>Trametes versicolor</i>	90 (7 days)	---	69 (7 days)	---	Mycellial Pellets	Meghna et al. 1995
Green Olive Wastewater	<i>Pleurotus ostreatus</i>	49 (30 days)	76 (30 days)	12 (30 days)	MnP, MnIP, Laccase	Static Liquid Culture	Aggelis et al. 2002
Olive Mill Effluent	<i>Penicillium spp.</i>	85 – 90 (20 days)	55 – 60 (20 days)	60 – 70 (20 days)	---	Static Liquid Culture	Robles et al. 2000
Paper Mill Effluent	<i>Trametes versicolor</i>	---	70 (3 h)	---	Laccase with mediator	Static Liquid Culture	Minussi et al. 2007
MSW	<i>Coriolus versicolor</i>	53 (10 days)	---	---	---	Static Liquid Culture	Fitz Gibbon et al 1998
Sugar Refinery Waste Water	<i>Phanerochaete chrysosporium</i>	55 (3 days)	63 (3 days)	48 (3 days)	---	Immobilised on PUF and Scouring Web	Guimaraes et al. 2005
MSW (6.25% v/v)	<i>Coriolus versicolor</i>	71.5 (10 days)	90 (10 days)	---	---	Shake Flask Technique	Kumar et al. 1998
Bleach Plant Effluent	<i>Rhizopus oryzae</i>	92 – 95 (24 h)	---	50 (24 h)	---	Immobilised on PUF	Nagarathnamma et al. 1999
Nitrocellulose Industry Effluent	<i>Aspergillus sp. Lentinus edodes</i>	83 – 95 (24-72 h)	56 – 79 (24-72 h)	---	---	Shake Flask Technique	Souza et al. 2005

MSW (10%)	(NIOCC #312)	75 (3 days)	50 (8 days)	50 (8 days)	---	Static Liquid Culture	Raghukumar & Rivonkar 2001
MSW (10%)	(NIOCC #312)	78 – 80 (7 days)	---	---	Glucose Oxidase	Immobilised on PUF	Raghukumar et al. 2004
TEA (10 %)	(NIOCC #312)	18 (3 days)	50 (3 days)	72 (3 days)	-	Static Liquid Culture	In this review
TEB (10 %)	(NIOCC #312)	37 (3 days)	22 (3 days)	79 (3 days)	-	Static Liquid Culture	In this review
Bleach Plant Effluent (2000 Color Units)	(NIOCC #312)	80 (8 days)	-	-	MnP	Static Liquid Culture	Raghukumar et al. 1996
BL (10%)	(NIOCC #2a)	40 (72 h)	14 (72 h)	50 (72 h)	-	Static Liquid Culture	D'Souza et al. 2006
MSW (10%)	(NIOCC #2a)	71 (48 h)	20 (48 h)	50 (48 h)	laccase	Static Liquid Culture	D'Souza et al. 2006
TEA (10%)	(NIOCC #2a)	64 (6 days)	87 (6 days)	82 (6 days)	laccase	Static Liquid Culture	D'Souza et al. 2006
TEB (10%)	(NIOCC #2a)	92 (3 days)	69 (3 days)	85 (3 days)	laccase	Static Liquid Culture	D'Souza et al. 2006

Legends to the Figures

Fig. 1. Schematic diagram illustrating the role and activity of various extracellular lignin-degrading enzymes produced by fungi (Modified from Field et al. 1993). MnP = manganese-dependent peroxidase; LiP = lignin peroxidase; VP = versatile peroxidase

Fig. 2. Schematic diagram illustrating the process of degradation of plant material in the marine habitat.

Fig. 3. Comparison of the efficiency of the two marine-derived fungi, NIOCC #312 and NIOCC #2a. The dyes and pollutants were added to the 6-day old cultures and % decolorization was estimated after 3 days. AB= Azure B; BG= Brilliant Green; CR= Congo Red; Poly-B= Poly-B 411; Poly-R= Poly-R 478; CV= Crystal Violet; RBBR= Remazol Brilliant Blue R; BL= Black Liquor; MSW= Molasses spent Wash; TEA= Textile effluent A; TEB= Textile effluent B. Standard deviation was < 5%.

Fig. 4. A) No correlation observed between MnP production and decolorization of MSW in NIOCC #312; B) Correlation observed between glucose oxidase production and decolorization of MSW in NIOCC #312; C) Correlation observed between MnP production and decolorization of Remazol Brilliant Blue R dye by NIOCC # 312.

Fig. 5. A) A 300-MHz Proton NMR spectra of the standard phenanthrene; B) intermediate degradation product, phenanthrene (*trans*)-9,10-dihydrodiol extracted from the fungal biomass (NIOCC #2a) on day 6. The numbers on peaks correspond to the numbers shown in the chemical structures in the insets. The insets in (A) and (B) show the structure of phenanthrene and phenanthrene (*trans*)-9,10-dihydrodiol respectively.

Fig. 6. Effect of phenanthrene on biomass production in A) NIOCC #312 and; B) NIOCC #2a.

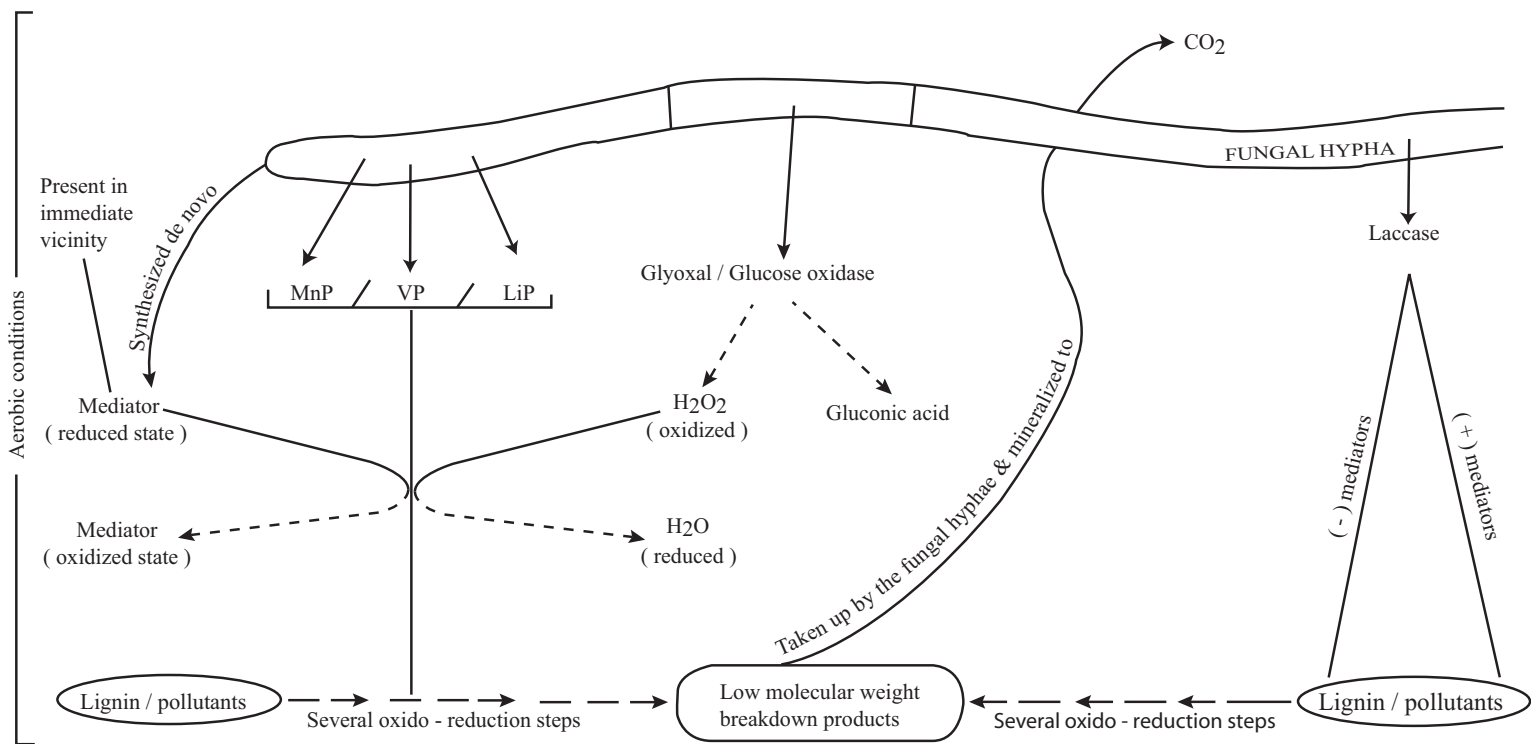


Fig.1

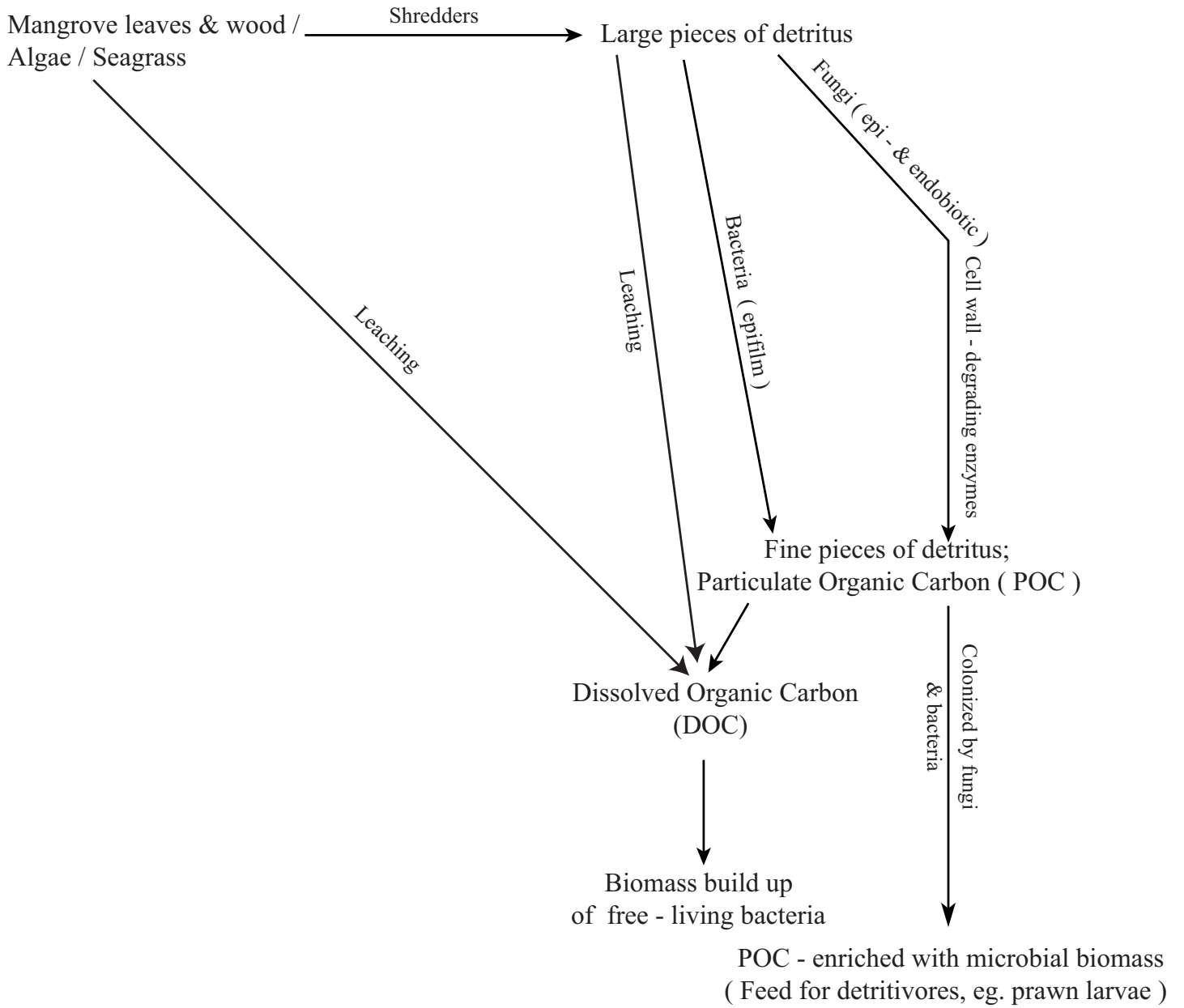


Fig. 2

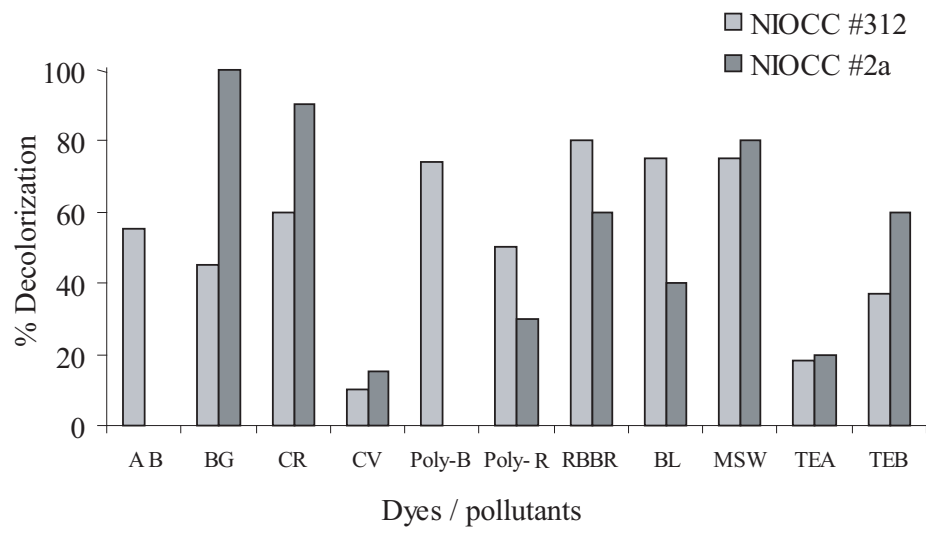


Fig. 3

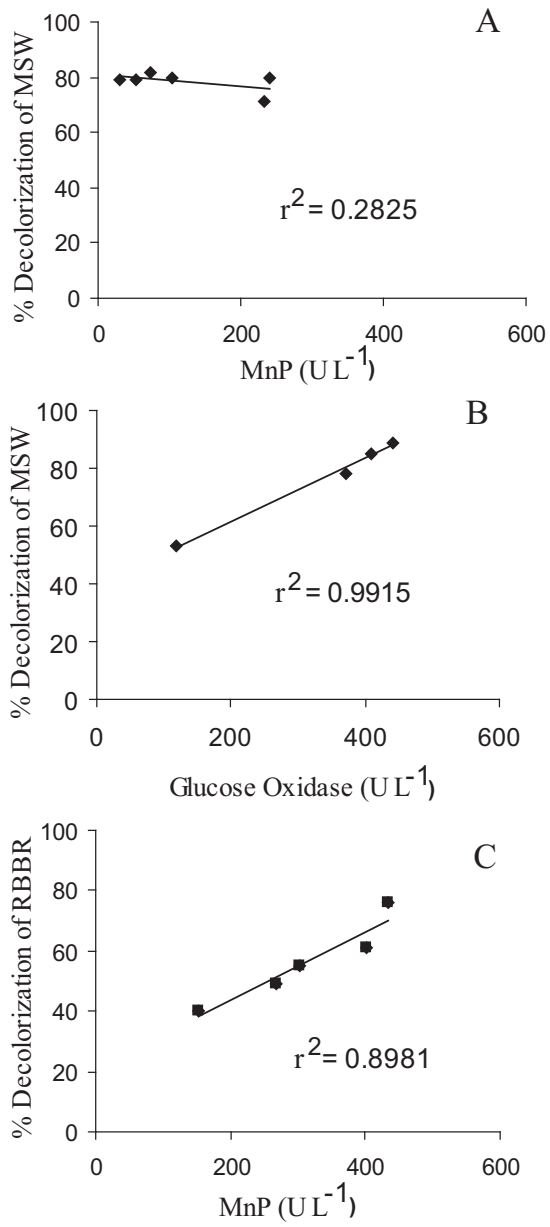


Fig.4

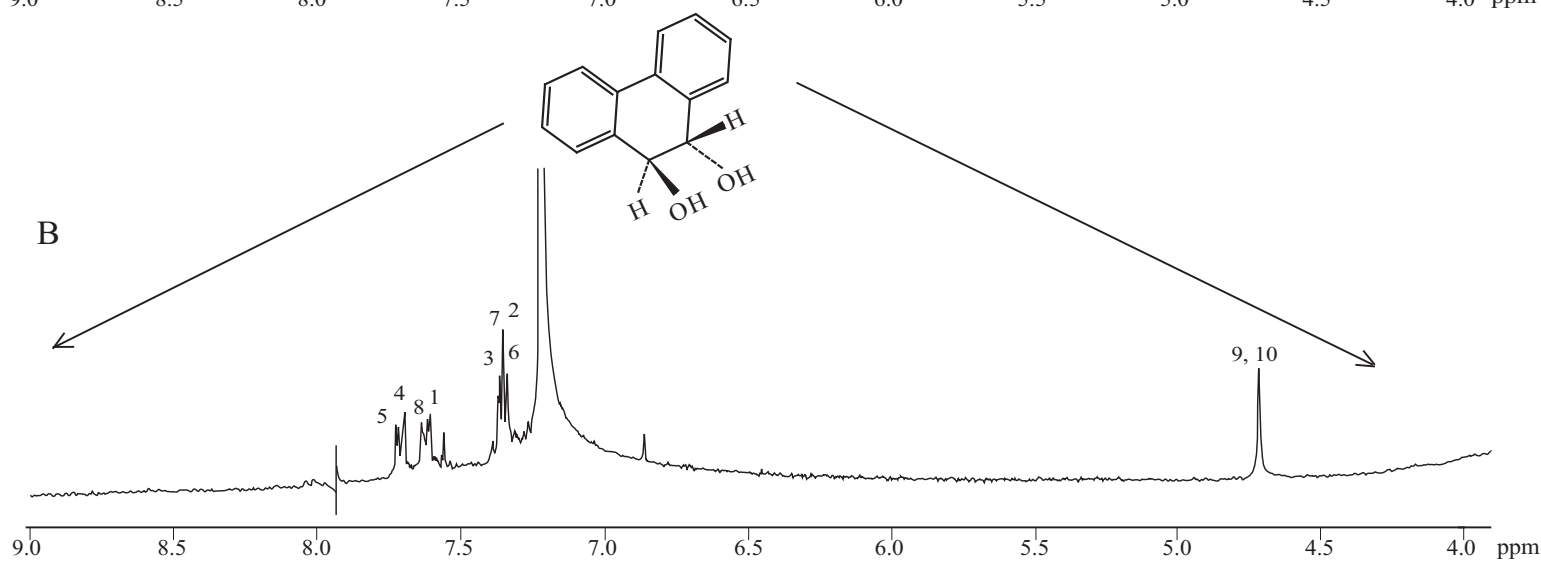
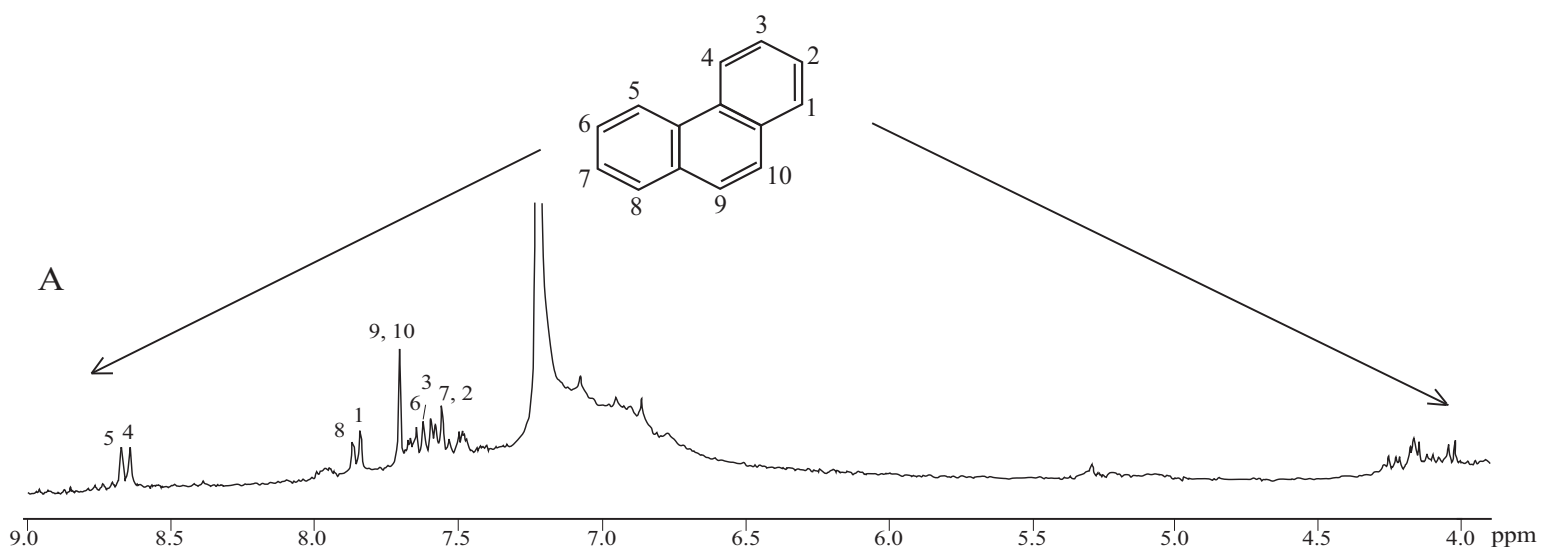


Fig. 5

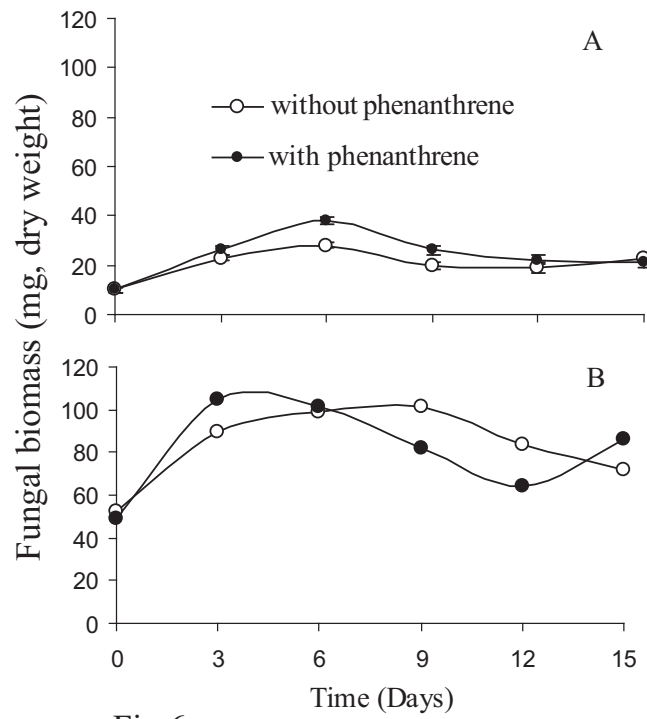


Fig.6