Chapter 1
Review of Literature
1.1 Industrial pollutants/effluents

Color is the first contaminant to be recognized in wastewater. These effluents dumped into the water bodies prohibit usage and affects its aesthetic and sentimental value. Paper and pulp mills, molasses based-alcohol distilleries, tanneries, dye-making units, and textile industries 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. Growing public awareness of the environment is forcing several industrial units to practice stringent pollution treatment on a top priority.

1.1.1 Paper and pulp mills

The pulp and paper industry is quite old. In India, more than 150 paper and board mills with an installed capacity of nearly 3 million tones year\(^{-1}\) are in operation (Subramanyam, 1990) of which 36 are the large mills with a production capacity >55 tones day\(^{-1}\), and the rest are small mills with production capacity <30 tones day\(^{-1}\) (Sastri, 1986). The large pulp-paper mills equipped with soda recovery discharge about 270 to 450 l effluent kg\(^{-1}\) of paper containing 40 to 50 g lignin kg\(^{-1}\) bleached paper produced. Contrary to that, the small paper mills without soda recovery discharge nearly 300 to 400 l of black liquor effluent containing 200 to 250 g lignin kg\(^{-1}\) of paper manufactured (Garg and Modi, 1999). More than 150x10\(^6\) tons of pulp is produced annually and about 50x10\(^6\) tons of lignin together with the chemicals used is released from the P&P industry indicating that a lot of efforts have to be undertaken to handle the enormous amounts of hazardous potential (Call and Mücke, 1997). One of the major problems of effluent discharge from the pulp and paper industry is its brown/black color, generally known as black liquor. The color of these wastewaters is primarily due to lignin and its derivatives, which are discharged in such effluents mainly from the pulping, bleaching, and chemical recovery stages of the plant. High
molecular-weight chlorinated lignins are generally not removed from the effluents. 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.

1.1.2 Textile dyes and textile mill effluent
The total annual world textile dye production is estimated at about 800 kt. In 1999 the value of the global dyestuff market was estimated at 6.6 billion US$, North America accounting for 1.2 billion US$, Central and South America for 0.7 billion US$, Western Europe for 1.2 billion US$ and Asia for 2.7 billion US$. India, the former USSR, Eastern Europe, China, South Korea and Taiwan consume approximately 600 thousand tons (kt) of dyes per annum (Ishikawa et al., 2000). The distribution of global dyestuff market has changed during the last decade, with Asia being the largest dyestuff market today (about 42%). Even though the dye industry is characterized by a large number of producers (about 2000 worldwide), just four Western companies accounted for nearly half of the market in 2000 (Wesenberg et al., 2003).

Dyestuffs can be classified according to origin, chemical and/or physical properties and characteristics related to the application process. About 15% of the dyes used for textile dying are released into processing waters (Mishra and Tripathy, 1993). Concern arises, as many dyes are made from known carcinogens such as benzidine and other aromatic compounds (Robinson et al., 2001). All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents, and microbial attack. These features unfortunately go with the perils of harmful effluent quality. They result into the reduced transmittance of sunlight, resulting in cessation of photosynthesis, leading to anaerobic conditions, which in turn result in the death of aquatic life causing foul smelling toxic waters. In addition to their visual effect
and their adverse impact in terms of chemical oxygen demand, many synthetic dyes are toxic, mutagenic and carcinogenic (Michaels and Lewis, 1985; Chung et al., 1992). Textile effluents are usually mutagenic or teratogenic to various microbiological and fish species (Daneshvar et al., 2003). Also, azo and nitro compounds have been reported to be reduced in sediments of aquatic bodies, consequently yielding potentially carcinogenic amines that spread in the ecosystem (Verma et al., 2003). The presence of dyes or their degradation products in water can also cause human health disorders such as nausea, hemorrhage, and ulceration of skin and mucous membranes (Solpan et al., 2003), and can cause severe damage to the kidney, reproductive system, liver, brain, and central nervous system (Kadirvelu et al., 2003).

1.1.3 Molasses-based effluents
Wastewaters containing molasses are generated by distilleries, fermentation industries, sugar mills, Pharmaceutical companies and other molasses-based industries. Molasses from sugarcane industry is the common raw material used in ethanol production due to its easy availability and low cost (Kalavathi et al., 2001). India is the second largest producer of ethanol in Asia. There are 319 distilleries in India with an installed capacity of 3.25 billion litres of alcohol (Uppal, 2004; Tewari et al., 2007). The Central Pollution Control Board (CPCB) categorizes distillery industry among 17 top polluting industries in India. For every one litre of alcohol produced, 10–15 l of spent-wash are generated and thereby a typical distillery producing ethanol from cane molasses generates nearly half million liters of spent-wash daily (Ghosh et al., 2002; Kumar et al., 1997). Approximately, 40 billion litres of spent-wash is generated annually in India alone for the production of 2.3 billion litres of alcohol. Distillery is one of the most highly polluting and growth-oriented industries in India with reference to the extent of water pollution and the quantity of wastewater generated. The Population equivalent of distillery waste based on BOD has been reported to be as
high as 6.2 billion, which means that the contribution of distillery waste in India to organic pollution is approximately seven times more than the contribution by the entire population (Kanimozhi and Vasudevan, 2010). These contain mostly dark brown colored recalcitrant compounds collectively termed as melanoidin polymers which are the product of Maillard reaction between the amino acids and carbonyl groups present in molasses (Wedzicha and Kaputo, 1992). With their high biochemical and chemical oxygen demand, these effluents are environmental hazards. When released in water bodies they cause oxygen depletion and associated problems, and/or if released in soil they reduce the soil alkalinity and manganese availability, inhibit seed germination and affect vegetation. Besides causing unaesthetic discoloration of water and soil, melanoidin pigments are also toxic to microorganisms present in soil and water (Mohana et al., 2009; Agarwal et al., 2010). Dark brown color of these effluents is highly resistant to microbial degradation and other biological treatments. Melanoids have recalcitrant compounds; thus the conventional treatment methods are not effective for complete color removal from this stream and color can even be increased during anaerobic treatments, due to re-polymerization of compounds (Satyawali and Balakrishnan, 2007). Anaerobic digestion of effluents produces dark brown sludge which is used as fertilizer and the colored waters are discharged after diluting them several folds with water. Thus ultimately fresh water resource which is a precious commodity in most parts of the world is wasted. The spent-wash is highly colored with an extremely high Chemical Oxygen Demand (COD) load and contains high percentage of dissolved organic and inorganic matter. The Biochemical Oxygen Demand (BOD) and COD, the index of its polluting character, typically range between 35,000–50,000 mg L⁻¹ and 80,000–1,00,000 mg L⁻¹ respectively (CPCB, 2003). Apart from high organic content, distillery wastewater also contains nutrients in the form of nitrogen, phosphorus and potassium that can lead to eutrophication of water bodies. Spent-wash disposal even after conventional treatment is hazardous and has a high pollution potential.
due to the accumulation of non-biodegradable recalcitrant compounds, which are mostly colored and in a highly complex state. Melanoidins have anti-oxidant properties causing toxicity to many microorganisms involved in wastewater treatment processes (Sirianuntapiboon et al., 2004a). Lowering of pH value of the streams, increasing organic load and obnoxious smell are some of the major problems due to distillery wastewater. The distillery wastewater poses a serious threat to water quality in several regions of the country. Disposal on land is equally detrimental causing a reduction in soil alkalinity and inhibition of seed germination. In addition to pollution, increasingly stringent environmental regulations are forcing distilleries to improve existing treatment and also explore alternative methods for effluent management (Kanimozhi and Vasudevan, 2010).

1.1.4 Polycyclic aromatic hydrocarbons
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.

1.2 Remedy to industrial pollutants
Several strategies including biological approaches besides physical and chemical methods are devised to restore polluted environments. 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. 2001b; Husain, 2006; Whiteley and Lee, 2006). Most commercial systems currently use activated carbon as sorbent to remove dyes in wastewater because of its excellent adsorption ability. Activated carbon adsorption has been cited by the US Environmental Protection Agency as one of the best available
control technologies (Derbyshire et al., 2001). However, although activated carbon is a preferred sorbent, its widespread use is restricted due to high cost. In order to decrease the cost of treatment, attempts have been made to find inexpensive alternative adsorbents. Also, adsorption is only a phase transfer and not an actual degradation.

1.2.1 Bioremediation of industrial pollutants

Bioremediation refers to processes that use microorganisms or their enzymes for the clean up of contaminated soils or waters, whereas, "Biodegradation" is the biologically mediated breakdown of chemical compounds. It is an umbrella term, encompassing most of the other jargon addressed in this section, and generally implies a series of biochemical reactions. When biodegradation is complete, the process is called "mineralization," i.e., the total breakdown of organic molecules into water, CO\text{2}, and/or other inorganic end products. In bioremediation, biological systems are used to transform and/or degrade toxic compounds or otherwise render them harmless. Bioremediation can involve indigenous microbial populations with or without nutrient supplementation, or it can involve inoculation of exogenous organisms into the site, whereas when exogenous organisms are added, the process is called "bioaugmentation." In either case, the goal is to disarm noxious chemicals without the formation of new toxins. Biotransformation is a step in the biochemical pathway which leads to the conversion of a molecule into a less toxic product. Biodeterioration is the breakdown of economically useful compounds but often the term has been used to refer to the degradation of normally resistant substances such as plastics, cosmetics, paint, wood products and metals. Biosorption may be simply defined as the removal of substances from solution by biological material. Such substances can be organic and inorganic, and in soluble or insoluble forms (Gadd, 2009).
The process of bioremediation can be monitored by measuring any of the following factors: (1) 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 (2) 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.

1.2.2 Biological methods for remediation

Biological treatment is often the most economical alternative when compared with other physical and chemical processes (Crini, 2006). Biological processes have attracted as a viable alternative to the known physico-chemical methods due to their cost, effectiveness and environmental benignity (McMullan et al., 2001; Chen et al., 2003). 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, in practice has not been as successful as physical and chemical methods (Pritchard et al., 1996). In practice, bioremediation strategies can be divided as:
A) Application of whole culture (in situ): Most of the experiments for decolorization and detoxification of various effluents and xenobiotics are carried out by addition of effluents to pre-grown, shallow, static cultures (Telke et al., 2009; Anastasi et al., 2010). Such cultures when immobilized on a variety of solid supports have been shown to decolorize various effluents (Chen et al., 2009).

B) Application of culture filtrate or enzyme(s): The use of enzymes in the degradation of organic compounds presents several advantages compared to the use of microorganisms such as their unique substrate-specificity and catalytic power, their capacity to act in the presence of many xenobiotic substances and/or under a wide range of environmental conditions, often unfavorable to active microorganisms (i.e. relatively wide temperature, pH and salinity ranges, high and low concentrations of contaminants); and their low sensitivity or susceptibility to the presence of predators and inhibitors of microbial metabolism (Gianfreda and Rao, 2004). Moreover, enzymes are able to reach substrates in pores with small dimensions, roughly 100 times smaller than bacteria (Quiquampoix et al., 2002).

Several limitations prevent the application of free enzymes/culture filtrate. The stability and catalytic ability of free enzymes are dramatically decreased by highly polluted wastewaters; besides, mediator by-products can inactivate the laccase. The use of immobilized enzymes can overcome some of these limitations and provide stable catalysts with long life times (Kunamneni et al., 2008).

C) Bio-sorption: It is a physico-chemical process and includes such mechanisms as absorption, adsorption, ion exchange, surface complexation and precipitation (Gadd, 2009). The term biosorption can describe any system where a sorbate (e.g. an atom, molecule, a molecular ion) interacts with a biosorbent (i.e. a solid surface of a biological matrix) resulting in an accumulation at the sorbate–biosorbent interface, and therefore a reduction in the solution sorbate
concentration. Biosorption is the process by which contents (color, metals, organic or inorganic ions etc.) are removed from aqueous solution by complexing to either living or dead biomass through functional sites that include: carboxyl, imidazole, sulphydryl, amino, phosphate, sulphate, thioether, phenol, carbonyl, amide and hydroxyl moieties. Both living and dead cells have been used for bio-adsorption (Fu and Viraraghavan, 2001). Decolorization by bioadsorption of dye wastewater using (dead or living) biomass, white-rot fungi and other microbial cultures was the subject of many studies reviewed in several recent papers (Aksu, 2005; Pearce et al., 2003; McMullan et al., 2001; Fu and Viraraghavan, 2001; Stolz, 2001; Robinson et al., 2001). Biosorption of dyes using ascomycetous or zygomycetous mycelia has been attempted by several groups (Corso and Almeida, 2009; Kumari and Abraham, 2007; Khalaf, 2008; Tigini et al., 2010).

1.3 **Fungi and remediation of industrial pollutants/effluents**

The organisms known as fungi, encompassing both Fungi and Stramenopila, share a unique nutritional strategy, i.e., their cells secrete extra-cellular enzymes which break down potential food sources, which are then absorbed back into the fungal colony. This way of life means that any discussion of fungal biodegradation must cover an extraordinary amount of catalytic capability. Fungi are heterotrophic eukaryotes that play a major role in the decomposition of dead plant tissues (cellulose and lignin) and to a lesser extent animal tissues such as keratin and chitin. The decomposition liberates nutrients back into the ecosystem. Fungi have evolved biologically and bio-chemically in a diverse manner that has allowed them to utilize various solid substrates. The decomposition of lingo-cellulose is probably the single most important degradative event in the Earth's carbon cycle. The utilization and transformation of the dead remains of other organisms is essential to the Earth's economy. An enormous ecological literature exists on the role of fungi as primary and secondary decomposers in these classic "cycles" of nature (Bennet et al., 2002).
The attributes mentioned below distinguish filamentous fungi from other life-forms and determine why they are good degraders and/or adsorbers (Bennet et al., 2002):

a) The mycelial growth habit gives a comparative advantage over single cells such as bacteria and yeasts, especially with respect to the colonization of insoluble substrates.

b) Fungi can rapidly ramify through substrates, literally digesting their way along by secreting a battery of extra-cellular degradative enzymes.

c) Hyphal penetration provides a mechanical adjunct to the chemical breakdown affected by the secreted enzymes.

d) The high surface-to-cell ratio characteristic of filaments maximizes both mechanical and enzymatic contact with the environment.

e) The extra-cellular nature of the degradative enzymes enables fungi to tolerate higher concentrations of toxic chemicals than would be possible if these compounds had to be brought into the cell.

f) Insoluble compounds that cannot cross a cell membrane are susceptible to attack.

g) Since the relevant enzymes are usually induced by nutritional signals independent of the target compound during secondary metabolism, they can act independently of the concentration of the substrate, and their frequently nonspecific nature means that they can act on chemically diverse substrates.

1.3.1 Lignin

Lignin (Latin lignum means wood), a group of abundant bio-polymers embodying some significant diversity, occupy a pivotal position in the carbon cycle of the biosphere. It is second to cellulose and the most abundant in terms of energy content (Boerjan et al., 2003; Lebo et al., 2001; Sjöström, 1993). It is a complex oxyphenyl propanoid polymer, found in all vascular plants including herbaceous
species, which provides rigidity, support, and protection to the plants. It is synthesized by one-electron oxidation of the precursors; p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, generating phenoxy radicals which then undergo non-enzymatic polymerization. These unspecific reactions create a high-molecular-weight, heterogeneous, three-dimensional polymer (Fig. 1.1). In gymnosperms, the primary lignin precursors are the two monolignol coniferyl and p-coumaryl alcohols, while in angiosperms sinapyl alcohol is also present. On the other hand, the lignins of grasses and cereals contain some covalently bound p-hydroxycinnamic (viz. p-coumaric and ferulic) acids, in addition to units derived from the three primary monolignol precursors (Garg and Modi, 1999).

Fig. 1.1: Structure of lignin complex. (a) Lignin monomers and type of lignin depending upon monomers; (b) simplified structure of lignin complex. Adapted from: Singh and Chen. (2008).

Lignin makes up 15 to 30% of the woody cells walls of gymnosperms (softwood) and angiosperms (hardwood). Lignin polymer comprises of a variety of monomers connected by various C–C and C–O–C non-hydrolyzable bonds with irregular arrangement of successive monomeric and intermonomeric bonds.
Lignin contains chiral carbons in both the L and R configuration, and this stereo irregularity renders it still more resistant to attack by most microorganisms. Both cellulose and lignin are rather rigid organic polymers which have been ‘invented and optimized’ by nature during the evolution process for constructive and long term preservation purposes. Harsh physico-chemical conditions have to be applied to attack or modify these two compounds.

Its bioconversion and biodegradation are of ecological significance and also have wide industrial applications (Boominathan and Reddy, 1992). Due to their phenolic nature, they are extremely resistant to microbial attack and bind together the cellulosic fibers to provide strength. Lignin and their chemical degradation products are optically inert. It is a highly irregular molecule having no precise structure, but contains a series of substructures occurring randomly. Lignin forms a matrix surrounding the cellulose, the most abundant natural polymer. Since this encrusting matrix significantly retards the microbial depolymerization of cellulose, the degradation of lignin is a significant step in the global carbon cycle. Furthermore, the presence of this intractable polymer is an obstacle to the efficient utilization of cellulose in a wide range of industrial processes (Gold and Alic, 1993).

Mammalian or other animal enzymes are not able to digest/breakdown lignin. However, some fungi and bacteria are able to degrade the polymer. This ability is significant as removal of this barrier is required to reach the nutrient source which is the cellulose. The type of degradation of lignin depends on the type of wood-decaying fungi which are classified as soft-rot, brown-rot and white-rot.

1.3.2 Wood-rot fungi

Depending upon their mode of attack and nature of the decay the wood-rot fungi are classified into three main Categories (Eriksson et. al., 1980; Eaton et al., 1980):
**Soft-rot fungi:** wood decay by soft-rot fungi results in softening of the tissues. Biochemical studies have shown that soft-rot decay results in lower methoxy content of wood lignin, thus making it more soluble. The ligninolytic system of soft-rot fungi does not have oxidative potential to attack the recalcitrant guaiacyl lignin but they can oxidize and mineralize syringyl lignin. These fungi degrade wood by forming microscopic cavities within the secondary cell wall. Soft-rot is more common in hardwood than in softwood. It has been suggested that the reason for this, is the quality differences in the lignin of hard- and soft-wood. The methoxyl content of hardwood lignin is usually higher about 21% than in softwood lignin where the methoxyl content is about 14%. A variety of molds belonging to ascomycetes and fungi imperfecti have been shown to decompose all major components of wood, including lignin; they are designated as soft-rot fungi (Eslyn et al., 1975; Yoon and Singh, 2000).

**Brown-rot fungi:** The members of this class are primarily cellulose and hemicellulose degraders and bring about only little changes in the wood lignin (Kirk, 1971; Crawford, 1981). Logs decomposed in this manner results into formation of brown powder consisting mainly of enzymatically liberated lignin. Colonization by such fungi is usually confined to the less lignified layers of the secondary cell wall. It was observed that erosion and thinning of cell wall pattern was similar to that caused by white-rot fungi. In brown rotted lignin, its methoxy content and aliphatic hydroxyl content decrease, while the carboxyl and phenolic hydroxyl contents greatly increase. Brown-rot fungi basically include several species of basidiomycetes. The characteristic brown color provided to brown-rotted wood is believed to be because of the formation of quinone-type chromophores produced during the auto-oxidation of O-diphenolic moieties. *Serpula lacrymans* and *Merulipora incrassate* are examples of fungi that cause brown-rot (Coggins, 1977).

**White-rot fungi:** The most potent and perhaps the most widespread naturally-found lignin degraders are thought to belong to white-rot fungi or closely related
litter-decomposing fungi, which include several hundred species of basidiomycetes and a few ascomycetes. There appears to be two basic differences between white-rot and brown-rot fungi: (1) the latter has poor activity toward synthetic and natural lignin degradation (Enoki et al., 1985); (2) they are unable to metabolize aromatic ring or aliphatic products of the aromatic ring cleavage. 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 (Fig. 1.2). Nevertheless, many extra-cellular ligninolytic enzymes produced by white rot fungi can catalyze the breakdown of lignin. These genes are differentially regulated in response to a variety of environmental signals, especially starvation. Degradation by white-rot fungi is largely an oxidative process. White-rot fungi produce various isoforms of extra-cellular oxidases including laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP), which are involved in the degradation of lignin in their natural lignocellulosic substrates.

![Fig. 1.2: Outlay of various modes of hydroxyl radical generation and agents involving lignin degradation. Adapted from: Shah and Nerud. (2002)](image-url)
White-rot basidiomycetous fungi are the only known organisms which are capable of degrading lignin extensively to CO$_2$ and H$_2$O in pure culture. Indeed, these organisms are able to degrade all of the major polymers in wood: cellulose, hemicellulose, and lignin. White-rot fungi cannot attack specifically on the lignin, because huge amount of energy is required to degrade lignin and thus more accessible energy source is also necessary (Ander and Eriksson, 1978). Lignin biodegradation is a key step for carbon recycling in terrestrial ecosystems, where white-rot basidiomycetes degrade this recalcitrant wood polymer enabling cellulose utilization by microbial populations.

1.4 Lignin Degrading Enzymes

Oxido-reductive 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 (LDEs) are one such group of oxido-reductive enzymes, which have practical application in bioremediation of polluted environment (Husain, 2006). 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). They oxidize lignin subunits using extra-cellular hydrogen peroxide generated by unrelated oxidases as co-substrate (Fig. 1.3). The LDEs share common features such as broad substrate specificity, high redox potential, and are mostly extra-cellular 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).

1.4.1 Lignin peroxidases (EC 1.11.1.14)

Lignin peroxidases (LiPs) catalyze the oxidation of non-phenolic aromatic lignin
moieties and similar compounds. LiPs are well known as part of the ligninolytic
system both of aphyllophoralic and agaricalic fungi (Glenn et al., 1983; Hatakka
et al., 1987; Hofrichter and Fritsche, 1997). The extra-cellular N-glycosylated LiP
with molecular masses between 38 and 47 K Da contain heme in the active site
and show a classical peroxidase mechanism (Tien et al., 1986). They are
glycosylated heme proteins that catalyze H$_2$O$_2$-dependent oxidation of a variety of
phenolic and non-phenolic model compounds, polycyclic aromatic hydrocarbons,
and other compounds that are resistant to microbial attack by one electron
oxidation mechanism followed by a series of non-enzymatic reactions, finally
yielding a variety of products. Here H$_2$O$_2$ gets reduced to H$_2$O 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
into 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). LiP catalyzes several oxidations in the
side chains of lignin and related compounds (Tien and Kirk, 1983) by one-
electron abstraction to form reactive radicals (Kersten et al., 1985). Also the
cleavage of aromatic ring structures has been reported (Umezawa and Higuchi,
1987). The role of LiP in ligninolysis could be the further transformation of lignin
fragments which are initially released by MnP. LiP are not essential for the attack
on lignin: several highly active WRF and litter-decaying fungi (e.g., Ceriopsis
subvermispora, Dichotomitus squalens, Panus tigrinus, Rigidosporus lignosus) do
not excrete this enzyme (Galliano et al., 1991; Hatakka, 1994; Maltseva et al.,
LiP have been used to mineralize a variety of recalcitrant aromatic compounds, such as three- and four-ring PAHs (Günther et al., 1998), polychlorinated biphenyls (Krcmár and Ulrich, 1998) and dyes (Chivukula et al., 1995). 2-Chloro-1, 4-dimethoxybenzene, a natural metabolite of WRF is reported to act as a redox mediator in the LiP-catalyzed oxidations (Teunissen et al., 1998).

1.4.2 Manganese Peroxidases (EC 1.11.1.13)

Manganese peroxidases of ligninolytic white-rot fungi constitute a second group of extra-cellular heme proteins that require free manganous ions for their activity. These enzymes are H₂O₂-dependent and first catalyze the oxidation of Mn (II) to Mn (III), which subsequently oxidizes the various phenolic compounds. In the latter reaction, phenoxy radical intermediate is formed through one-electron oxidation of phenol by Mn (III). The most common ligninolytic peroxidases produced by almost all white-rot basidiomycetes and by various litter-decomposing fungi are manganese peroxidases (MnP). These are glycosylated glycoproteins (Nie et al., 1999) with an iron protoporphyrin IX (heme) prosthetic group (Glenn and Gold, 1985), molecular weights between 32 and 62.5 kDa (Hofrichter, 2002) and are secreted in multiple isoforms (Leisola et al., 1987; Urzúa et al., 1995). MnP preferentially oxidize Mn²⁺ into Mn³⁺ (Glenn et al., 1986), which is stabilized by chelators such as oxalic acid (Wariishi et al., 1992), itself also excreted by the fungi (Galkin et al., 1998; Kuan and Tien, 1993; Takao, 1965). Chelated Mn³⁺ acts as a highly reactive (up to 1510 mV) (Cui and Dolphin, 1990), low molecular weight, diffusible redox-mediator.
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\begin{align*}
(\text{Fe}^{3+})\text{Pox} + \text{H}_2\text{O}_2 & \rightarrow (\text{Fe}^{(IV)}=\text{O})\text{Pox}^* + \text{H}_2\text{O} \\
\text{Compound I} & \\
(\text{Fe}^{(IV)}=\text{O})\text{Pox}^* + \text{A} & \rightarrow (\text{Fe}^{(IV)}=\text{O})\text{Pox} + \text{A}^* \\
\text{Compound II} & \\
(\text{Fe}^{(IV)}=\text{Pox} + \text{A}) & \rightarrow (\text{Fe}^{3+})\text{Pox} + \text{A}^* \\
\text{Compound II} & 
\end{align*}
\]

**Fig. 1.3:** Reaction mechanism for heme peroxidases. The heme peroxidase is a redox process consisting of three distinct steps. The first step is the reaction of the resting enzyme \([\text{Fe}^{3+}]\text{Pox}\) with \(\text{H}_2\text{O}_2\) in a two-electron transfer which results in the formation of Compound I. Compound I has one reducing equivalent at the oxylferric iron \([\text{Fe}^{(IV)}=\text{O}]\) and the other forms a cation radical \([\text{Pox}^*]\). Compound I is then reduced by the substrate \(\text{A}\) in two sequential one-electron steps through Compound II. Adapted from: Conesa et al. 2002.

### 1.4.3 Laccases (EC 1.10.3.2)
Laccase is a benzenediol:oxygen oxidoreductase (a multi-copper enzyme), ubiquitous from bacteria, e.g., *Azospirillum lipoferum* and actinomycetes like *Streptomyces*, to fungi to plants and even in insects (Baldrian, 2006). This enzyme had been reported more than a hundred years ago (Bertrand, 1896; Yoshida, 1883), but the significance and broad studies over the role of this enzyme in wood degradation had been conducted in the last few decades.

### 1.4.4 Characteristics of laccases
The high-redox potential laccases occur mainly in basidiomycetes, especially white-rot fungi (Gutierrez et al., 2006; Rebrikov et al., 2006; Quarantino et al., 2007; Cherkashin et al., 2007; Hernandez-Luna et al., 2008), the low-redox potential laccases seem to be widely distributed in moulds (Jung et. al., 2002), bacteria, insects, and plants. In fungi, laccases carry out a variety of physiological roles including morphogenesis, fungal plant pathogen/host interaction, stress defense, and lignin degradation (Thurston C.F., 1994 and Gianfreda et. al., 1999).
1.4.5 Oxidation mechanism of laccase

Fungal laccases are monomeric, dimeric or tetrameric glycoproteins with four copper atoms, per monomer located at the catalytic site (Fig. 1.4). Type 1 (T1) copper is responsible for the oxidation of the substrate and imparts the blue color to the enzyme. Laccase often sports a high degree of glycosylation, which confers a degree of self-resistance to attack by proteases (Yoshitake et al., 1993). The redox potential of the T1 copper site is directly responsible for the catalytic capacity of the enzyme. The mechanism of interaction between a laccase T1 site and its substrate seems to be identical among fungal laccases (Smirnov et al., 2001). In its native state, the enzyme holds copper atoms in the mono-valent state as Cu'. When molecular oxygen binds at the trinuclear cluster formed by T2 and T3 copper, the four copper atoms are oxidized (Cu^2+) in two steps, while passing through a peroxide-level intermediate (Solomon et al., 2001). Oxygen is then reduced to divalent oxygen, and subsequently becomes water. Oxidation of substrate(S) is always carried out by T1 copper. Electrons are transferred from the T1 site to the tri-nuclear cluster by a His-Cys-His tri-peptide motif. Each substrate is oxidized by a successive one electron oxidation step. The capture of four electrons by the substrate(s) returns the enzyme to its native state. The stoichiometric ratio corresponding to the molar ratio of substrate/dioxygen transformation is generally 4/1, i.e., four electrons withdraw from four substrate molecules per one dioxygen reduced. If substrate molecules donate more than one electron, a lower ratio (or decimal values) may be observed. It is assumed that laccases operate as a battery, storing electrons from the four individual oxidation reactions of four molecules of substrate, in order to reduce molecular oxygen to two molecules of water. The oxidation of a reducing substrate by laccase typically involves the loss of a single electron and the generation of a free radical (Solomon et al., 2001; Xu, 1999). The redox potential of of T1 copper site is directly responsible for the catalytic capacity of the enzyme (Smirnov et al., 2001).
### Reduced From

<table>
<thead>
<tr>
<th>$T_1$</th>
<th>$Cu^{1+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_2$</td>
<td>$H_2O$</td>
</tr>
<tr>
<td>$T_3$</td>
<td>$Cu^{1+}$ $Cu^{1+}$</td>
</tr>
</tbody>
</table>

### Oxidized "Resting" Form

<table>
<thead>
<tr>
<th>$T_2$</th>
<th>$Cu^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_3$</td>
<td>$OH$</td>
</tr>
<tr>
<td>$T_1$</td>
<td>$Cu^{2+}$</td>
</tr>
</tbody>
</table>

**Catalytic Cycle**

- $+4e^-$ rapidly
- $-H_2O$
- $O-O$ bond cleavage

**Fig. 1.4:** Catalytic cycle of laccase showing the mechanism of four-electron reduction of a dioxygen molecule to water at the enzyme copper sites. Adapted from: Soloman et al. (2001)

Many laccases are characterized by the presence of one type-1, one type-2, and two type-3 copper ions. The substrates are oxidized by the type-1 copper, and the extracted electrons are transferred to the type-2/type-3 copper site, where molecular oxygen is reduced to water (Baldrian, 2006). Laccases are divided into "low-redox potential" and "high-redox potential" laccases depending on the structure and properties of the copper center.

### 1.4.6 Laccase substrates
Laccases are able to catalyze direct oxidation of ortho and para-diphenols, amino-
phenols, polyphenols, polyamines, and aryl diamines as well as some inorganic
ions (Solomon et al., 1996). 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 H₂O₂ as the co-substrate but rather molecular
oxygen.

Laccases have a wide substrate range, which can serve industrial purposes
and/or bioremediation processes. The simple requirements of laccase catalysis
(presence of substrate and O₂), as well as its apparent stability and lack of
inhibition (as has been observed with H₂O₂ for peroxidase), make this enzyme
both suitable and attractive for biotechnological applications. Laccases are more
stable in their extracellular role as they are often produced as highly glycosylated
derivatives where the carbohydrate moieties increase their hydrophilicity. For
phenolic substrates, oxidation by laccase results in formation of an aryloxyradical,
an active species that is converted to a quinone in the second stage of the
oxidation. Quinone intermediates can spontaneously react with each other to form
soluble or insoluble colored oligomers, depending on substrate and environmental
parameters (Walker, 1988). Laccase can decarboxylate phenolic and
methoxyphenolic acids (Agematu et al., 1993), and also attacks methoxyl groups
through demethylation (Leonowicz et al., 1984). Dehalogenation of substituents
located in the ortho or para position may also occur in the case of substituted
compounds (Schultz et al., 2001). Catalytic activity of laccase is usually measured
with a susceptible laccase substrates, such as azinobis(3-ethylbenzathiazoline-6-
sulfonic acid) (ABTS), 2,6-dimethoxyphenol, syringaldazine or guaiacol. The
most specific substrates of theses is N-bis(3,5-dimethoxy-4-hydroxybenzylidene
hydrazine) or syringaldazine (Thurston, 1994). Relatively higher stability of
laccases in extra-cellular fluids makes them suitable for the bioremediation.
Laccases exhibit an extraordinary natural substrate range (phenols, polyphenols,
anilines, aryl diamines, methoxysubstituted phenols, hydroxyindols,
benzenethiols, inorganic/ organic metal compounds and many others) which is the major reason for their attractiveness for dozens of biotechnological applications (Nyanhongo et. al., 2002, Xu, 2005, Riva 2006). The half life at 50°C of the purified enzyme from *Trametes sp.* ranged from 50-70 h (Smirnov et al., 2001). It is usual for laccases to be present as a several isozymes in a single species. Some of these are constitutively expressed while others may be inducible. The pattern or presence of these isozymes may also be dependent over the culture age and substrate used (Moldes et al., 2004). Laccases are known to decolorize certain azo dyes without direct cleavage of the azo bond through a highly non-specific free radical mechanism, and thus do not form toxic amines (Chivukula et al., 1995). The interest towards the laccases has been increased after the discovery of their ability to oxidize xenobiotic and non-phenolic compounds. Baldrian, (2006) has reported about hundred compounds identified as laccase substrates.

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).

1.4.7 Laccase mediators

The downside of laccases however, is that the redox potential although varying between different laccase isozymes (0.5-0.8 V), cannot be compared with that of the peroxidases, especially LiP. This redox potential is not high enough for oxidation of many of the xenobiotic compounds. This led to the discovery of mediator system (LMS). The discovery of “mediators” – small molecules that can extend the enzymatic reactivity of laccase towards several “uncommon” substrates – stimulated interest in laccases for detoxification and industrial
purposes (Bourbonnais and Paice, 1990; Call and Mücke, 1997). These are small molecules which can act as redox intermediates between the active site of the enzyme and a non-phenolic substrate. 3-Hydroxyanthranilic acid (3-HAA) was the first natural mediator for laccases described. The few common examples of Laccase — mediatoes are ABTS, 1-hydroxybenzotriazole (HBT) vanillic acid etc. The discovery of 1-hydroxybenzotriazole (HBT), an effective laccase mediator in pulp processing (Call, 1994) lead to a new class of mediators with NOH as the functional group, which is oxidized to a reactive radical (R—NO). LMS enlarges substrate range being able to oxidize compounds with redox potential (E°) higher than that of laccase (typically, laccase E° at the T1 site is in the range +475 to +790 mV but the LMS allows to oxidize molecules with E° above +1100 mV) (Fernández-Sánchez et al., 2002; Johannes and Majcherczyk, 2000). Besides, the mediator acts as a diffusible electron carrier enabling the oxidation of high molecular weight biopolymers such as lignin, cellulose or starch (Alcalde, 2007). Hence, the steric issues that hinder the direct interaction between enzyme and polymer are overcome by the action of the redox mediator.

Thus, laccase and LMS find potential application in delignification and biobleaching of pulp (Bourbonnais et al., 1997; Smith et al., 1997; Camarero et al., 2004; Ibarra et al., 2006), treatment of wastewater from industrial plants (Bergbauer et al., 1991; Berrio et al., 2007) enzymatic modification of fibers and dye-bleaching in the textile and dye industries (Abadulla et al., 2000; Kunamneni et al., 2008), enzymatic crosslinking of lignin-based materials to produce medium density fiberboards (Widsten et al., 2004), detoxification of pollutants and bioremediation (Keum & Li, 2004; Bollag et al., 2003; Gianfreda & Rao et al., 2004; Alcalde et al., 2006; Zumarraga et al., 2007), detoxification of lignocellulose hydrolysates for ethanol production by yeast (Jonsson et al., 1998; Larsson et al., 1999) enzymatic removal of phenolic compounds in beverages-wine and beer stabilization, fruit juice processing (Cantarelli et al., 1989; Servili et al., 2000; Minussi et al., 2002) and construction of biosensors and biofuel cells.
Laccases have been intensively studied with a focus on their industrial applicability (Bajpai, 1999; Gianfreda et al., 1999; Rodriguez et al., 1999; Yaropolov et al., 1994), molecular genetics (Cullen, 1997; Karahanian et al., 1998; Ong et al., 1997; Collins and Dobson, 1997) and cloning (Hatamoto et al., 1999).

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 CO$_2$ 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 bio-bleaching 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). The interaction between laccases and other extra-cellular enzymes in pollutant oxidation has not been studied extensively, but is presumed to be advantageous in terms of broader substrate range, decreased inactivation by free radicals and further mineralization of toxic compounds.

### 1.4.8 Lignin degrading enzymes in Bioremediation

Many workers divide bioremediation (*in situ*) strategies into three general categories: (i) the target compound is used as a carbon source, (ii) the target compound is enzymatically attacked but is not used as a carbon source (co-metabolism), and (iii) the target compound is not metabolized at all but is taken
up and concentrated within the organism (bioaccumulation). Although fungi participate in all three strategies, they are often more proficient at co-metabolism and bioaccumulation than using xenobiotics as sole carbon sources (Bennet et al., 2002).

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.

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. Acevedo et al., (2010) demonstrated degradation of PAHs by the application of free and immobilized MnP. 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 also been implicated in PAHs degradation.

Involvement of different ligninolytic enzymes in biodegradation of synthetic dyes and simulated textile waste-waters has been reported (Murugesan et al., 2007; Park et al., 2007; Casas et al., 2009; Faraco et al., 2009; Niebisch et al., 2010). Color removal from MSW was shown to be MnP-dependent in *Phanerochaete chrysosporium* (Dehorter and Blondeau, 1993) and laccase dependent in *Trametes versicolor* (González et al., 2008). 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).

### 1.5 Advantages of Marine-derived Fungi in Bioremediation

Although many ecological roles for fungi in the terrestrial ecosystem have been described and thoroughly studied, the ecology of fungi in the marine environment has been more difficult to study. The following section will highlight several
examples that demonstrate the importance of fungi in marine ecosystems and in turn for bioremediation.

Mangrove plants and sea-grasses contain 50% lignocellulosic material as structural polymers and are the major contributor's of lignocellulose substrate in coastal marine environment (Benner and Hodson, 1985). Mangrove leaves, twigs, wood pieces, and sea-grasses fallen into the intertidal zone are colonized by epibiobiotic bacteria and epi- and endo-biotic fungi. 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). Efficiency of marine-derived fungi in treatments of industrial effluents has largely remained unexplored.

Halo-tolerant marine fungal species have evolved unique metabolic mechanisms that are responsive to salt concentrations. For fungi to grow in the marine environment, they must have osmo-regulatory mechanisms that signal the production of polyols and amino compounds in conjunction with increasing the concentration of cytoplasmic ions. Marine-derived fungi grow and produce degradative enzymes in seawater media and thus may be useful in treating wastewaters with high salt content. 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 sea water ranges from 7.5–8.2. 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 grow in the presence of saline and alkaline conditions perhaps are well suited for treatment of such effluents.

A basidiomycete Phlebia sp., strain MG-60 isolated from mangrove stands was reported as a hypersaline tolerant lignin-degrading fungus which participated in bio-bleaching of pulp and decolorization of dyes (Li et al., 2002) in the presence of different concentrations of sea salts. Purified laccase from the marine fungus NIOCC #2a was not inhibited in the presence of NaCl up to 0.3 M concentration and retained 75% of its activity in the presence of half strength sea water (D’Souza-Ticlo et al., 2009).

1.5.1 Lignin-degrading ability of marine fungi

Mineralization of $^{14}$C (ring)-labeled synthetic lignin to $^{14}$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 $^{14}$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 $^{14}$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}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}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}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.

### 1.5.2 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 sea-grass 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 seawater. 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 sea-grass (Thalassia hemprichii) of the Lakshadweep island, India, produced all three LDEs, also does not confirm to any of the above categories (Raghukumar et al., 1999).

Another Marine-derived fungus isolated from Chorao Island, Goa showed hyper-production of laccase (D’Souza et al., 2006). 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 sea-water media. 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, bio-bleaching 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 sea-water 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).

### 1.5.3 Application of Marine-derived fungi in remediation

The ecological features and characteristics of marine-derived fungi discussed above make them potentially better candidates for bioremediation than their terrestrial counterparts. Unfortunately, very few attempts have been made to exploit this potential.

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). In another study marine adapted strains of *Aspergillus* and *Mucor* depleted pyrene and benzo[a]pyrene substantially (Passarini et al., 2010). 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 (Raghukumar et al., 1999; D’Souza et al., 2006).

Marine fungi *Sordaria fimicola* (NIOCC #298) and *Halosarphelia ratnagiriensis* (NIOCC #321), which produced MnP and laccase, brought about 65–75% decolorization of bleach plant effluent within 8 days (Raghukumar et al., 1996). Also, NIOCC #312 decolorized molasses spent wash effectively (Raghukumar and Rivonkar, 2001).

In this thesis, marine-derived fungi have been isolated and screened for the production of lignin degrading enzymes. These fungi and their enzymes were subjected to the decolorization and detoxification of various industrial effluents. The biological methods of remediation are usually time-consuming. Also, degradation products may lead to increase in toxicity in some cases. Attempt was
made to make the remediation process more effective and fastidious by the application of combined approach using several techniques. Detoxification studies were carried out to assess the reduction in toxicity. LDEs are known to occur as several isoforms. Homology studies were done to investigate the presence of some novel enzyme-coding sequences. Isolation of laccase coding sequence from a selected isolate and its expression in suitable host was attempted.

I propose to study the Potential of marine-derived fungi and their enzymes in bioremediation of industrial pollutants, with the following objectives:

- Isolation of fungi from various marine habitats, specifically for lignin degrading enzymes.
- Screening these fungi for decolorization of pollutants and laccase produced by different fungi will be examined for its homology.
- To study the influence of various parameters on decolorization and detoxification of colored effluents by selected marine fungi producing these enzymes.
- Isolation of a laccase gene from the best isolate and express it in a compatible yeast species.