Potential of marine-derived fungi and their enzymes in bioremediation of industrial pollutants

Thesis submitted for the degree of

Doctor of Philosophy

in

Marine Sciences

to the

Goa University

by

Ashutosh Kumar Verma

Work carried out at

National Institute of Oceanography,

Dona Paula, Goa-403004, India

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STATEMENT

As per requirement, under the University Ordinance 0.19.8 (vi), I state that the present thesis titled "***Potential of marine-derived fungi and their enzymes in bioremediation of industrial pollutants***" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities or suggestions have been availed of.

Ashutosh Kumar Verma

CERTIFICATE

This is to certify that the thesis titled **"*Potential of marine-derived fungi and their enzymes in bioremediation of industrial pollutants*"** submitted for the award of the degree of Doctor of Philosophy in the Department of Marine Sciences, Goa University, is the bona fide work of **Mr Ashutosh Kumar Verma**. The work has been carried out under my supervision and the thesis or any part thereof has not been previously submitted for any degree or diploma in any university or institution.

Place:

Date:

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Ph.D. Supervisor,
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National Institute of Oceanography,
Dona Paula, Goa, India.

Acknowledgments

This is the best opportunity for me to convey my sincere regards for all those people who enabled me to accomplish thesis work successfully.

It is difficult to overstate my gratitude to my Ph. D. supervisor, Dr. Chandralata Raghukumar. Her enthusiasm and great efforts to explain things clearly and patiently, made my work even more interesting. She encouraged me to choose this topic for my thesis and I am very grateful to her for valuable guidance and constant encouragement throughout this study. She always supported and taught me experimental and writing skills. Throughout my thesis-writing period, she provided encouragement, sound advice, good teaching, good company and lots of good ideas. She as a person is always ready to hear and solve the problems of her students in whatever way possible whilst allowing the room to work in their own way. I feel proud and very fortunate of getting opportunity to work in her guidance, as one simply could not wish for a better or friendlier supervisor. Working with her was the most memorable achievement for me and I attribute the present work to her encouragement and constant effort and without her this thesis, too, would not have been completed. I also thank Dr. S. Raghukumar, for his valuable and thoughtful suggestions regarding my work. At several instances, he provided me with insights and guidance to recognize my mistakes. Both of them have influenced me a lot with their enthusiastic and positive approach. Their confidence imbibing attitude and endless endeavors through which I have gained and learned a lot building up my future and personality.

I wish to thank Dr. S. R. Shetye, Director, NIO for giving me opportunity to work and providing the infrastructure facilities in the institute.

I am thankful to my Co-guide Dr. S. Ghadi, Department of Biotechnology, Goa University for all his support throughout my study.

I would also like to extend my appreciation to all my FRC members, Dr. G. N. Nayak, Dean, Department of Marine Science, Goa University, Dr. H. Menon, Head, Department of Marine Sciences, Goa University and VC's nominee Dr. N. Ramaiah, Scientist, NIO for all their critical comments and for their periodic assessment of my work. I would also thank Dr. D. J. Bhat, Head, Department of Botany, Goa University for helping me in the identification of fungi.

I humbly acknowledge Dr. N. Ramaiah, DU leader, Microbial Ecology Laboratory who provided me the facilities for completion of the work as and when required.

I am thankful to Dr. M.P. Tapaswi, Head Librarian, NIO, for providing some important references whenever required. I am also grateful to Mr. Arun Mahale, and other staff specially Mr. Javali of DTP section to help me in modification of some of the figures in this thesis.

I express my sincere thanks to Dr Chandrakant Govind Naik for his efforts in spectrometric analysis. His help and contribution is notable in the completion of this thesis.

I am thankful to Dr. Yogesh Shouche, Scientist, NCCS, Pune for extending the sequencing facility in his lab. I am indebted to my dear friend Pankaj Verma for his great help and useful suggestions throughout my thesis.

I acknowledge the financial assistance provided by CSIR (Council for Scientific and Industrial Research) during my research work.

I am thankful to all my friends in the Marine Biotechnology and Microbial Ecology Lab, NIO for all their timely help. Special mention is required of Dasu and I owe everlasting gratefulness to him, who was always there to help me in all the possible ways. I thank Cathrine, Samir, Varada, Donna and Purnima, for making the lab a wonderful place to work. They provided a stimulating and fun environment. I thank all my friends from BOD, COD, GOD and POD for the help provided during the study.

I am thankful to Atul Ltd., Gujrat for the supply of textile mill effluents, Jeypore Sugar Co. Ltd., Andhra Pradesh and Emmellen Biotech Pharmaceuticals Ltd., Maharshtra for molasses-based effluents.

I am deeply indebted to all my family members for their support and constant encouragement and showing trust in me.

And last but not the least; I am thankful to almighty for giving me all the strength to achieve this goal.

Ashutosh Kumar Verma

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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 150×10^6 tons of pulp is produced annually and about 50×10^6 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. Melanoidins 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, where as, "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_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).

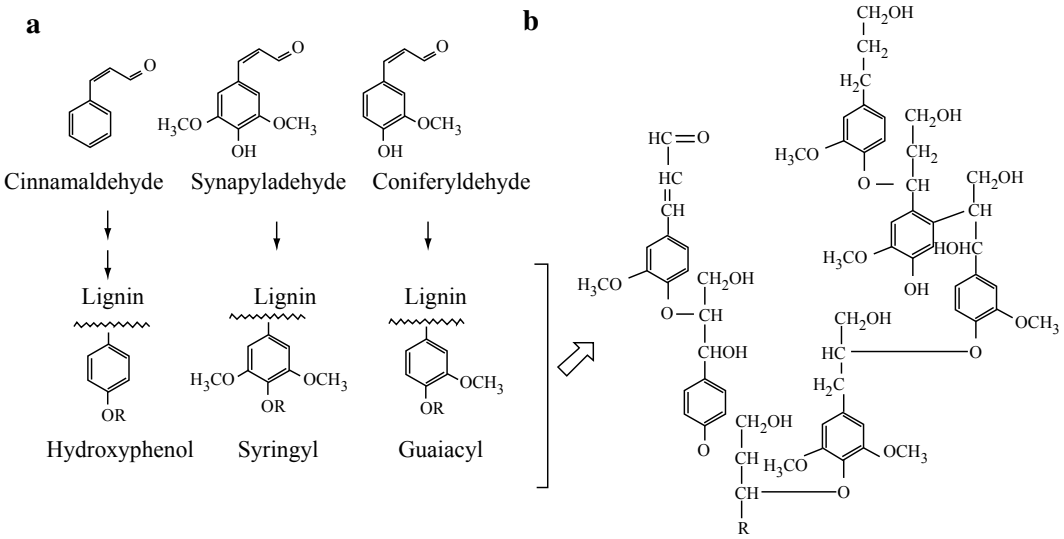


Fig. 1.1: Structure of lignin complex. (a) Lignin monomers and type of lignin depending upon monomers; (b) simplified structure of lignin complex. Adapted from: Singh and Chen, (2008).

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 CO_2 and H_2O 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.

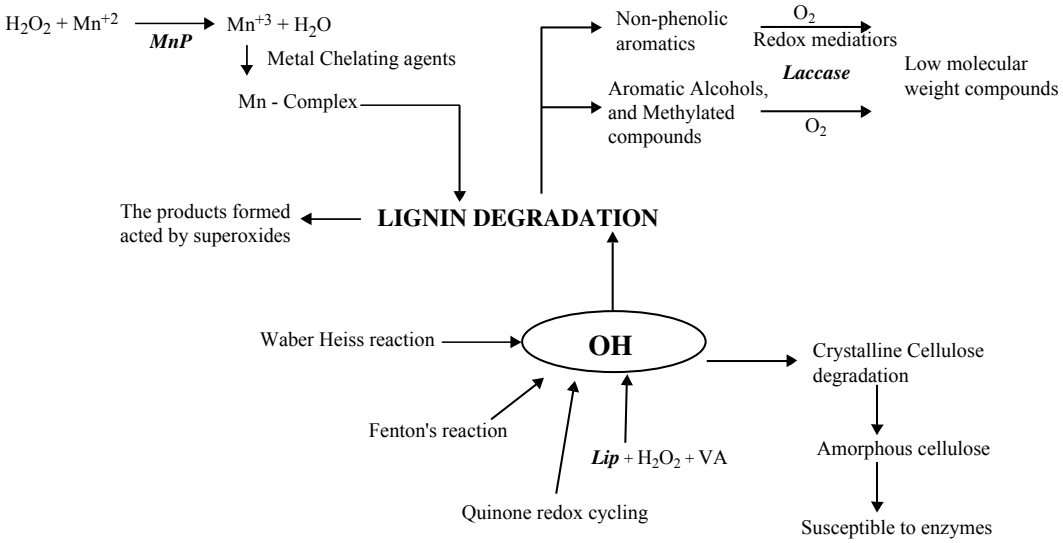


Fig. 1.2: Outlay of various modes of hydroxyl radical generation and agents involving lignin degradation. Adapted from: Shah and Nerud, (2002)

White-rot basidiomycetous fungi are the only known organisms which are capable of degrading lignin extensively to CO_2 and H_2O 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_2O_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_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 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.,

1991; Peŕié and Gold, 1991). 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 H_2O_2-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 Mn^{2+} into Mn^{3+} (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 Mn^{3+} acts as a highly reactive (up to 1510 mV) (Cui and Dolphin, 1990), low molecular weight, diffusible redox-mediator.

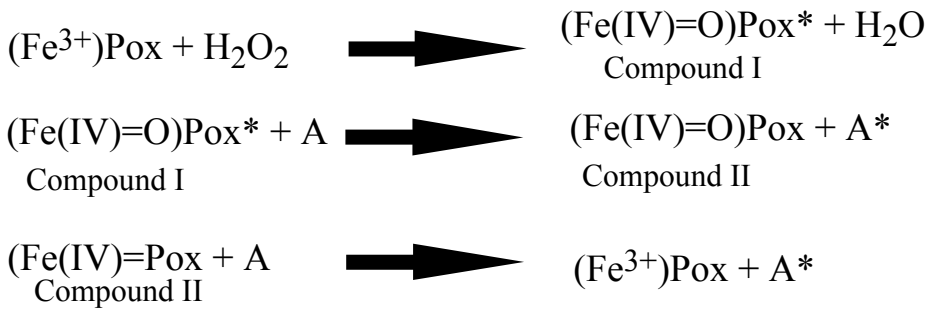


Fig. 1.3: Reaction mechanism for heme peroxidases. The hemeperoxidase is a redox process consisting of three distinct steps. The first step is the reaction of the resting enzyme [(Fe^{3+})Pox] with H_2O_2 in a two electron transfer which results in the formation of Compound I. Compound I has one reducing equivalent at the oxyl-ferric iron [Fe(IV)=O] and the other forms a cation radical [Pox*]. Compound I is then reduced by the substrate (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; Quaratino 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).

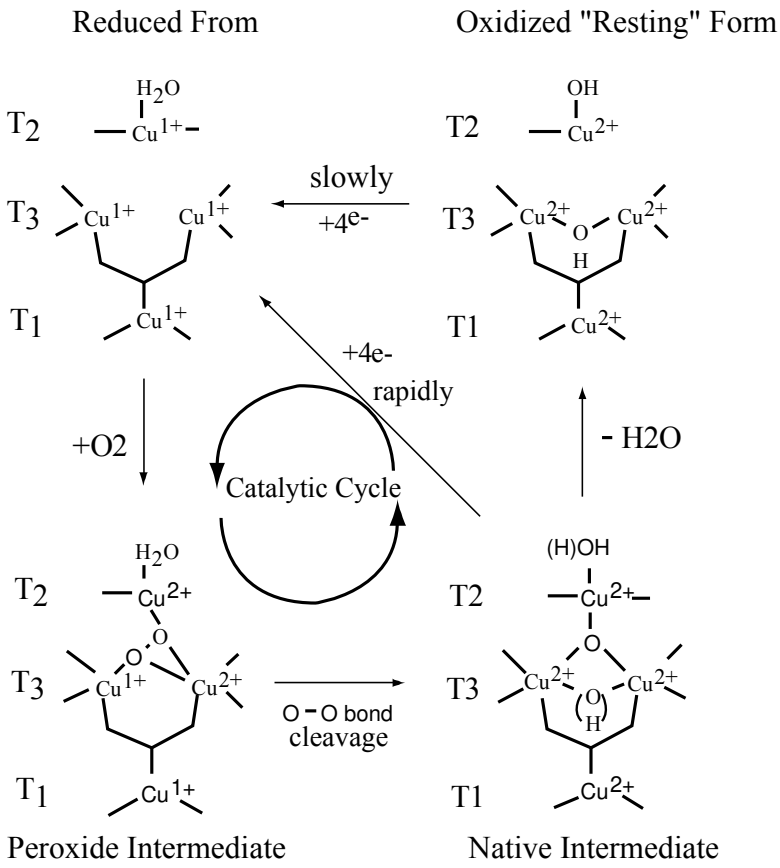


Fig. 1.4: Catalytic cycle of laccase showing the mechanism of four –electron reduction of a dioxygen molecule to water at the enzyme copper sites. Adapted from: Soloman et al. (2001)

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 H_2O_2 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 O_2), as well as its apparent stability and lack of inhibition (as has been observed with H_2O_2 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

(Ghindilis A, 2000). Laccases have been intensively studied with a focus on their industrial applicability (Bajpai, 1999; Gianfreda et al., 1999; Rodríguez 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) demostrated 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 epi-biotic 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 macro-organisms 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 from10–34 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 of17 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 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-weekperiod. 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. Phenantherene 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 *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). 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 there 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 asses 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.

2.1 *Introduction*

2.1.1 *Occurrence and role of fungi in terrestrial and aquatic ecosystems*

The most acceptable estimate of the number of species of fungi on Earth is about 1.5 million and according to a study only 7% of these are known (Hawksworth, 2004). These heterotrophic eukaryotes have a worldwide distribution, and grow in a wide range of habitats, including extreme environments such as deserts, areas with high salt concentrations (Vaupotic et al., 2008), ionizing radiation (Dadachova et al., 2007) as well as in deep sea sediments (Raghukumar and Raghukumar, 1998; Singh et al., 2010) and anoxic sediments (Jebaraj et al., 2010). Other examples of aquatic fungi include those living in hydrothermal areas of the ocean (Le Calvez et al., 2009). Although often inconspicuous, fungi occur in every environment on the earth and play vital roles in most ecosystems.

Along with bacteria, fungi are the major decomposers in most terrestrial (and some aquatic) ecosystems, and therefore play a pivotal role in biogeochemical cycles (Gadd, 2007) and in many food webs with the virtue of there diverse enzyme system. As decomposers, they play an essential role in nutrient cycling, especially as saprotrophs and symbionts, degrading organic matter to inorganic molecules, which can then re-enter anabolic metabolic pathways in plants or other organisms (Lindahl et al., 2007). They are important in the cycling of carbon that is sequestered in wood and other plant tissues.

Beneath the quiet waters of a small lake, bog, swamp or within the waters of a running brook or river, unseen but active, a distinctive group of fungi lives and reproduces. The filamentous bodies of these fungi invade submerged substrates (dead stems and leaves of herbaceous and woody plants). Penetrating their substrates, aquatic fungi release enzymes that break down the ligno-cellulose of plant cell walls, the pectins that hold cells together, and starch stored in plant tissues. The fungi then use the resulting simple sugars and amino acids to grow. In

carrying out their enzymatic activities, these fungi contribute to the decomposition of dead plant material and serve as food for invertebrate grazers.

The distribution of fungi in the marine environment has not been studied well when compared with the studies on fungi in fresh-water and terrestrial ecosystems. The marine environment includes oceans, estuaries, mangroves, salt marshes and lagoons where the salinity ranges from 5 to 35 psu (practical salinity unit). Marine fungi are not taxonomic entity but are categorized into ecological groups based on their ability to occupy specific habitats and substrata (Hyde et al., 2000; Kis-Papo, 2005). Obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat, whereas facultative marine fungi are from freshwater or terrestrial milieus with the ability to grow and possibly also sporulate in the marine environment (Kohlmeyer and Kohlmeyer, 1979). Hyde et al. (2000) suggested that the ability to germinate and to form mycelium under natural conditions should be used as a criterion for the definition of a marine fungus. Majority of the fungi classified as obligate marine are parasites on plants and animals. Also, they exist as saprobes on dead organic matter (Kis-Papo, 2005). Marine fungi are distinct from their terrestrial and freshwater counterparts, both in their taxonomy, morphology and adaptation to an aquatic habitat (Jones, 2000).

2.1.2 *Marine fungi*

Fungi from marine habitats were first described around the middle of the 19th century in France by Duriers and Montagne, (1846–1850). However, the study of marine fungi as a distinct ecological branch of mycology was started by Barghoorn and Linder, (1944) describing 10 new genera and 25 species of lignicolous marine fungi. Afterwards, fungi have been isolated and enumerated to some extent from mangrove leaves, sea-grasses, and salt-marsh grass (Sathe and Raghukumar, 1991; Newell, 1993; Raghukumar et al., 1995; Alias and Jones,

2000a, b; Besitulo et al., 2010). Marine fungi comprise an estimated 1500 species, excluding those that form lichens (Jones and Mitchell, 1996). There are sources of overestimation of species biodiversity within and among habitats because of several reasons. Since most of the species are identified by analyzing morphological features and within marine habitats, we do not adequately know the meiosporic/mitosporic connections hence the same fungus may be counted twice. Synonymies among species also occur, probably in many cases due to the lack of published keys and compilations of species descriptions. Among habitats, different taxonomic specialists may work at different ends of an ecotone and the same fungal species may be counted twice in the overall estimation of fungal diversity (Shearer et al., 2007). Several recent papers have addressed the issue of the diversity and numbers of marine fungi (Jones, 1995; Jones and Mitchell, 1996; Jones and Alias, 1997). Kis-Papo, (2005) compiled all the publications and listed 467 higher marine fungi from 244 genera. They may be divided into a majority of Ascomycota (97%), a few Basidiomycota (~2%) and anamorphic fungi (<1%). In another assessment 465 marine meiosporic and mitosporic acomycetes and 10 basidiomycetes were reported (Shearer et al., 2007). Jones et al. (2009) has presented the most recent figure of the number of higher marine fungi. This includes 424 species of Ascomycota belonging to 251 genera, 94 species of anamorphic fungi (of 61 genera) and 12 species of Basidiomycota (of 9 genera).

The largest order of marine ascomycetes is the Halosphaeriales. Jones, (1995) presented 43 genera and some 133 species of this order while in another study, the figure was 45 genera and 119 species (Kis-Papo, 2005). This is approximately half of the total number of marine ascomycetes (Kohlmeyer and Volkmann-Kohlmeyer, 1991).

In a major study, 200 higher marine fungi were encountered from fifty five mangroves and their associates (Jones and Alias, 1997). A review literature indicated that 625 fungal species have been reported from mangrove environments (Schmit and Shearer, 2003) but these also included those growing

on the terrestrial parts of mangrove trees. Out of these, only 230 species occurred on intertidal or submerged mangrove substrata.

A total of 112 species of xylophillus basidiomycetes, distributed in 63 genera, 27 families and 9 orders from mangroves have beed reported (Baltazar et al., 2009). Of these Polyporaceae is the most represented family with 33 species.

2.1.3 *Fungi in Mangroves and coastal waters*

The mangroves cover approximately one-fourth of the entire tropical coastline and extended over 15.5 million ha worldwide yielding a global biomass of 6.7 Gton dry weights (Twilley et al., 1992; Alongi, 2002). Mangroves are one of the most productive natural ecosystems (Kohlmeyer and Volkmann-Kohlmeyer, 1993) and are important in soil building and global cycling of carbon dioxide and sulphur (Bandaranayake, 1998). In a study it was estimated that fungi associated with decaying sea grass and mangrove plants contribute about 3% of the biomass g^{-1} (dry weight) of detritus (Raghukumar et al., 1999). Marine fungi which are found on attached parts of mangroves and are repeatedly isolated or collected on mangle substrata from the sea or an estuary, with the ability to sporulate under these conditions are called as manglicolous fungi (Jones and Hyde, 1990). Manglicolous fungi constitute the second largest group of marine fungi (Hyde and Jones, 1988). Mangroves are woody plants that grow at the interface between land and sea in tropical and sub-tropical latitudes where they are adapted to the conditions of high salinity, extreme tides, strong winds, high temperatures, muddy-anaerobic soils and high microbial and faunal competition (Alongi, 2002). Hence the fungi present in these habitats are also unique physiological groups adapted to the peculiar environment. They are distinct in their physiology, morphology and adaptations to an aquatic habitat (Meyers, 1996). The drift woods of marine environment are inhabited by only specific group of fungi, which colonize the woods because of their genetics potentiality, a battery of enzyme producing abilities and adaptability to the changing physico-chemical

condition of the environment. In addition, mangrove is a unique habitat where there are diverse niches for fungi and their distribution pattern changes with varying geographical regions. Also, extent of salinity, kind of substrates, position of inter-tidal region, nature of floor, pH and oceanic region affect the occurrence and diversity of marine fungi in the mangrove ecosystem (Fig. 2.1). In addition, they are dependent on the nature of the substrate and temporal regions that favor the colonization, growth and substrate possession.

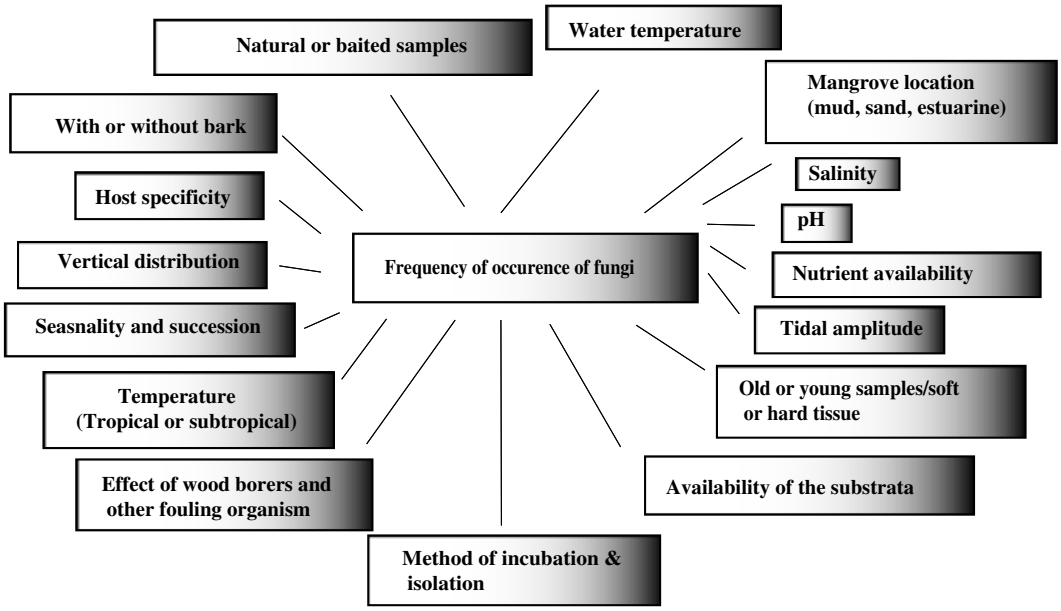


Fig. 2.1: Factors which influence the frequency of occurrence of mangrove fungi. Adapted from: (Alias and Jones, 2009).

The fungi colonizing submerged parts of mangroves are mostly saprobes (Kohlmeyer, 1969; Jones and Alias, 1997). They play a major role in the microbial processes occurring in the mangroves especially the mangrove food web and the surrounding coastal area, to release nutrients which can again be used by plants and animals as organic sources for metabolism (Raghukumar, 2004). Mangrove plants, found in the tropical and subtropical estuarine belts contain

about 50% lingo-cellulosic structural polymers and about 50% soluble organics which include tannins and phenolics (Benner and Hodson, 1985). The fungi involved in the degradation and mineralization of these kinds of substrates are termed as lignicolous or lignin-degrading fungi.

Endophytic fungal association with mangrove plants confers protection from adverse environmental conditions and allows them to successfully compete with saprobic fungi decomposing senescent parts (Kumaresan and Suryanarayanan, 2002). These fungi inhabit at least for some time in their lifecycle, the inner tissues of plants without causing any external symptoms (Petrini, 1991; Hirsch and Braun, 1992). Mangrove plants provide a hostile environment for endophytes, by the presence pf phenolics like tannins, which are known to inhibit the growth of litter or soil fungi (Kumaresan et al., 2001). Hence they are adapted to grow in the presence of phenolic compounds. It has been demonstrated that endophytes of various plants are able to utilize most substrates present in the cell walls of the hosts with the virtue of their enzymes (Carroll and Petrini, 1983; Sieber-Canavesi et al., 1991; White et al., 1991). After the tissue becomes senescent, endophytic fungi release an array of non-specific enzymes necessary for the degradation of lignocellulosic material (Maria et al., 2005).

Many mangrove plants have high concentration of salts in their leaves and few species often excrete salts which get crystallized on their leaf surface (Tomlinson, 1986). Therefore halotolerant foliar endophytic marine fungi have evolved unique metabolic mechanisms that are responsive to salt concentrations. Bugni and Ireland, (2004) suggested that marine fungi have osmoregulatory mechanisms that signal the production of polyols and amino compounds in conjunction with increasing concentration of cytoplasmic ions.

2.1.4 *Marine fungi from coastal waters of India*

The report on 18 marine fungi along the coastal waters of the Tamil Nadu by Raghukumar, (1973) was the first major publication on this group from India.

Afterwards, several new genera and species of marine and mangrove fungi have been reported from the west coast of India (Patil and Borse, 1982; Hyde and Borse, 1986a, b; Borse, 1987; Raghukumar et al., 1988; Borse and Hyde, 1989; Hyde et al., 1992).

Twenty six manglicolous marine fungi comprising 20 Ascomycetes, 1 Basidiomycete and 5 Mitosporic fungi were isolated from the mangrove forests of Kerala, South India. Based on the percent frequency of occurrence, *Lulworthia grandispora* (13.19%), *Dactylospora haliotrepha* (12.09%), *Savoryella lignicola* (10.99%) and *Cirrenalia pygmea* (10.99 %) were the most frequent species (Nambiar and Raveendran, 2009). A total of 165 marine fungi encompassing 111 ascomycetes, 1 basidiomycete and 53 mitosporic fungi were reported from Indian mangrove (Sridhar, 2009). A total of 33 fungi (20 Ascomycota, 1 Basidiomycota, 12 Mitosporic fungi) were recorded from the estuarine systems in the coastal waters of Goa, India (Vishwakiran et al, 2001). Altogether 88 species belonging to 47 genera of higher marine fungi were isolated from woody substrates along the west coast of India (Prasannarai and Sridhar, 2001).

2.1.5 *Potential of marine fungi for lignocellulose degradation*

Since marine fungi grow in unique and extreme habitats, they have the capability to produce unusual metabolites and enzymes with physiological novelty. In a study, Raghukumar et al. (1995) found that the sea grasses and mangrove plants are the major contributors of lignocellulose in the highly productive costal marine environments. Fungi play an important role in degradation of lignocellulose in these ecosystems (Raghukumar et al., 1994). Studies on the involvement of the fungi and their enzymes in the breakdown of mangrove leaves and wood have also been conducted (Hyde, 1990). They are the major decomposers of woody and herbaceous substrata entering marine eco-systems. Their importance lies in their ability to degrade lignocellulose with the aid of extracellular enzymes. Rohrmann and Mlitoris, (1992) reported that about 85% of marine ascomycetes tested

produced endoglucanase. Several marine isolates showed xylanolytic and cellulolytic activity in a study (Ragukumar et al., 1994). Pointing et al. (1998) reported that 15 fungi they tested produced cellulolytic enzymes. Most of the marine fungi have been identified from substrata containing lignocellulose, and therefore it is not surprising that several genera have been implicated in wood decay activity within marine and estuarine environments. Morphological decay features suggesting soft rot and white rot decay have been observed in wood samples colonized by the marine fungi (Mouzouras, 1989). It is clear that a comprehensive study of lignocellulolytic marine fungi is necessary to better understand their role in the environment. From existing studies it seems likely that several species may be cellulolytic, with some also capable of lignin degradation. These marine fungi are probably soft rot and white rot degraders of wood, and participate in the turnover of an abundant biopolymer. Furthermore, examining the physiology of lignocellulolytic marine fungi may reveal strains with novel commercial uses since ligninolytic fungi have a variety of potential biotechnological applications including bioremediation (Hyde et al., 1998; Raghukumar, 2008). A marine derived fungus isolated from mangrove detritus produced xylanase that was thermo-stable at 55 ºC and active at pH 8.5. Several of the industrial effluents have alkaline pH and high salt content and therefore, marine fungi may be ideally suited for the bioremediation of such effluents (Raghukumar, 2002). A marine derived isolate NIOCC #312 was able to decolorize the bleach plant effluent at pH 4.5 as well as at 8.5 (Raghukumar, 2008).

2.1.6 *Isolation and Screening of fungi producing lignin degrading enzymes*

Culturable bacteria from natural substrates is relatively easy to obtain on the basis of colony count of the unicells of typical isolate, whereas, sampling for fungi is much more difficult due to their comparatively slower filamentous growth habit. There may be a large mass of fungal mycelium present, but the methods adapted

from bacteriology may not detect it. Moreover, dormant fungal spores may produce numerous colonies while thriving non-sporulating colonies may not be recovered at all. On Petri-plates, colonies representing many species are usually isolated; these are then sub-cultured and tested further. With few exceptions, this approach leads to the contamination by bacteria. In general, fungi are slower growing and produce fewer propagules than do bacteria. In addition, fungi are less likely than bacteria to have the capacity to use xenobiotics as sole carbon sources. Many fungi need a supplemental carbon source to sustain growth, i.e., their degradative potential is co-metabolic (Bennett et al., 2002).

Thus, the key to successful isolation of fungi for xenobiotic degradation is two fold:

(i) The recognition that fungi are easily outgrown by bacteria.

(ii) The recognition that they produce many potent bio-degradative enzymes capable of degrading toxic pollutants yet does not use these breakdown products to sustain growth.

To successfully isolate fungi with potential for bioremediation, it is necessary to impose imaginative enrichment conditions, including the careful selection of supplementary carbon and nitrogen sources along with bacteriostatic agents.

Qualitative assays are powerful tools used in screening fungi for lignocellulose degrading enzyme production. Such tests give a positive or negative indication of enzyme production. They are particularly useful in screening large numbers of fungal isolates for several classes of enzyme. Different workers used different media and physio-chemical parameters for isolating lignin degrading enzyme producing fungi. Dion, (1951) used various phenolic compounds for screening of laccase-producing *Polyporus versicolor* which resulted in visual color change in the presence of phenolic materials. Westermark and Eriksson, (1974) used agar plates containing lignin for screening laccase-producing fungi. To test for laccase activity, plates were flooded with guaiacol which gives an intense red to brown color after oxidation by laccase.

Arora and Sandhu, (1985) used lignin-guaiacol agar medium for screening such fungi. Field et al., (1992) demonstrated that a polymeric dye, red poly (vinylamine) sulfonate anthrapyridone (poly R-478), could be used for screening white-rot fungi which have the ability to degrade anthracene and benzo[a]pyrene. Kiiskinen et al., (2004) cultivated fungi on solid media containing indicator compounds (such as RBBR, poly R-478, guaiacol, and tannic acid) that enabled the detection of ligninolytic enzymes as specific color reaction. Marine-derived fungi selected by their capacity to decolorize/tolerate RBBR dye was used for the detection of lignolytic enzymes (da Silva et al., 2008). Four marine fungi namely, *Nia vibrossa* (basidiomycete), *Julella avicenniae* (ascomycete), *Lignincola laevis* (ascomycete) and *Stagonospora* sp. (a mitosporic fungus) were notable in their ability to completely decolorize such polymeric dyes whilst utilizing either glucose or cellulose as a primary carbon source (Pointing et al., 1998). 2, 2′azinobis- (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) is also suitable for screening LDEs because its one-electron oxidation product is soluble in water, stable, and intensely green (D'Souza-Ticlo., 2008).

2.1.7 *Identification of fungi*

Fungal taxonomy is a dynamic, progressive discipline that consequently requires changes in nomenclature. In mycology, species have historically been distinguished by a variety of methods and concepts. The fungus comprises of a telomorph (sexual state) and one or more anamorphs (asexual state). Traditional fungal classification is possible when the fungus is at its telomorphic stage. Classification based on morphological characteristics, such as the size and shape of spores or fruiting structures, has traditionally dominated fungal taxonomy. Schmit and Lodge, (2005) listed the advantages of the classical methods for fungal identification. These are generally less expensive and need less specialized equipments. Further, the data from various studies can be combined in order to perform meta-analysis, which can be used to determine the biological and

environmental factors that influence fungal community structure at large scales. Fungi that have not produced spores under the given environmental conditions (e.g., lack of appropriate light, moisture, temperature, or specific nutrient conditions) are grouped under the term "non-sporulating fungi." Many of these organisms never sporulate in culture (mycelia sterilia), but some represent non-sporulating colonies of common fungi (for example *Cladosporium*, *Alternaria*, or even *Aspergillus*). As all the fungi are capable of producing a non-sporulating state, the distribution of non-sporulating fungi is cosmopolitan in nature. Identification of these non-sporulating fungal species is not possible using standard microscopic techniques which require information on spore-bearing structures. Hence this is the major limitation of phenotypic approaches and many fungi will remain unclassified when taxonomists solely rely on phenotypic characteristics. Additionally, more taxonomic expertise is required for classical methods than molecular methods. Species may also be distinguished by their biochemical and physiological characteristics, such as their ability to metabolize certain bio-chemicals, or their reaction to chemical tests. Although some of these are very useful for identifying poorly differentiated fungi, in most cases they are only complementary tools of morphological data. The biological species concept discriminates species based on their ability to mate. If an organism looses sexual and asexual reproductive structures, accurate taxonomic assignment is quite difficult even at the level of phylum (Sugiyama, 1998). Conversely, nucleic acids, as genotypic characters, are ubiquitous and are not dependent on the expression of reproductive structures (Taylor, 1993). Revolutionary technical advances have led to the use of molecular studies for phylogenetic classification. The advent of polymerase chain reaction (PCR) has allowed the analysis of phylogeny precisely and authentically. The application of molecular biological tools, such as gene cloning, nucleic acid sequencing and proliferation of high performance computers, and improvement of molecular evolutionary analysis programs to study diversity has greatly enhanced the resolution and added robustness to

estimates of genetic diversity within various taxonomic groups (Hibbett et al., 2007). The use of ribosomal DNA (rDNA) fingerprints has become one of the most useful techniques to aid in fungal identification and to study phylogenetics. The nuclear ribosomal gene cluster is comprised of three regions coding for the small (18S) and large (28S) subunit ribosomal RNA genes which are interrupted by the internal transcribed (ITS) spacer 1 and 2 including 5.8S rDNA (Fig. 2.2). These genes can be used to compare organisms at several levels as they evolve as a single unit even though the rates of evolution vary within individual regions of the ribosomal RNA gene cluster (Hibbett, 1992). The small (18S) and large (28S) subunit is useful for the reconstruction of long-term speciation events above species ranks while the rapidly evolving ITS1 and ITS2 region facilitates the recognition of species and subspecies (White et al., 1990).

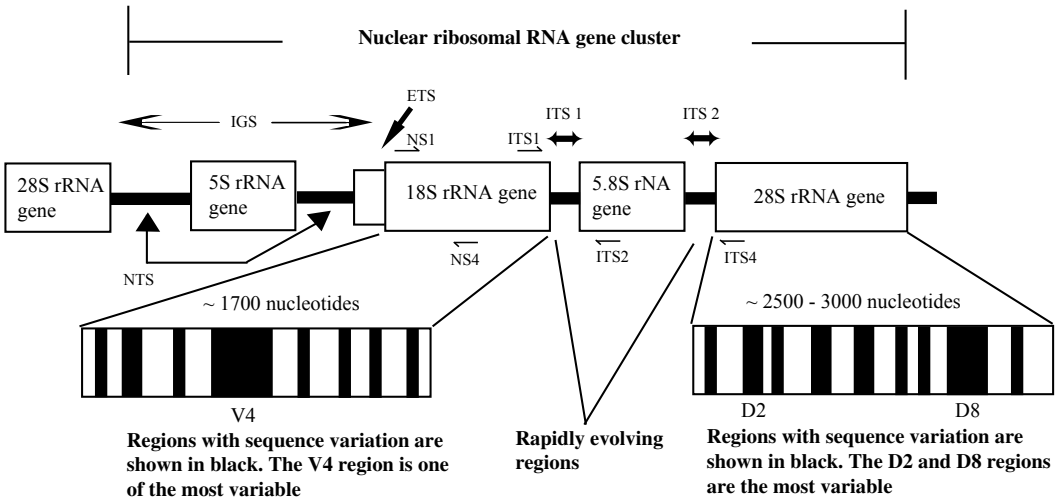


Fig. 2.2: Schematic representation of the fungal rRNA gene. The cluster comprises three main gene (5.8S, 18S and 25S or 28S rRNA molecules) interspersed between spacer regions (IGS – intergenic spacer, NTS – non-transcribed spacer, ETS – externally transcribed spacer). The spacer regions are the least conserved and evolve at a faster rate than the genes. The ITS regions contains variable sequences that also have a fast rate of evolution. These spacers are useful in determining the evolutionary relationships at the species and below level. The genes comprise variable (the V and D regions) and conserved sections. The least conserved of these are the V4 region in the 18S rRNA gene, and the D2 and D8 regions in the 28S rRNA gene. The

primers used to amplify the various rDNA regions are represented as one direction (5' to 3') arrows. Modified from: (Mitchell and Zuccaro, 2006)

The fungi have been historically considered to be plants and mycology is traditionally a sub-discipline of botany because of their apparent lack of motion. Whittaker, (1969) recognized them as a kingdom and phylogenetic analyses of both ribosomal DNA and protein-coding genes suggest that fungi are more closely related to animals than plants (Wainright et al., 1993; Lang et al., 2002). Now they are placed with the animals in the monophyletic group of opisthokonts (Shalchian-Tabrizi et al., 2008). Analyses using molecular phylogenetics support a monophyletic origin of the fungi (Hibbett, 2007). The taxonomy of the fungi is in a state of constant flux, especially due to recent research based on DNA comparisons. These current phylogenetic analyses often overturn classifications based on classical and sometimes less discriminative methods based on morphological features and biological species concepts obtained from experimental matings.

There is no unique generally accepted system at the higher taxonomic levels and there are frequent name changes at every level, from species upwards. Efforts among researchers are now underway to establish and encourage usage of a unified and more consistent nomenclature (Celio et al., 2006). The major phyla (sometimes called divisions) of fungi have been classified mainly on the basis of characteristics of their sexual reproductive structures. Currently, seven phyla are proposed: Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Ascomycota, and Basidiomycota (Hibbett, 2007). Around 100,000 species of fungi have been formally described by taxonomists so far, but the global biodiversity of the kingdom fungus is not fully understood (Mueller and Schmit, 2006).

2.2 *Objectives*

Isolation of fungi from detritus and decaying wood from marine habitats, screening them for lignin degrading enzyme production and to study the influence of various parameters for the production of these enzymes and decolorization of industrial effluents. Identification of these fungi by the application of molecular methods was done.

2.3 *Material and methods*

2.3.1 *Sampling*

The collection of detritus, drift wood and attached wood of mangrove trees for the isolation of fungi was carried out at various mangrove swamps namely, Choraõ, Cumbarjua, Nerul, Oxel and Britona of Goa, India (73° 30' – 74º 10' E and 15° 25' – 15º 40' N) (Fig. 2.3). Each sampling was done during the low-tide and physico-chemical conditions prevalent were recorded. These included dissolved oxygen content, pH, temperature and salinity of the ambient sea-water. Salinity was determined by measurement of the conductivity of the water sample with Autosal, Laboratory Salinometer (Guildline instruments Ltd., Canada). For pH determination, a pH meter (LabIndia, India) was used. Temperature was measured with a Celsius thermometer.

The decaying wood and leaf matter were rinsed with the ambient estuarine water to remove attached soil particles and other extraneous matter. They were then placed in sterile transparent zip-lock bags and transported to the laboratory. The decomposing sea-grass leaves were collected from a coral lagoon of Kavarati Island in the Lakshadweep Archipelago, Arabian Sea.

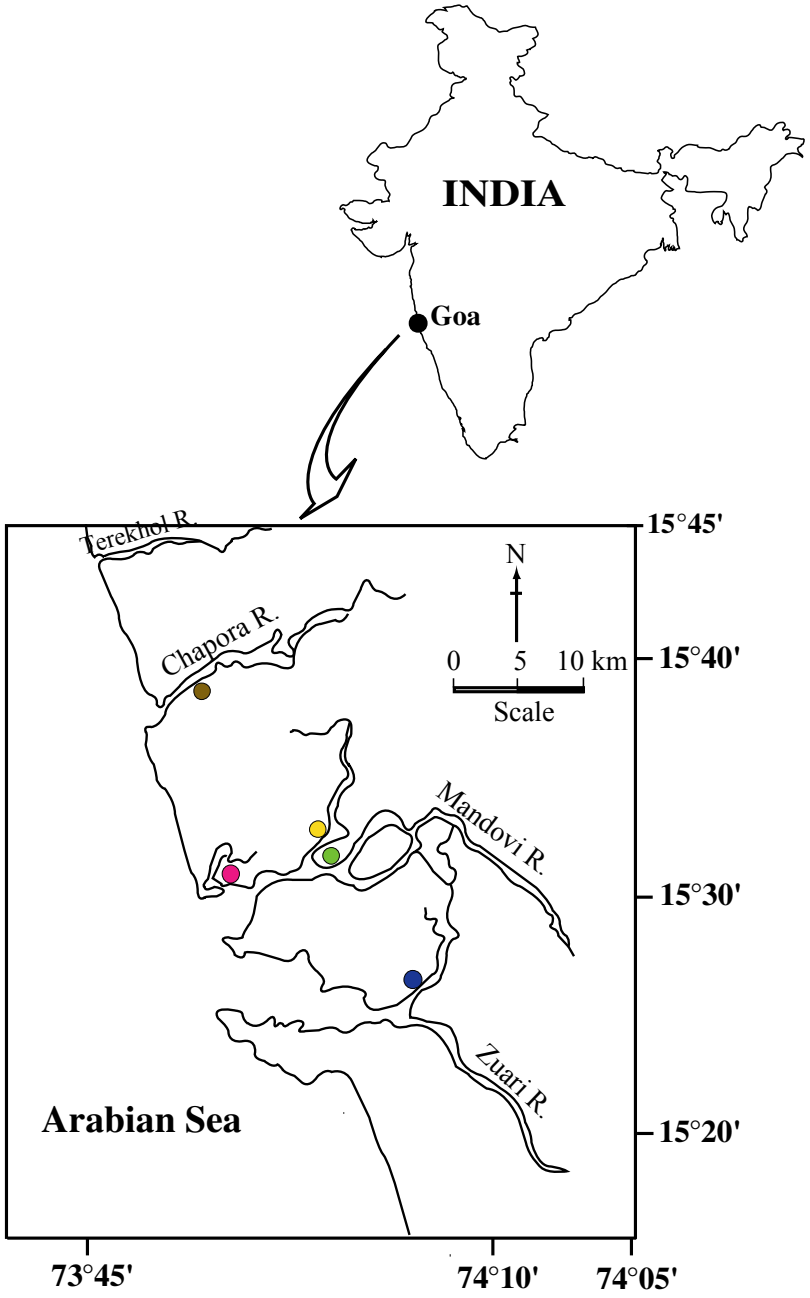


Fig. 2.3: Geographical locations of sampling sites.

● Choraõ ● Cumbarjua ● Nerul ● Oxel ● Britona

2.3.2 *Isolation of fungi*

Fungi from detritus were isolated by particle plating method (Damare et al., 2006). Isolation from decaying wood material was carried by moist chamber incubation method (D'Souza et al., 2006) and single spore isolation method (Choi et al., 1999). They were maintained in Boyd & Kohlmeyer (B & K) medium (Appendix 6.1.2) prepared with sea water of 15–17 psu, containing 10% antibiotic solution (Appendix 6.2).

***Particle-plating method*:** It is a selective isolation technique which minimizes the recovery of common saprobic fungi (Bills and polishook, 1994). The decaying leaves were cut into pieces of ~1 cm^2 under sterile conditions and surface sterilized for 5 min with a mixture of 0.5% sodium hypochlorite and 0.1% detergent prepared in sterile distilled water. The solution was then drained and the leaf pieces washed 3-4 times with sterile sea-water. These pieces were homogenized using sterile mortar and pestle and passed through sterile stainless steel sieves of 200 µm and 100 µm in succession, followed by washing with sterile sea-water. The particles retained on the 100 µm sieve were then plated on B&K medium prepared with 50% sea-water and fortified with 10% antibiotic solution to prevent bacterial growth. The antibiotic solution contained 400,000 U of procaine penicillin and 1g of streptomycin sulphate in 100ml of sterile distilled water. The plates were incubated at room temperature.

For the isolation of slower growing wood decay fungi, 1% (w/v) of benomyl, a benzimidazole fungicide, was added in the medium (Maloy, 1974).

***Single-spore isolation method*:** Decaying wood pieces after incubation in a moist chamber for over a fortnight were thoroughly examined for the presence of fruiting bodies of sporulating fungi using a stereo-microscope. A sterile glass needle was used to transfer a single fruiting body onto the slide, containing a drop

of sterile sea-water. This was then placed under the low power of a stereo-microscope and crushed to release the spores. By capillary action, the drop was sucked into a sterile Pasteur pipette, which was then released into a Petri-plate containing B&K medium with 10% antibiotic solution, which was gently tapped so that the drop flows downwards in a straight line. The plates were examined under the microscope for the presence of isolated spores, the area on the plates on which isolated spores were present were marked and incubated at room temperature and examined regularly till germination, under the light microscope. The germinated spores were excised and transferred onto fresh plate containing B&K medium.

***Moist chamber incubation method*:** This method was applied to isolate the non-sporulating fungi as well as fungi which produce spores that tightly adhere to each other within wood. Decaying wood pieces were incubated in glass Petri-plates containing tissue paper towel moistened with sterile sea-water until visible mycelia were detected. The hyphae or the cluster of spores were directly spotted / transferred onto plates containing B&K medium with 10% antibiotic solution.

2.3.3 *Screening for Lignin-degrading fungi*

The fungi isolated from the above methods were qualitatively screened for the presence of lignin degrading enzymes using model compounds and dyes. Excessive pigment producing isolates were not selected for analysis.

Qualitative assay for Lignin-degrading enzymes

Preliminary screening of the isolates for the presence of lignin-degrading enzymes was carried out on B&K agar medium containing model compounds such as guaiacol, ABTS (2, 2'-azinobis-3-ethylbenthiazoline-6-sulfonate) and Poly-R 478 (Poly-R). The production of an intense brown color under and around the fungal colony in guaiacol supplemented medium and deep green to deep purple color in

ABTS-supplemented medium indicated presence of laccase activity Decolorization of Poly-R from pink to yellow or colorless indicated presence of lignin peroxidase and/or manganese peroxidase (D'Souza et al., 2006).Positive isolates were routinely checked for purity by light microscopy.

2.3.4 *Biomass estimation*

Biomass estimation was carried out to asses the ability of isolated and screened fungi to grow in saline conditions. Selected isolates were grown in B&K medium prepared in distilled water (D/W) and half strength sea-water with salinity of 15-17 psu (practical salinity units). The mycelia were harvested on day 9 by centrifugation of the culture at 5000 rpm for 10 min and washed twice with D/W to remove any precipitated salts. They were lyophilized and the dry weight was measured and expressed in g L^{-1}.

2.3.5 *Identification of isolated fungi*

As most of the screened isolates were anamorphs, they were identified by amplification and sequence analysis of their partial 18S and complete Internal Transcribed Spacer (ITS) regions of rRNA gene.

Isolation of DNA from the cultures

DNA was isolated from all the screened filamentous fungi. The fungi were grown in B&K (broth) medium for 4-5 days. Mycelia were harvested, lyophilized and crushed in a mortar and pestle to a fine powder. Isolation of DNA was carried out following the modified form of standard procedure (Stoeck and Epstein, 2003). The above samples were incubated at 65ºC for two hours in a high salt extraction buffer containing 100 mM Tris-HCl buffer with 8 pH containing 100 mM Na_2EDTA, 100 mM $NaPO_4$, 1.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate, Proteinase K, 100 µg mL^{-1} for obtaining fungal DNA.

PCR amplification of 18S and ITS regions of rRNA gene

For amplification of 18S and ITS regions of the fungal SSU rDNA, specific primers were used. Partial region (~ 1100bp) of 18S rDNA was PCR-amplified using a universal fungal specific primer sets, NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3'). Full length of Internal transcribed spacer (ITS) region was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The conditions for PCR included an initial hot start incubation (5 min at 94ºC) followed by 34 cycles (denaturation at 94º C for 30 sec, annealing at 55º C for 30 sec and extension at 72º C for 1 min) and a final extension at 72ºC for 5 min.

Sequencing and phylogenetic analyses

Fresh PCR products were purified by using gel extraction kit (Sigma, Genosys, USA) and sequenced at National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3730 DNA Analyzer, Applied Biosystems, USA). Forward and reverse sequences were assembled using Chromas Pro version 1.34 (Technelysium Pvt. Ltd, Tewantia, Queensland, Australia). Sequences obtained with ITS and 18S rDNA primers were analyzed separately. Multiple alignments were done for all the sequences along with closely matching sequences in Clustal W (Thompson et al., 1994). Gaps and ambiguously aligned sequences were removed from further analyses. A phylogenetic analysis was conducted using distance setting (Maximum Parsimony) in MEGA 4 (Kumar et al., 2008) with 1,000 bootstrap replicates. Phylogenetic trees were constructed with sequences obtained with ITS and 18S rDNA primer sets individually.

2.3.6 *Quantitative assay for lignin-degrading enzymes*

Few fungal isolates, which were positive for production of LDEs during qualitative assay, were subjected for quantitative enzyme estimation. The fungi were grown in B & K broth (20 ml in 100 ml Erlenmeyer flasks) for 7 days. The fungal biomass after rinsing to remove the residual medium was homogenized in sterile sea water in Omni Macro-homogenizer (No. 17505, Marietta, GA, USA) for 5 s. The mycelial suspension (10%, v/v) was used for inoculating 20 ml of low nitrogen (LN) medium (Appendix 6.1) prepared with half strength sea-water (D'Souza-Ticlo et al., 2006). The activity of the lignin-degrading enzymes, laccase, Manganese peroxidase (MnP) and lignin peroxidase (LiP) was measured (Appendix 6.4.1, 6.4.2, 6.4.3) in the culture supernatants on day 6, 9 and 12.

2.3.7 *Decolorization of dyes by fungi*

The isolates positive for LDE production and belonging to different class of fungi were tested for decolorization of various reactive dyes. Synthetic dyes namely, Reactive Blue 140, Reactive Yellow 145, Reactive Green 19, Reactive Blue 163, Reactive Blue 4, Reactive Blue 140 base, Reactive Blue 160 base and Reactive Red 11 were kindly provided by Atul Pvt. Ltd, Gujrat, India. Reactive Black 5 was purchased from Sigma-Aldrich, USA. Synthetic dyes were each added separately at 300 and 3000 mg L^{-1} to 6 day-old cultures raised in B&K broth. The day of addition of dyes was considered as day 0 for color measurements. Decolorization of these dyes in the culture supernatants was monitored at absorbance maxima of respective dye after appropriately diluting with D/W on day 0, 3, 6 and 9. Decrease in absorbance with respect to that of control (0 day sample) was used for calculating % decolorization.

2.3.8 *Statistical analyses*

The significance of the results obtained was evaluated by two way analysis of variance (ANOVA) using Excel (Microsoft, USA) program for statistical significance.

2.4 *Results*

The physico-chemical conditions prevalent at the time of sampling are mentioned in Table 1.1. Salinity varied in different sampling stations ranging from 21-33 psu. The pH was mostly around 7 and temperature varied from 21-30 ºC. Dissolved oxygen (DO) was in the range of 4.6-10.6 at different sampling sites.

Table 2.1: Physico-chemical conditions of the estuarine water prevalent at the sampling sites.

Sampling Station	Date	Temperature (˚C)	pH	Dissolved Oxygen (mg L^{-1})	Salinity (psu)
Choraõ	23.11.2006	27	7.5	4.6	23
Cumbarjua	24.11.2006	24	7.9	7.3	26
Nerul	03.12.2006	27	7.4	9.8	21
Oxel	05.12.2006	21	7.1	10.6	33
Britona	06.12.2006	23	7.0	5.9	29
Kavarati	09.03.2007	30	--	--	

Fungi were isolated from mangrove detritus, decaying leaves and sea grass by the particle-plating method whereas; single spore isolation and moist chamber incubation method was applied for isolation from decaying wood. These isolates were subjected to screening for LDE production. An intense brown color due to oxidation of guaiacol (Fig. 2.4B) and deep green or deep purple color from ABTS oxidation (Fig 2.4C & 2.4D) indicated laccase activity. Transformation of Poly-R 478 dye was indicative of peroxidase activity (Fig. 2.4E & 2.4F).

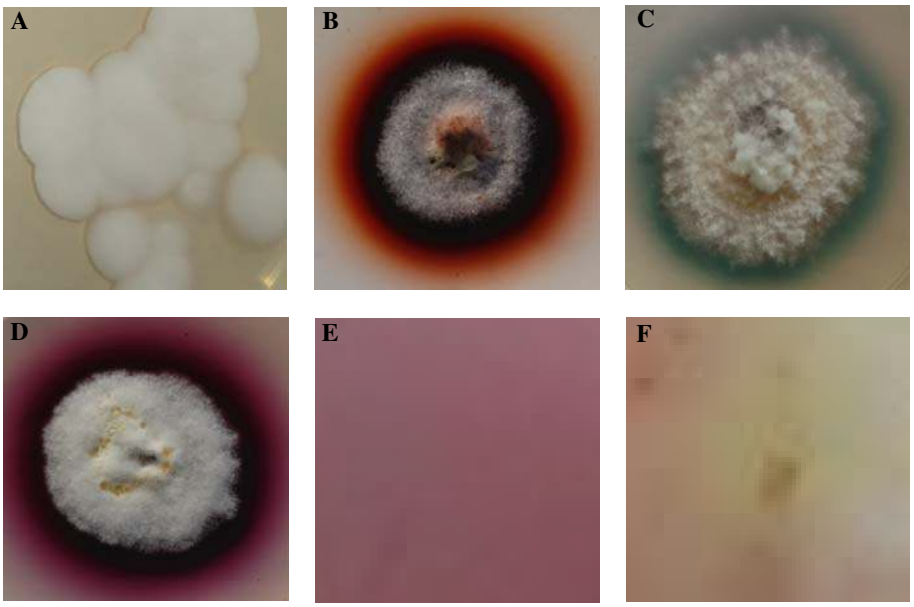


Fig. 2.4: Qualitative representation of laccase activity: A) Isolate with no activity; B) Guaiacol at 4mM; C & D) ABTS at 2mM. Qualitative determination of Peroxidase activity: E) Poly-R 478; F) Isolate showing decolorization/Transformation.

About 50 isolates were screened and those which were positive for the LDEs during qualitative plate assay are listed in Table 2.2. Laccase and Peroxidase production during screening was compared with previously characterized isolates, NIOCC #2a and #312 respectively (Raghukumar et al., 1999; D'Souza et al., 2006). Laccase producing fungi were observed to be dominating in the mangroves. Only four isolates decolorized Poly-R indicating rare occurrence of peroxidases in fungi from these environments.

Table 2.2: Positive isolates for lignin degrading enzymes screened by qualitative plate assay

Sampling Station	Isolate (NIOCC)	Guaiacol Oxidation (Laccases)	ABTS Oxidation (Laccases)	Poly R-478 Decolorization (Peroxidases)
Choraõ	#1V	+++	+++	-
	#4V	+++	++	-
	#9V	+++	+++	+
	#C10	++	+	-
	#11V	++	++	-
	#17V	+++	++	-
	#31V	+++	+++	-
	#38V	++	+	-
	#C3	+++	+++	-
	#C2	++	+	-
	#15V	+++	+++	+
Cumbarjua	#2V	+++	++	-
	#6V	+	++	-
	#13V	+++	++	-
	#14V	+++	+	-
	#32V	++	+	-
Britona	#5V	++	+++	-
	#8V	+++	+++	-
	#16V	++	+++	-
	#DV2	+++	+++	++
	#27V	+	+	-
	#29V	+	+	-
	#50V	++	+	++
Oxel	#18V	+++	+++	-
	#19V	++	+++	-
	#21V	+	+	-
Nerul	#20V	+++	+++	-
	#28V	+++	+++	-
	#36V	+	+	-
	#2V2	++	+	-
Kavarati	#3V	+	+	-
	#23V	+	+	-
	#26V	++	+	-
Choraõ*	#2a	+++	+++	+
Kavarati**	#312	+	+	+++

+ And – Sign indicates Intensity of Oxidation and Decolorization

***Guaiacol and ABTS Oxidation were compared with NIOCC #2a, a positive control for laccase**

****Poly R-478 Decolorization was compared with NIOCC #312, a positive control for peroxidases**

The production of biomass was determined in B&K broth prepared with D/W and half strength sea-water. The growth was significantly more in the medium prepared with half strength sea-water (*p* value = 0.000429) (Fig. 2.5).

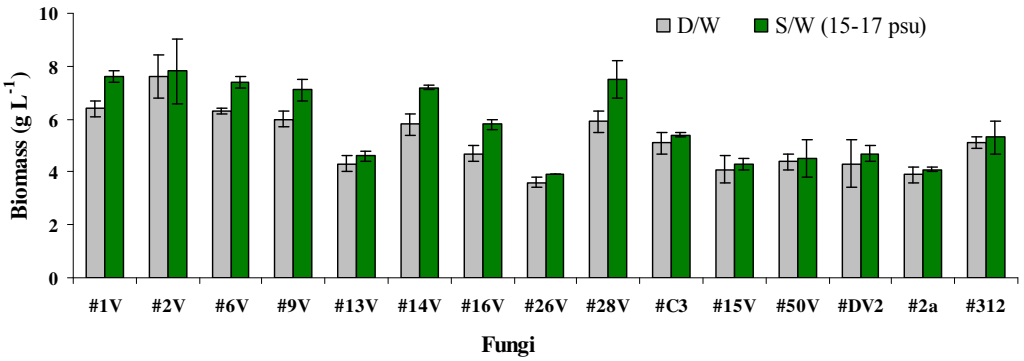


Fig. 2.5: Production of biomass in B&K medium prepared with D/W and half strength sea-water.

The fungi could not be identified by classical morphological taxonomic methods since no reproductive structures were observed; hence rDNA sequence comparison was used for identification. Two separate stretches of rDNA from isolates were analyzed namely, the partial 18S and complete ITS1-5.8S-ITS2 regions. The sequences obtained from all the isolates have been deposited in NCBI Gene Bank and accession numbers obtained (Table 2.3). Most of the fungi belonged to the phylum Ascomycota and clustered in the classes Sordariomyctes, Dothidiomycetes and Eurotiomycetes. The orders of these isolates were Pleosporales, Eurotiales, Xylariales, Diaporthales, Lulworthiales, Hypocreales and Incertae sedis. Maximum numbers of ascomycetes were of Lulworthiales (Table 2.3; Fig. 2.6, 2.7).

Only 3 isloates namely, NIOCC #15V, NIOCC #50V and NIOCC #DV2 belonged to the phylum Basidiomycota. Sequence analysis of 18S of #15V identified it to be *Coriolopsis byrsina.* However, ITS sequence analysis of this isolate showed 97% homology to an uncultured fungus clone. In a similar way, #50V clustered with genera *Phanerochaete* on the basis of 18S sequence while ITS sequence analysis showed maximum similarity to an uncultured fungus clone. Another basidiomycete, #DV2 was 99% identical to *Trametes vesicolor* and 99% to *Lenzites* sp. on the basis of 18S and ITS sequence analysis (Table 2.3; Fig. 2.6 & Fig. 2.7).

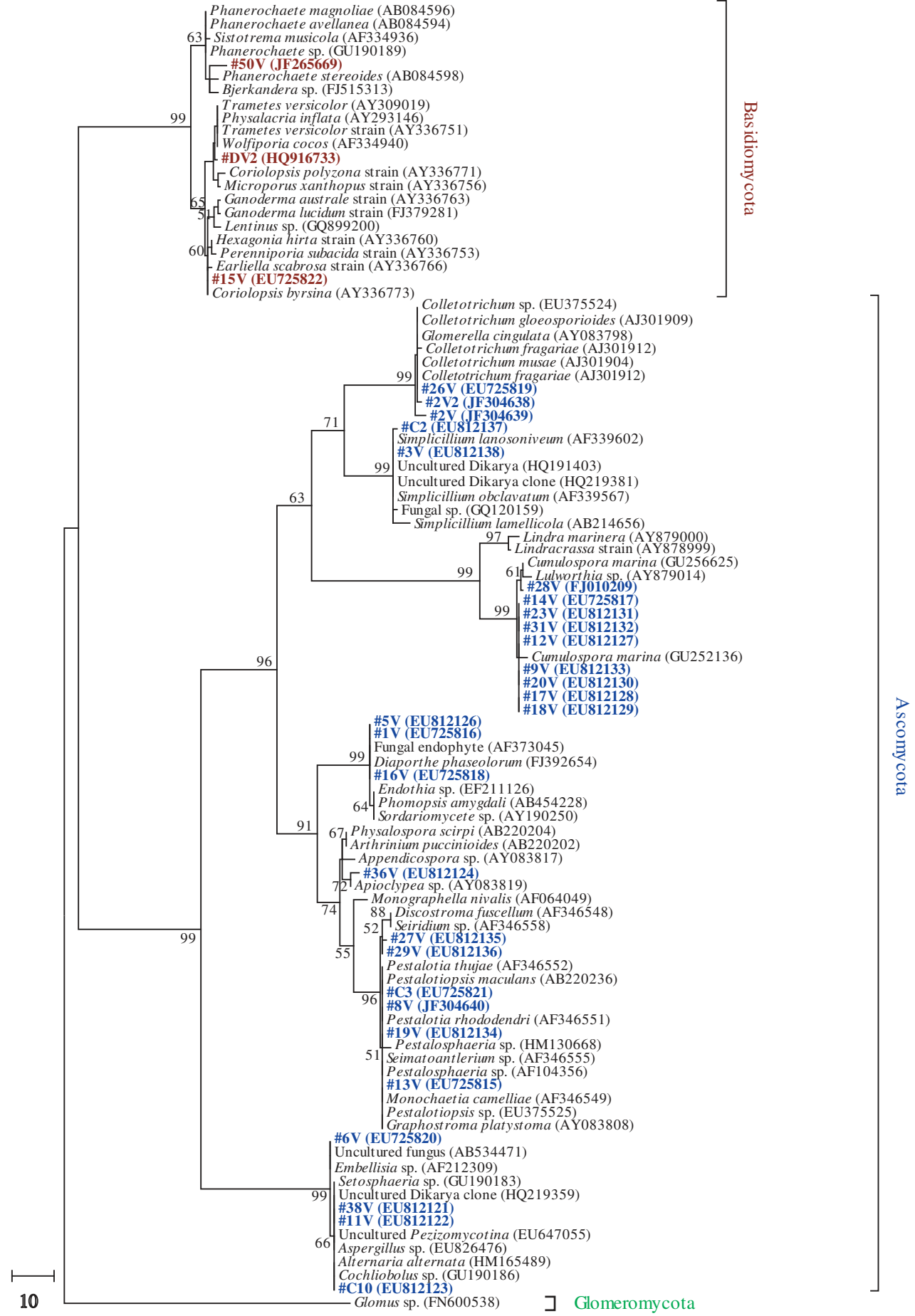


Fig 2.6: Maximum parsimony Phylogenetic tree based on fungal 18S gene sequences. Gen Bank accession no. are given within brackets. Fungi isolated during present study are marked brown (Basidiomycota) and blue (Ascomycota). *Glomus* sp. belonging to phylum Glomeromycota was used as an outgroup.

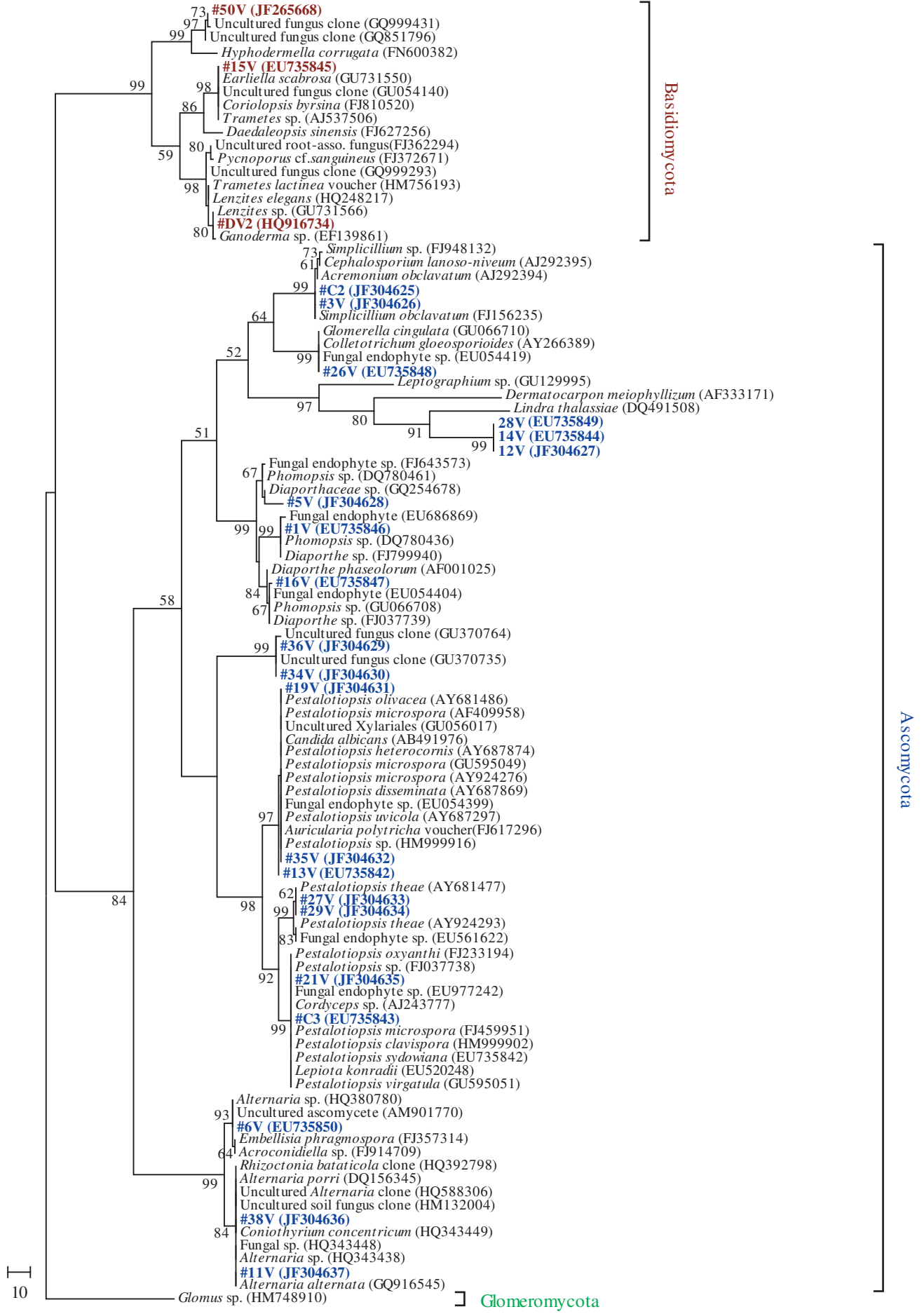


Fig 2.7: Maximum parsimony Phylogenetic tree based on fungal ITS gene sequences. Gen Bank accession no. are given within brackets. Fungi isolated during present study are marked brown (Basidiomycota) and blue (Ascomycota). *Glomus* sp. belonging to phylum Glomeromycota was used as an outgroup.

Table 2.3: Closest identified relative of the isolates during Blastn search with rRNA gene sequences

Isolate #		18S Sequence			ITS sequence			Phylum
Designation (NIOCC)	**Gen Bank Accession nos. of 18S and ITS sequences**	**Identity (Max % identity)**	**Gen Bank Accession No. of closest relative**	**Q- coverage (Error value)**	**Identity (Max % Identity)**	**Gen Bank Accession No. of closest relative**	**Q- coverage (Error value)**	
#15V	18S: EE059806 ITS: FJ010208	*Coriolopsis byrsina* (100 %)	AY336773	100% (0)	Uncultured fungus clone	GU054140	97% (0)	**Basidiomycota**
# 50V	18S: EU725822 ITS: EU735845	*Phanerochaete* sp. (99%)	GU190189	99% (0)	Uncultured fungus clone	GQ851796	99% (0)	
#DV2	18S: EU725818 ITS: EU735847	*Trametes versicolor* (99%)	AY336751	100% (0)	*Lenzites* sp. (99%)	GU731566	95% (0)	
#1V	18S: EU725821 ITS: EU735843	*Diaporthe phaseolorum* (100%)	FJ392654	100% (0)	*Diaporthe* sp. (99%)	FJ799940	99% (0)	**Ascomycota**
#2V	18S : JF304639	*Colletotrichum* sp. (99%)	EU375524	100% (0)	----	----	----	
#2V2	18S : JF304638	*Colletotrichum* sp. (99%)	EU375524	100% (0)	----	----	----	
#3V	18S: EU812138 ITS: JF304626	Uncultured Dikarya clone	HQ219381	100% (0)	*Acremonium obclavatum*	AJ292394	99% (0)	
#5V	18S: EU812126 ITS: JF304628	*Diaporthe phaseolorum* (100%)	FJ392654	100% (0)	*Diaporthaceae* sp. (96%)	GQ254678	96% (0)	
#6V	18S: EU725820 ITS: EU735850	*Embellisia* sp. (100%)	AF212309.2	100% (0)	*Alternaria* sp. (100%)	HQ380780	100% (0)	
#8V	18S: JF304640	*Pestalotiopsis maculans* (100%)	EU725821	100% (0)	----	----	----	
#9V	18S: EU812133	*Cumulospora marina* (99%)	GU256625	100% (0)	----	----	----	
#11V	18S: EU812122 ITS: JF304637	Uncultured Dikarya clone	HQ219359	100% (0)	Uncultured soil fungus clone (99%)	HM132004	100% (0)	

Table 2.3: contd.

Isolate #		18S Sequence			ITS sequence			Phylum
Designation (NIOCC)	**Gen Bank Accession nos. of 18S and ITS sequences**	**Identity (Max % identity)**	**Gen Bank Accession No. of closest relative**	**Q- coverage (Error value)**	**Identity (Max % Identity)**	**Gen Bank Accession No. of closest relative**	**Q- coverage (Error value)**	
#12V	18S: EU812127 ITS: JF304627	Cumulospora marina (99%)	GU256625	100% (0)	*Lindra thalassiae* (96%)	DQ491508	30% (4e-65)	**Ascomycota**
#13V	18S: EU725815 ITS: EU735842	*Pestalotiopsis maculans* (100%)	AB220236	100% (0)	*Pestalotiopsis uvicola* (98%)	AY687297	100% (0)	
#14V	18S: EU725817 ITS: EU735844	*Cumulospora marina* (99%)	GU256625	100% (0)	*Lindra thalassiae* (96%)	DQ491508	28% (0)	
#16V	18S: EU725818 ITS: EU735847	*Diaporthe phaseolorum* (100%)	FJ392654	100% (0)	*Diaporthe* sp. (99%)	FJ037739	99% (0)	
#17V	18S: EU812128	*Cumulospora marina* (99%)	GU256625	99% (0)	----	----	----	
#18V	18S: EU812129	*Cumulospora marina* (99%)	GU252136	99% (0)	----	----	----	
#19V	18S: EU812134 ITS: JF304631	*Pestalotiopsis* sp. (100%)	EU375525	100% (0)	*Pestalotiopsis* sp. (98%)	HM999916	100% (0)	
#20V	18S: EU812130	*Cumulospora marina* (99%)	GU256625	100% (0)	----	----	----	
#21V	18S: EU812125 ITS: JF304635	*Pestalosphaeria* sp. (100%)	HM130668	100% (0)	*Pestalotiopsis* sp. (100%)	FJ037738	100% (0)	
#23V	18S: EU812131	*Cumulospora marina* (99%)	GU256625	100% (0)	----	----	----	
#26V	18S: EU725819 ITS: EU735848	*Colletotrichum gloeosporioides* (100%)	DQ916151	100% (0)	*Glomerella cingulata* (99%)	GU066710	100% (0)	
#27V	18S: EU812135 ITS: JF304633	*Pestalotiopsis* sp. (99%)	EU375525	100% (0)	*Pestalotiopsis theae* (99%)	AY681477	99% (0)	

Table 2.3:contd.

Isolate #		18S Sequence			ITS sequence			Phylum
Designation (NIOCC)	**Gen Bank Accession nos. of 18S and ITS sequences**	**Identity (Max % identity)**	**Gen Bank Accession No. of closest relative**	**Q- coverage (Error value)**	**Identity (Max % Identity)**	**Gen Bank Accession No. of closest relative**	**Q- coverage (Error value)**	
#28V	18S: FJ010209 ITS: EU735849	*Lulworthia* sp. (99%)	AY879014	100% (0)	*Lindra thalassiae* (96%)	DQ491508	28% (9e-67)	**Ascomycota**
#29V	18S: EU812136 ITS: JF304634	*Pestalotiopsis* sp. (99%)	EU375525	100% (0)	*Pestalotiopsis theae*	AY924293	99% (0)	
#31V	18S: EU812132	*Cumulospora marina* (99%)	GU256625	100% (0)	----	----	----	
#32V	18S: JF319442	*Cumulospora marina* (99%)	GU256625	99% (0)	----	----	----	
#34V	ITS: JF304630	----	----	----	Uncultured fungus clone	GU370735	98% (0)	
#35V	ITS: JF304632	----	----	----	Fungal endophyte sp.	EU054399	100% (0)	
#36V	18S: EU812124 ITS: JF304629	*Apioclypea* sp. (99%)	AY083819	100% (0)	Uncultured fungus clone	GU370735	100% (0)	
#38V	18S: EU812121 ITS: JF304636	Uncultured Dikarya clone	HQ219359	100% (0)	Uncultured *Alternaria* clone	HQ588306	100% (0)	
#C2	18S: EU812137 ITS: JF304625	Uncultured Dikarya clone (99%)	HQ219381	100% (0)	*Acremonium obclavatum*	AJ292394	100% (0)	
#C3	18S: EU725821 ITS: EU735843	*Pestalotiopsis* sp. (100%)	EU375525	100% (0)	*Pestalotiopsis sydowiana*	EU076923	99% (0)	
#C10	18S: EU812123	Uncultured Dikarya clone	HQ219359	100% (0)	----	----	----	

---- Sequences were either ambiguous or not of good quality

The quantitative production of LDEs was determined in isolates which were selected by qualitative plate assay. Two isolates, namely NIOCC #2a and NIOCC #312 which were isolated and characterized previously (Raghukumar et al., 1999; D'Souza et al., 2006) were also included for this study. Among Ascomycetes, NIOCC #13V was the best laccase producer whereas; NIOCC #2a produced maximum laccase titer among basidiomycetes (Fig 2.8 A). Production of MnP was better in #50V, #DV2 and #312 than other candidates. Several isolates which were found to be negative for peroxidase production during qualitative plate assay also showed minimal MnP production (Fig. 2.8 B). Production of LiP was very low in all of these isolates. Overall, basidiomycetes produced considerably higher amounts of these enzymes than ascomycetes (Fig. 2.8 C). Maximum production was on day 9 for all the enzymes.

Potential of few LDE positive isolates for decolorization belonging to different class and genera was assessed quantitatively by testing them for color removal from reactive dyes at two different concentrations of 300 mgL^{-1} and 3000 mgL^{-1}. Of these, Reactive Yellow 145 and Reactive red 11 belonged to the Azo group whereas; all others were of anthraquinonic type. The isolates which efficiently decolorized these dyes are shown in Fig. 2.9. Irrespective of the enzyme production, all the dyes except Reactive Yellow 145 and Reactive Red 11 were effectively decolorized at both the concentrations of 300 mg L^{-1} and 3000 mg L^{-1} (Fig. 2.10). There was the significant difference in decolorization at two different concentrations of the dye (p value = 1.95E-20). Also, the color removal achieved by the individual isolates was significantly different from each other (p value = 9.87E-23). Only #15V and #312 were able to remove color by more than 60% in Reactive Yellow 145 at 300 mg L^{-1} concentration of dye; whereas, #312 was best for the decolorization of Reactive Red 11. Color removal from Reactive Blue 140 base and Reactive Blue 160 base was equally good by ascomycetes as well as basidiomycetes. Among ascomycetes, #6V was most efficient in removing

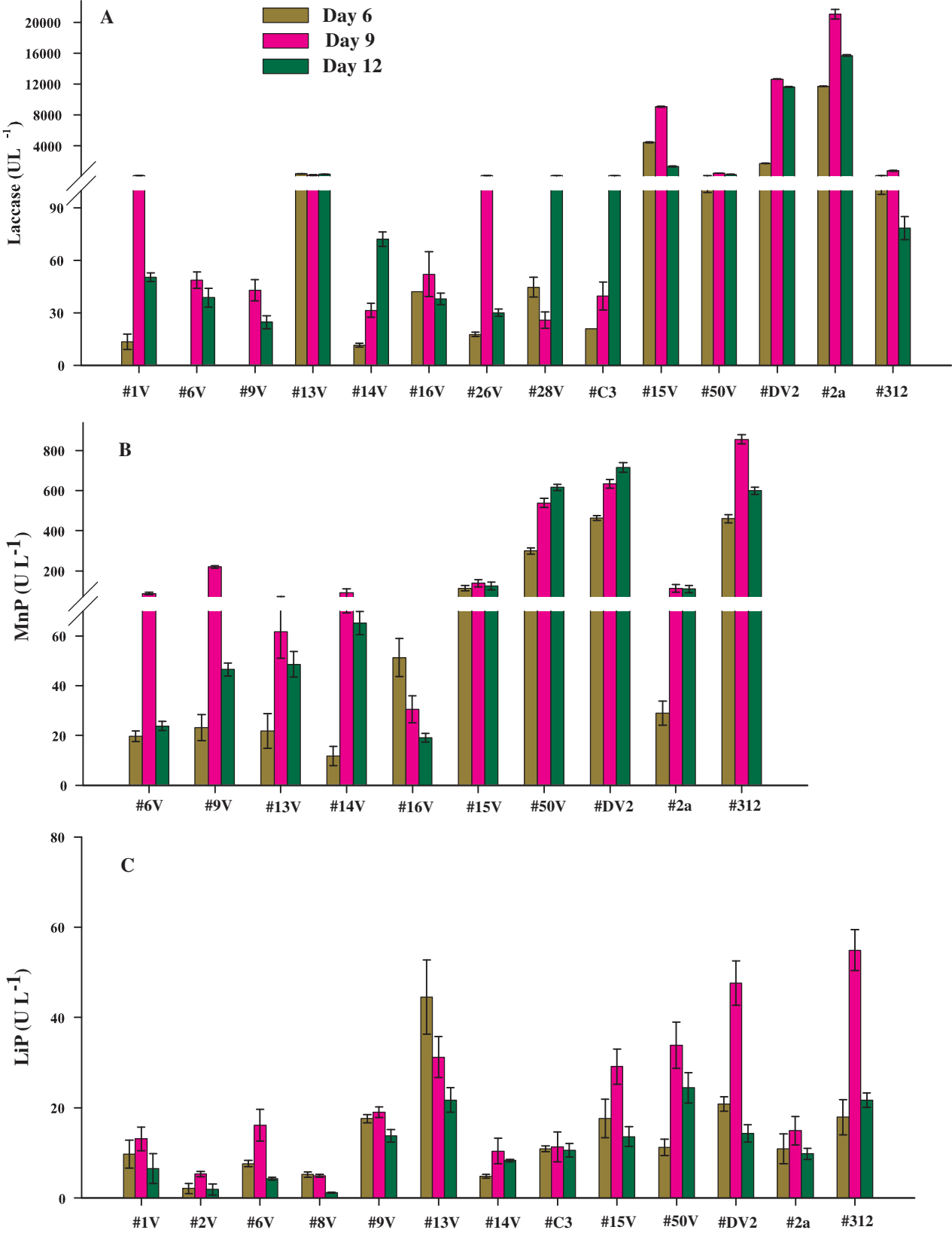
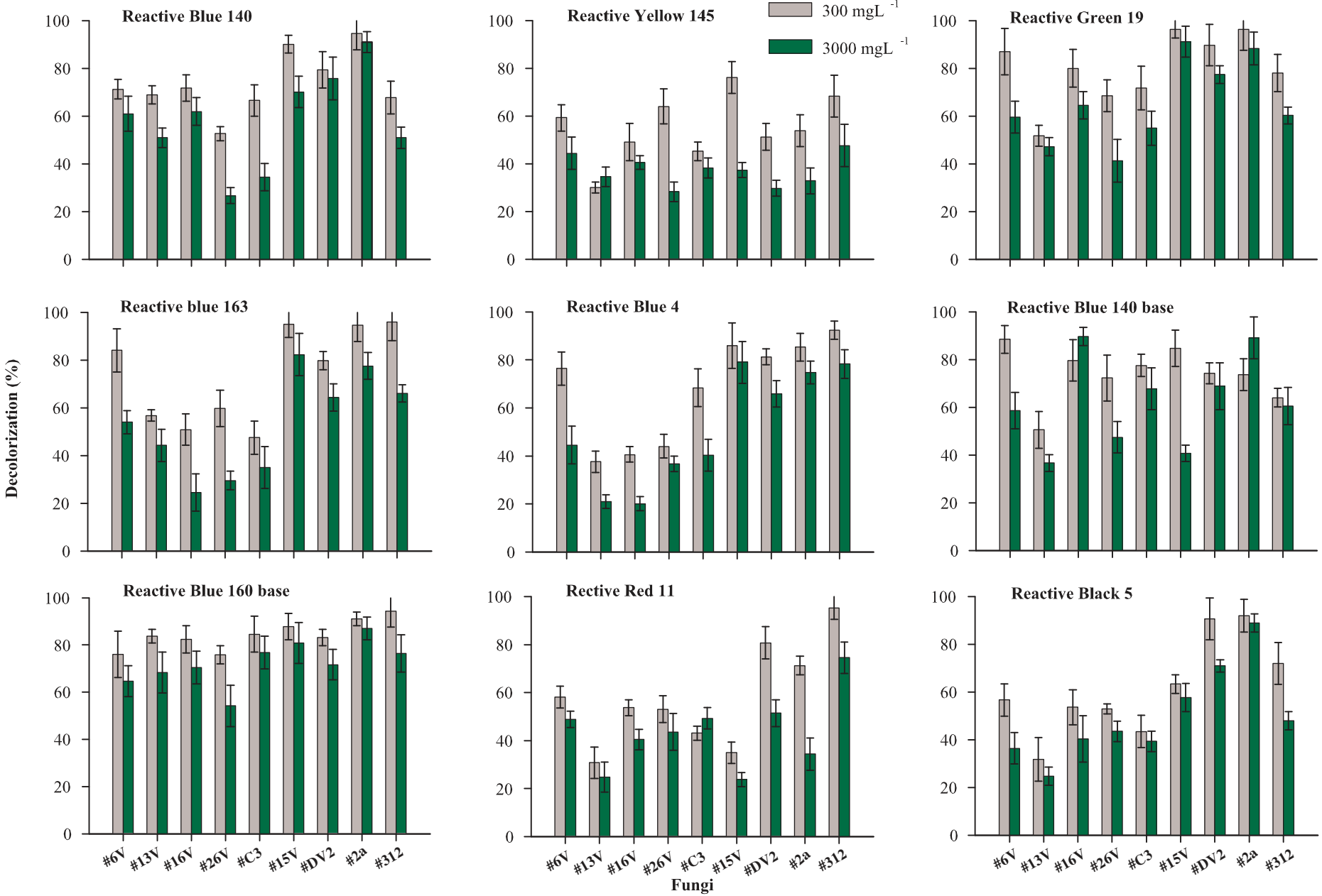


Fig. 2.8: LDE production by several isolates in LNmedium. NOCC #2a and #312 were isolated during previous studies (D'Souza et al., 2006; Raghukumar et al., 1999) and used as reference cultures.

color from large number of dyes. In general the color removal by basidiomycetes was significantly higher than ascomycetes (Table 2.4).

Fig. 2.9: Decolorization of Reactive dyes by representative fungi.



Fig, 2.10: Decolorization of reactive dyes at two different concentations using various isolates, estimated on day 3, 6 and 9. NOCC #2a and #312 were used as reference isolates.

Table. 2.4: The isolates which showed maximum and minimum color removal

Dye	Isolate showing maximum color removal				Isolate showing minimum color removal			
	Concentration of Dye							
	300 mg L^{-1}		3000 mg L^{-1}		300 mg L^{-1}		3000 mg L^{-1}	
	Phylum of the isolates							
	Asco mycota	Basidio mycota	Asco mycota	Basidio mycota	Asco mycota	Basidio mycota	Asco mycota	Basidio mycota
Reactive Blue 140	#16V (72%)	#2a (95%)	#16V (62%)	#2a (91%)	#26V (53%)	#312 (68%)	#26V (27%)	#312 (51%)
Reactive Yellow 145	#26V (64%)	#312 (68%)	#6V (44%)	#312 (48%)	#13V (30%)	#DV2 (51%)	#26V (28%)	#DV2 (30%)
Reactive Green 19	#6V (87%)	#15V (97%)	#16V (65%)	#15V (91%)	#13V (52%)	#312 (78%)	#26V (41%)	#312 (60%)
Reactive Blue 163	#6V (84%)	#312 (96%)	#6V (54%)	#15V (82%)	#C3 (48%)	#DV2 (80%)	#16V (25%)	#DV2 (64%)
Reactive Blue 4	#6V (76%)	#312 (92%)	#6V (45%)	#15V (79%)	#13V (38%)	#DV2 (81%)	#16V (20)	#DV2 (66%)
Reactive Blue 140 base	#6V (89%)	#15V (85%)	#16V (90%)	#2a (89%)	#13V (51%)	#2a (74%)	#13V (37%)	#15V (41%)
Reactive Blue 160 base	#C3 (85%)	#312 (94%)	#C3 (77%)	#2a (87%)	#26V (76%)	#DV2 (83%)	#26V (54%)	#DV2 (72%)
Reactive Red 11	#6V (58%)	#312 (95%)	#C3 (49%)	#312 (75%)	#13V (31%)	#15V (35%)	#13V (25%)	#15V (24%)
Reactive Black 5	#6V (57%)	#2a (92%)	#26V (44%)	#2a (89%)	#13V (32%)	#15V (63%)	#13V (26%)	#312 (48%)

% Decolorization values are given within brackets.

Isolates which showed maximum and minimum decolorization of each dye has been represented with green and red respectively

P values obtained by one way analysis of variance (ANOVA) between ascomycetes and basidiomycetes for: (I) maximum decolorization achieved at the dye concentration of 300 mgL^{-1} was 0.007683, (II) maximum decolorization achieved at the dye concentration of 3000 mgL^{-1} was 0.006056, (III) minimum decolorization achieved at the dye concentration of 300 mgL^{-1} was 0.00722 and (IV) minimum decolorization achieved at the dye concentration of 3000 mgL^{-1} was 0.004074

2.5 *Discussion*

2.5.1 *Sampling and isolation of fungi*

Mangrove vegetation is widely distributed along the two estuarine river systems, Mandovi on the north and Zuari in the south of Goa, India (Fig. 2.3). Several studies have focused on detritus from coastal marine macrophytes, since these contribute an enormous amount of organic matter to adjacent waters. The litter fall in the Chorao mangroves of Goa, India has been estimated as nearly 500 g C m^{-2} yr^{-1}. The detritus input into Mandovi and Zuari estuaries through the 2000 hectares of mangroves in Goa may amount to 10.1 x 103 tons of C yr^{-1} (Wafar et al., 1997). Fungi play an important role in the decomposition of organic matter in mangroves, as they possess a wide spectrum of enzymes, especially lignocellulose degrading enzymes. The litter-decomposing fungi are those basidiomycetous and ascomycetous fungi that together with bacteria participate in the decomposition of leaf litter to CO_2 and humus (Dix and Webster, 1995). Their normal habitat is the ground litter layer of soil and the humus layer of forest and grasslands. Mangroves harbor a rich diversity of mycota mostly saprophytic; however some of them also live as endophytes. Also, such sites are suitable for the rich growth of decomposers as detritus would have been submerged for long periods (D'Souza-Ticlo et al., 2006). The composition of substratum greatly affects fungal diversity. Temperature and salinity in particular, are important while discussing the occurrence and distribution of fungi in the mangroves. Care must be taken in collecting wood, i.e., to collect only material that has been in the marine habitat for several weeks as indicated by the attack of marine fouling or boring organisms (Jones and Hyde, 1988). Presence of calcareous shells and bore-holes on the wood samples indicate that they were submerged in the marine conditions for a considerable period. In turn, probability of getting marine-derived

lignicolous fungi increases (Vrijmoed, 2000). Decaying substrates rich in lignin are the most suitable candidates for the isolation of lignicolous fungi. In the present study, sampling of mangrove detritus was always carried out during low tide since it allowed collection of decaying leaves and woody samples that remained submerged during high tides. This enabled isolation of truly marine form (Besitulo et al., 2002).

The unique physico-chemical properties of the marine environment are likely to have conferred marine fungi with special physiological adaptations that could be exploited in biotechnology (Raghukumar, 2008). These parameters especially salinity in the estuarine/mangrove ecosystems keeps on fluctuating as they are regularly flushed with fresh water from the rivers. This happens due to the cyclic occurrence of low and high tide along with the seasonal variations in the tropical areas. These conditions lead to the abundance and diversity of species in the mangroves including facultative and obligate marine fungi. Lorenz and Molitoris, (1992) demonstrated that salinity optimum for growth in some marine fungi show upward shift with increasing incubation temperature. Terrestrial fungi generally grow best at pH 4.5-6.0, whereas facultative marine fungi were demonstrated to grow and produce various extra-cellular enzymes at pH 7-8 (Raghukumar et al., 1994; 1999; 2004; Damare et al., 2006b).

2.5.2 *Screening for lignin-degrading fungi*

Worldwide, there is little published information on the lignin-degrading ability of the obligate and facultative marine fungi. Several studies have been conducted to assess the ability of marine fungi to degrade lignocellulose (Leightley, 1980; Suhirman and Jones, 1983; Mouzouras et al., 1988; Mouzouras, 1989). Moreover, the presence of MnPs, LiPs and laccasses in facultative and obligate marine fungi has not been investigated except for few reports (Kamei et al., 2008; D'Souza et al., 2006; Raghukumar et al., 1999).

Laccase is a common component of fungal ligninolytic systems and produced by a diverse variety of taxa (Mayer and Staples, 2002). It seems to be a common enzyme in marine fungi. For example, Rohrmann and Molitoris, (1992) showed that laccase was present in 18 of 21 (ca. 86%) marine ascomycetes, based on assays with different substrates, such as 1- naphthol, benzidine and syringaldazine. When using the ABTS method, Raghukumar et al. (1994) and Pointing et al. (1998) found 11 of 17 (65%) and 14 of 15 (93%) marine fungal isolates were laccase producers, respectively. Similarly, in our study 64% of the isolates were found to be laccase producers in the qualitative plate assay (Table 2.2).

Decolourization of Poly-R dye was proposed as an approach for detection of ligninolytic ability because of its good correlation with the lignin-degrading system in *Phanerochaete chrysosporium* (Gold et al., 1988). However, the specific mechanism is not fully understood. Kuwahara et al., (1984) suggested that manganese peroxidase was at least partly responsible for the Poly-R decolourization, while Ollikka et al. (1993) found lignin peroxidase to be involved. These ligninolytic peroxidases have been reported from a variety of genera of white-rot basidiomycetes (Hatakka, 1994; Tuor et al., 1995). Pointing et al. (1998) reported that three of 15 marine ascomycetes decolorized Poly-R, irrespective of the presence or absence of sea-water in the medium. In another study, none of the fungi exhibited an ability to decolourize Poly-R-478 dye, indicating the lack of ligninolytic peroxidases (Luo et al., 2005). In the present study, Poly-R decolourization ability during plate assay, was observed in only four of the marine-derived fungi tested (Table 2.2), implying the deficient production of peroxidase in mangrove fungi.

Analysis of growth and production of degradative enzymes in sea-water media is the best way to test the adaptation of marine or marine-derived fungi to their environment. Marine fungi showed improved growth and LDE production in

agar media (Raghukumar et al., 1994; Pointing et al, 1998) or in liquid media containing sea-water (D'Souza et al., 2006; Raghukumar et al., 1999). A basidiomycetous fungus *Phlebia* sp. (strain #MG-60 was identified as a hypersaline-tolerant lignin-degrading fungus (Li et al., 2002; Li et al., 2003a, b). To confirm that the isolates screened for the present study are marine adapted, they were tested for the growth in media prepared with D/W and half strength sea-water. These fungi showed significantly more biomass in saline conditions as shown in Fig. 2.5.

Most of the isolates, positive for the LDE production were anamorphs. Also, D'Souza-Ticlo, (2008) reported lower laccase production and decolorization potential in the sporulating isolates than anamorphs. This may be due to the consumption of energy for spore formation which in turn results into the lower LDE production.

2.5.3 *Identificaton of fungi*

Although the outline of the Kingdoms seems relatively stable, the classification of fungi at the lower levels - class, order, family and genus, is being shaken up by an influx of molecular data. The classification based on the ideas first proposed by the great biologist, Anton de Bary, in the 19th century, and refined by many subsequent generations of mycologists using morphological, anatomical and developmental characteristics is still relevant. But it now seems that a true phylogenetically based classification has become possible as a result of molecular studies.

Phylogenetic trees inferred from 18S rDNA sequence divergence indicate the existence of two divisions, Ascomycota and Basidiomycota, among the higher fungi (Sugiyama, 1998). Dikaryotic fungi possess more diverse enzyme system and can penetrate deeper into woody substratum (Mouzouras et al., 1988). Marine fungi occur as endophytes in mangrove and estuarine plants and comprise mainly

anamorphic species (Alias and Jones, 2009). Ascomycota is the largest phylum within the fungi and pezizomycotina is the largest subphylum of Ascomycota. It seems mangrove ecosystem studied here, is also dominated by the same group (Fig. 2.6 & Fig. 2.7).

The field of marine mycology began about 65 yrs ago with a widely cited paper by Barghoorn and Linder, (1944). The genus *Lulworthia* was introduced by Sutherland, (1916) for a scolecosporous ascomycete on living thalli of the rock-weed *Fucus vesiculosus* at the coast of Lulworth in Dorset. This is the largest genera of marine Ascomycota (Kohlmeyer et al., 2000). Also, the order Lulworthiales has been mostly reported from mangrove habitats (Pang et al., 2011). Most of the isolates belonging to the order Lulworthiales may be novel since the similarity to their nearest relative was less or equal to 96% during ITS sequence analysis (Fig. 2.7).

Another genus, *Lindra*, of marine pyrenomycetes with filamentous ascospores was described by Wilson, (1956). However, in another study, it was proposed that the *Lindra/Lulworthia* clade represents an independent transition from terrestrial to marine environment (Spatafora et al, 1998). It is distinguishable from *Lulworthia* by the absence of mucus-filled apical chambers in the ascospores (Kohlmeyer, 1980; Nakagiri, 1984). *Lindra* is the only genus in the Lulworthiales that does not have apical spore chambers filled with mucus (Campbell et al., 2005). Leightley, (1980) reported *Lulworthia grandispora* as the dominant fungus on mangrove timbers. We also obtained highest number of strains belonging to these two genera (Table 2.3; Fig 2.6 & Fig. 2.7). Another order which was found to be prominent was xylariales dominated by the genera, *Pestalotiopsis*.

As one hypothesis, marine ascomycetes are described as primary or secondary inhabitants of marine waters. Primary marine species are believed to be derived from ancestral lineages that originated in the marine environment. Secondary marine species represent the reintroduction of fungi into the marine environment and share a more recent common ancestory with terrestrial lineages

(Kohlmeyer, 1986). Kholmeyer and Kholmeyer, (1979) recognized 43 species of higher fungi, including 23 ascomycetes, 17 deuteromycetes, and 3 basidiomycetes from the mangroves. Hyde, (1990) listed 120 species from 29 mangrove forests around the world. These included 87 ascomycetes, 31 deuteromycetes, and only 2 basidiomycetes. Alias, (1996) listed 339 fungi known from mangroves with the majority (151 species in 84 genera) of ascomycetes. The basidiomycetes were the least represented group with only 3 genera, while 37 species in 29 genera were mitosporic fungi. In total 66 fungal species, 57 ascomycetes and only 2 basidiomycetes were recorded from the mangroves of Del Carmen, Siargao Island, Philippines (Besitulo et al., 2010). Sakayaroj et al. (2010) recorded 42 endophytes in seagrass of which 98% accounted for ascomycetes and their anamorphs. The abundance of ascomycete group of fungi on marine and mangrove substrates has been reported by Hyde and Jones, (1988), and this might be due to their spores showing adaptation to the marine ecosystem by way of production of appendages, which provide buoyancy in water, entrapment and adherence to substrates, as reported in mangrove wood (Aleem, 1980; Kohlmeyer, 1981; Rohrmann and Molitoris, 1986; Ravikumar and Vittal, 1996), driftwood (Prasannarai and Sridhar , 1997; Prasannarai et al., 1999) and animal substrates (Ananda et al., 1998). The reports of earlier researchers including some recent studies (Maria and Sridhar, 2002; Gayatri et al., 2006; Raveendran and Manimohan, 2007; Nambiar et al., 2009) also supported the result of the maximum number of ascomycetes in mangroves and brackish water ecosystems. In the present study also only 3 species of basidiomycete were obtained (Table 2.3; Fig. 2.6, 2.7). The basidiomycetes in the mangrove forests mostly colonize wood (Kohlmeyer and Volkmann Kohlmeyer, 1991), whereas; mitosporic fungi usually colonize leaves and other organic material in the sediment (Schimit and Shearer, 2003). All the basidiomycetes obtained were isolated from the wood by single spore isolation method.

Primer sets for the amplification of two different segments of rRNA gene were chosen in the present study because 18S rDNA is supposed to be conserved in nature and thus discrimination between closely related species is difficult to be resolved. On the other hand, ITS region of rRNA gene is known to give better resolution of taxonomic species, but phylogenetic analyses of unknown sequences cannot be resolved due to its less conserved nature (O'Brien et al., 2005). Therefore, to overcome this problem and get a better diversity estimate, both of these segments were amplified in this study.

Both sporulating as well as non-sporulating fungi could be identified by molecular-based identification system in the present study. However, ITS and 18S sequences produced different results for identification at the level of species for many of the fungi (Table 2.3; Fig. 2.6, 2.7). This indicates that identification up to species level should either be based on more than one technique or by using several fungal specific primers amplifying different segments of gene(s) as reported by Pang and Mitchell, (2005). Insufficient database in particular, for ITS sequences also is one of the reasons for reduced diversity assessment (Zachow et al., 2009; Anderson et al., 2003).

The pleomorphy is a marked characteristic of the higher fungi therefore dual nomenclature is permitted. However, molecular analysis of rRNA gene sequences does not support the deutereomycetes and are phylogenetically assigned to either the Ascomycota or Basidiomycota (Sugiyama, 1998).

2.5.4 *Quantitative assay for lignin-degrading enzymes*

Interest in fungal ligninolytic enzymes has intensified in recent years because of their potential applications. Marine fungi, as a group of organisms physiologically adapted to the marine environment, might become a source of extra-cellular enzymes with good tolerance to saline conditions. Laccases of terrestrial origin have been found susceptible to fluoride, chloride and bromide ions (Koudelka and

Ettinger, 1988; Xu, 1996; Garzillo et al., 1998). Luo et al. (2005) proposed that these halides likely act on the copper ions located at the catalytic centres of the laccase. Kamei et al. (2008) reported that the hyper-saline conditions up-regulated the specific MnP isozymes of a marine adapted strain of a *Phlebia sp.* at the transcriptional level. They proposed that this strain might be evolutionarily adapted to saline-rich conditions for the sufficient expression of its lignin degradation ability. Marine fungi were well adapted for the saline conditions as seen in present study where they were able to produce these enzymes in the medium prepared with half strength sea-water (Fig 2.8).

A range of lingo-cellulolytic enzymes have been reported from some marine-derived fungi (Rohrmann and Molitoris, 1992; Raghukumar et al., 1994; Pointing et al., 1998; D'Souza et al., 2006). Raghukumar et al. (1994) reported the presence of laccases showing activity in both acidic and alkaline conditions in marine fungi. Mabrouk et al. (2010) reported presence of laccase activity in the marine-derived fungal isolates. None of these fungi showed the presence of other two LDEs namely, MnP and LiP. In the present study also, laccase was the most prominent enzyme produced by several isolates during quantitative assay (Fig. 2.8). Bonugli-Santos et al. (2010) reported the presence of LDEs in three marine-derived fungi. Two of these belonged to the ascomycetes and one to the zygomycetes. In a study, endophytic fungi from mangroves namely *Pestalotiopsis* sp. and *Glomerella* sp. produced an array of lingo-cellulose degrading enzymes (Kumaresan and Suryanarayanan, 2002). In my study, several ascomycytes belonging to different genera produced considerable amounts of laccase and MnP but it was significantly less than basidiomycetes (Fig. 2.8).

2.5.5 *Decolorization of synthetic dyes*

The biological methods of color removal are governed by many physical and physiological factors (Nyanhongo et al., 2002; Gadd, 2008).The basidiomycetes

have a diverse system of extra-cellular enzymes with the aid of which they degrade xenobiotics. All the isolates were able to decolorize synthetic dyes at the concentration of 300 mg L^{-1}, to the varying extent. At the higher concentration of 3000 mg L^{-1}, the 3 basidiomycete cultures showed better removal of color than ascomycetes (Table 2.4, Fig. 2.9, 2.10). Laccase was the major LDE produced by #15V, #DV2 and #2a and anthraquinonic dyes being substrates of laccase were better decolorized than azo dyes by these isolates. Whereas, maximum color removal from azo dye reactive red 11 was achieved by #312 having MnP as the major LDE. Since ascomycetes lack prominent enzyme system as in basidiomycetes, the color removal by these isolates may be due to sorption of the color over the mycelia, rather than actual degradation. The mechanism involved in the color removal by ascomycetes needs further investigation.

Fig 2.6 The evolutionary history was inferred using Maximum parsimony. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale; with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 782 positions in the final dataset, out of which 173 were parsimony informative. Phylogenetic analyses were conducted in MEGA4.

Fig 2.7 The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale; with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 307 positions

in the final dataset, out of which 166 were parsimony informative. Phylogenetic analyses were conducted in MEGA4.

3.1 *Introduction*

3.1.1 *Various colored effluents and their characteristics*

Several industries generate complex wastewater with various types of coloring agents. These include paper and pulp industry, molasses-based sugar mills and alcohol distilleries, synthetic dye manufacturing & textile industry and leather-based industry.

Natural pigments used for coloring textiles have been replaced by ‘‘fast colors’’ which do not fade on exposure to light, heat and water. These features unfortunately go with the perils of harmful effluent quality. There are more than 8000 chemical products associated with the dyeing process listed in the color index, while over 1, 00,000 commercially available dyes exist with over 7×10^5 metric tones of dyestuff produced annually (Zollinger, 1987). About 15% to 20% of the dyes used for textile dying are released into processing waters (Mishra and Tripathy, 1993; Cooper, 1995). Besides being unaesthetic, these effluents are mutagenic, carcinogenic and toxic (Chung et al., 1992), mainly due to the fact that many dyes are made from known carcinogens, such as benzidine, naphthalene and other aromatic compounds. The textile industry generates huge amounts of colored waste-waters, which contribute enormously to water deterioration (Banat et al., 1996; Vijayaraghavan et al., 2008). Unfortunately, conventional treatment techniques are not always effective towards textile effluents that are one of the most difficult-to-treat wastewaters on account of their considerable amount of suspended solids, high chemical demand and the massive presence of weakly biodegradable and often toxic substances. Therefore, efficient, eco-friendly and cost-effective remedies for wastewater treatment are needed (Vijayaraghavan et al., 2008).

Wastewaters containing molasses are generated by distilleries, fermentation industries, sugar mills and other molasses-based industries. These contain melanoidin polymers which are the product of Maillard reaction between the amino acids and carbonyl groups in molasses when it is subjected to the high

temperatures. 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. 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 large quantity of the bleach plant effluents from the paper and pulp industries are intensely colored waste-water generated at various stages of processing (Eriksson and Kirk, 1986). The color of these waste-waters is contributed mainly by the lignin and its derivatives, which are discharged in such effluents mainly from the pulping, bleaching and recovery stages of the plant. Since the pulp produced corresponds to only approximately 40-45% of the original weight of the wood, the effluents are highly loaded with organic matter (Ali and Sreekrishnan, 2001). These effluents are characterized by the presence of high biochemical oxygen demand (BOD), chemical oxygen demand (COD), chlorinated compounds, suspended solids, fatty acids, tannins, resin acids, lignin and its derivatives, sulfur and related compounds, etc. Polychlorinated dibenzodioxins and dibenzofurans are recalcitrant and tend to persist in nature. Thsese persistant organic pollutants (POPs) have been classified as priority pollutants by the United States Environmental protection Agency (USEPA, 1998). These components are known as DNA damaging agents and have been shown to induce inherited genetic defects and cancer (Loprieno, 1982; Brusick, 1987;

Easton et al., 1997). The dioxins have been named as 'known human carcinogens' by the World Health Organisation (WHO, 1997).

3.1.2 *Various methods for treatment of colored effluents*

Color is the first contaminant and visible indication of pollution. The methods dealt with the remediation of these kinds of effluents can be broadly divided into physical chemical and biological. Physical methods of treatment of colored effluents include adsorption over various substrates such as activated charcoal, irradiation, coagulation, ultra-filtration, reverse osmosis etc. Various chemical methods including oxidative processes like use of ion exchange, Fenton's reagent, ozonation, electrochemical oxidation and precipitation are also applied for the remediation (Anjaneyulu et al., 2005).

Biological treatment is often the most economical alternative when compared with other physical and chemical processes. Biodegradation methods such as fungal decolorization, microbial degradation, adsorption by (living or dead) microbial biomass and bioremediation systems are commonly applied to the treatment of industrial effluents because many microorganisms such as bacteria, yeasts, algae and fungi are able to accumulate and degrade different pollutants (McMullan et al., 2001; Fu and Viraraghavan, 2001; Banat et al., 1996).These processes have potential to mineralize several components present in effluents to harmless inorganic compounds like CO_2, H_2O and the formation of a lesser quantity of relatively insignificant amount of sludge (Mohan et al., 2002).

Chemical and physical methods for treatment of dye wastewater are not widely applied to textile industries because of exorbitant costs and disposal problems. The involvement of white-rot fungi in waste-water treatment is gaining importance because of the potential to degrade a vast range of xenobiotics. The ability of fungal enzymes to transform a wide variety of hazardous chemicals aroused interest in using them in bioremediation (Whiteley and Lee, 2006). The enzyme based methods for the remediation of effluents have minimal impact on

ecosystems and low energy requirements. Treatment with basidiomycetous fungi or their lignin-degrading enzymes namely, lignin peroxidase, manganese-dependent peroxidase and laccases have been widely reported (Wesenberg et al., 2003; Blánquez et al., 2008). These act on a broad range of substrates and hence are able to degrade several xenobiotics (Kim and Nicell, 2006) including synthetic dyes (Wesenberg et al., 2003). Biological treatments involving white-rot fungi (WRF) have also attracted increasing interest since several studies revealed their potential to combine decolorization and toxicity reduction. Among eukaryotic organisms, white-rot fungi belong to the best xenobiotic degraders. For instance, they and their laccases are well known for their remarkable bio-transformation or biodegradation abilities towards a broad variety of both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants (Bennet et al., 2002). Laccases have the potential of degrading dyes of diverse chemical structure (Blánquez et al., 2004; Hou et al., 2004) including synthetic dyes employed in the industry. Intrestingly, Novozyme (Novo Nordisk, Denmark) launched a new application of laccase enzyme in denim finishing: DenLite®, the first industrial laccase. A formulation based on lignin mediator system (LMS) capable of degrading indigo in a very specific way was launched by Zytex (Zytex Pvt. Ltd., Mumbai, India) on the trade name of Zylite (Couto and Herrera, 2006). 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). Bioremediation of melanoidin-containing waste waters using white-rot fungi and their lignin-degrading enzymes with some success have been reported (Gold and Alic, 1993; Thakker et al., 2006). Enzymatic treatment could also be used prior to other biological treatments to remove specific chemicals. Waste-water may also be treated with the enzyme containing extra-cellular fluid of a fungal growth culture without any purification step (Tekere et al., 2005).

LDE-producing profiles and their involvement in remediation vary from organism to organism. For instance, Laccase was the main enzyme involved in dye decolorization by cultures of *Phlebia tremellosa* (Kirby et al., 2000; Robinson et al., 2001b) and by *Pleurotus sajor-caju* (Chagas and Durrant, 2001). These isolates lacked LiP or MnP activity (Kirby et al., 2000). MnP could only be detected when the culture medium was supplemented with $MnCl_2$. In the presence of LiP and/or MnP in addition to laccase (*Pleurotus ostreatus*, *Schizophyllum commune*, *Sclerotium rolfsii*, *Neurospora crassa*) seemed to increase by up to 25% the degree of decolorization of individual commercial triarylmethane, anthraquinonic, and indigoid textile dyes using enzyme preparations (Abadulla et al., 2000). On the contrary, MnP was reported as the main enzyme involved in dye decolorization by *Phanerochaete chrysosporium* (Chagas and Durrant, 2001) and LiP for *Bjerkandera adusta* (Robinson et al., 2001b). LiP was also considered as the principal decolorizing enzyme in cultures of *Phanerochaete chrysosporium* (Kirby et al., 1995). These studies support the thought that LDE's play significant roles in dye metabolism by WRF (McMullan et al., 2001). 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. On the contrary, Font et al. (2006) demonstrated that laccase and not MnP or LiP plays the major role in decolorizing and detoxifying effluents from pulp and paper industry. Uptake effects or sorption of color by WRF mycelia without real degradation are generally minimal (Glenn and Gold, 1983). These effects are, rather, seen in applications of non-WRF, such as *Aspergillus niger*, whose (dead) biomass could be used as an adsorbent (Fu and

Viraraghavan, 2000; Sumathi and Manju, 2000) and serve as part of a technical solution in water pollution control.

Another, green technology to deal with this problem include sorption of colored effluents on bacteria and fungal biomass (Dönmez, 2002; Fu and Viraraghavan, 2002; Prigione et al., 2008a, b) or low-cost non-conventional adsorbents (Crini, 2006; Ferrero, 2007). Adsorption through activated carbon or organic resin is the most common practice, in spite of the high costs involved. Color removal by bio-sorption is an alternative to the economically disadvantageous physical and chemical methods of treatment (Namasivayam et al. 1996). Biosorption, an alternative to physico-chemical treatment is recommended by several researchers for treatment of colored effluents. The main attractions of biosorption are high efficiency, cost effectiveness and substantial removal of color from large volumes (Aksu, 2005; Gadd, 2009). In the last decade, several researches have shown that biosorption can be regarded as a valid alternative to traditional methods and to microbial or enzymatic biodegradation (Vijayaraghavan et al., 2008). It is a physico-chemical process and includes mechanism such as adsorption, absorption, ion exchange, surface complexation and precipitation (Gadd, 2008). In addition, the process of biosorption is reported to be governed by type of membrane lipids (Kennedy and Pham, 1995), pH and hydrophilicity (Bayramoğlu and Arica, 2007).

Biological sorbants include plant, fungal, and bacterial biomass, either live or dead (Robinson et al. 2001b). Live or dead microbial biomass of algae, yeast, bacteria and fungi has been used for this purpose (Satyawali and Balakrishnan, 2008). The use of biomass for wastewater treatment is increasing because of its availability in large quantities and at low price. Microbial biomass is produced in fermentation processes to synthesize valuable products such as antibiotics and enzymes. In such processes, a large amount of biomass is generated, which can be used in sorption of pollutants. Biomass has a high potential as a sorbent due to its physico-chemical characteristics. Microbial

bioadsorbants have been used for removal of heavy metals (Gadd, 2009), dyes (Prigione et al., 2008a, b) and hazardous organic pollutants (Aksu, 2005).

In fungal decolorization, fungi can be classified into two kinds according to their life state: living cells to biodegrade and biosorb effluents, and dead cells (fungal biomass) to adsorb effluent (Fu and Viraraghavan, 2001a). Among the different bio-sorbents tested against dyes so far, dead fungal biomass has proved to be particularly suitable, presenting several advantages over the live: they are not affected by toxic wastes, do not require nutrients and do not release toxins or propagules (Crini, 2006; Prigione et al., 2008a; Anastasi et al., 2009; Tigini et al., 2011).

Bioadsorption potential of microbial exopolymeric substance (EPS) is well known (Wingender et al., 1999). Basidiomycetous fungi are reported to produce large amount of EPS (Maziero et al., 1999; Smith et al., 2002). 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 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).

Despite the established benefits of biosorption and the huge amount of publications on this topic, applications at industrial level are virtually absent probably because of the still low robustness of biomass-based systems. Thus, some authors suggested that the attention should be focused on biomass modifications, alteration of bioreactors configuration and physico-chemical conditions to enhance biosorption (Gadd, 2009). Moreover, recent literature

indicates the need to generate performance data on real or simulated industrial effluents since many biotic and abiotic factors can affect the biosorption process (Aksu 2005; Gadd, 2009; Kaushik and Malik, 2009). Nevertheless, most of the studies on biosorption focus on dye removal from single dye solutions and only few with multi-component solutions have been carried out so far (Khalaf, 2008; Prigione et al., 2008b).

Another method reported in treatment of industrial and domestic wastewater is application of ultrasound (sonication) which is an advanced oxidation method (Sangave et al., 2007). Sonication/sonochemical methods are relatively new and involve aqueous solutions containing the organic pollutants to be subjected to cavitation using ultrasound. Sonication causes formation of gaseous bubbles or vaporous cavities in a liquid. These subsequently collapse violently causing increase in temperature and pressure locally at several points in a reactor resulting in the formation of reactive hydrogen atoms and hydroxyl radicals. Also, acoustic cavitation results in the formation, growth and subsequent collapse of micro-bubbles or cavities occurring in extremely small interval of time (milliseconds), releasing large magnitudes of energy (Gogate and Pandit, 2004). These two combine to form hydrogen peroxide which promotes oxidation reactions and is responsible for the destruction of refractory compounds. The advantage of using sonication rests with the simplicity of its use. Sonication is generally performed as a pre-oxidation step before biological treatment as it is reported to increase biodegradability (Sangave and Pandit, 2006). However, its effect on decolorization of industrial effluents has not been reported.

3.1.3 *Enzymes responsible for remediation of colored effluents*

Ligninolytic white-rot fungi produce lignin peroxidase (LiP; EC1.11.1.14), manganese peroxidase (MnP; EC.1.11.1.3), laccase (benzenediol:oxygen oxidoreductase; EC.1.10.3.2), versatile peroxidase (VP; EC. 1.11.1.16) and H_2O_2-generating enzymes, in various combinations, which play an important role in the

degradation of lignin (Boominathan and Reddy, 1992). They are the major lignin degrading enzymes of white rot fungi (WRF) involved in lignin and xenobiotic degradation (Pointing, 2001; Wesenberg et al., 2003). Lignin degrading enzymes (LDEs) of white-rot basidiomycetous fungi have been extensively studied for the degradation of recalcitrant compounds and variety of industrial effluents (Asgher et al., 2008).

Laccase is an important enzyme in the lignin-degrading enzyme complex of ligninolytic fungi. It belongs to the group of polyphenol oxidases and is predominantly present in fungi and higher plants. The most extensively studied are the extra-cellular laccases from lignin degrading basidiomycetes. Several ascomycetous and hyphomycetous fungi also produce laccase (Baldrian, 2006). Laccases have been lately reported to be produced by several marine and marine-derived fungi (Raghukumar et al. 1994, 1999; Pointing et al. 1998; Pointing and Hyde 2000; D'Souza-Ticlo et al. 2009).The typical substrates of laccase are substituted mono-phenols, poly-phenolic compounds and phenolic groups of lignin polymer. Fungal laccases are considered as ideal catalysts due to their broad substrate specificity and their few requirements. It is a copper oxidase capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water. Since laccase has broad substrate specificity, it can be used in the degradation of several xenobiotics including synthetic dyes and industrial effluents (Fu and Viraraghavan, 2001). Besides, bioremediation potential, they have gained importance recently due to a number of diverse applications such as delignification of lignocellulosics and cross-linking of polysaccharides, food technological uses, personal and medical care applications, biosensors and analytical applications (Galhaup et al., 2002).

The major drawback of the laccases is its low redox potential, therefore, mention of laccase-mediators while discussing about them is essential. 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 usual substrates of laccase (Fig. 3.1).

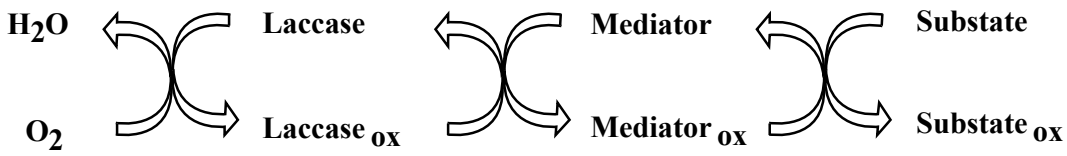


Fig. 3.1: Oxidation of non-substrate compounds by laccase by the aid of mediator

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). Lack of correlation between laccase activity and degradation of xenobiotic compounds further supports the role of LMS (Johannes and Majcherczyk, 2000). The ability of laccase to oxidize non-phenolic substrates with mediators has been demonstrated (Rocherfort et al., 2002; Shleev et al., 2003; Rocherfort et al., 2004). 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, and 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 (Fig. 3.2).

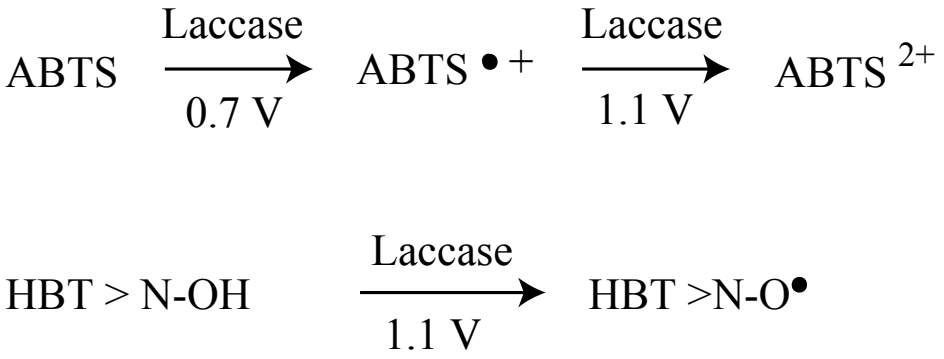


Fig. 3.2: Redox potentials of the oxidation reactions of ABTS and HBT by laccase. Adapted from: Kunamneni et al., 2008

Laccase-mediator system has found applications in paper pulp delignification (Camarero et al., 2007), degradation of polycyclic aromatic hydrocarbons (Johannes and Majcherczyk, 2000) and industrial dyes (Camarero et al., 2005).

The two major glycosylated heme proteins that catalyze H_2O_2-dependent oxidation produced by WRF are MnP and LiP. The important difference between these two is in the nature of the reducing substrate. The oxidation of lignin and other phenols by MnP is dependent on free manganous ion. Manganese peroxidase catalyzes the H_2O_2-dependent oxidation of lignin. The enzyme oxidizes Mn(II) to Mn(III), which diffuses from the enzyme surface and in turn oxidizes the phenolic substrate (Gold and Alic, 1993).

. Lignin peroxidase catalyzes the H_2O_2-dependent oxidation of a wide variety of non-phenolic lignin model compounds and aromatic pollutants. It is the strongest known fungal peroxdase and has been much studied (Kirk and Farrell, 1987; Hammel, 1997). These reactions include benzylic alcohol oxidations, side-chain cleavages, ring-opening reactions, demethoxylations, and oxidative dechlorinations. All of these reactions are consistent with a mechanism involving the initial one-electron oxidation of susceptible aromatic nuclei by an oxidized enzyme intermediate to form a substrate aryl cation radical. The ability of LiP to oxidize lignin nonspecifically to generate cation radicals which undergo a variety

of non-enzymatic reactions accounts for the variety of metabolic products observed such as veratryl alcohol to veratryl aldehyde (Gold and Alic, 1993)

Versatile Peroxidase has been recently described as a new family of ligninolytic peroxidases, together with LiP and MnP obtained from *Phanerochaete chrysosporium* (Camarero et al., 1999; Martínez, 2002). It is a heme containing structural hybrid between MnPs and LiPs, since they can oxidize not only Mn^{2+} but can also oxidize veratryl alcohol, phenolic, non-phenolic and high molecular weight aromatic compounds including dyes in manganese-independent reactions (Kamitsuji et al., 2004; Pogni et al., 2005; Shrivastava et al., 2005). Interestingly, these enzymes exhibited both LiP and MnP-like activity and they could oxidize Mn^{2+} to Mn^{3+} at around pH 5.0 while aromatic compounds at around pH 3.0, regardless of the presence of Mn^{2+} (Husain, 2009). Therefore these enzymes were called as hybrid MnP - LiP peroxidases or VP.

LDE are essential for lignin degradation, however for lignin mineralization they often combine with other processes involving additional enzymes. Therefore, in addition to peroxidases and laccases, white rot fungi produce a variety of oxidases that are capable of generating H_2O_2, presumably for utilization by extra-cellular peroxidases during the degradation of lignin. Such auxiliary enzymes (by themselves unable to degraded lignin) include glyoxal oxidase, an extra-cellular, idiophasic, copper-containing enzyme; glucose oxidase; veratryl alcohol oxidase; and methanol oxidase (Gold and Alic, 1993). Also, superoxide dismutase for intracellular production of H_2O_2, and cellobiose dehydrogenase involved in feedback circuits and linking ligninolysis with cellulose and hemicellulose degradation in nature are reported (Leonowicz et al., 1999). Oxalate producing, oxalate decarboxylase (ODC), NAD-dependent formate dehydrogenase (FDH) and P450 mono-oxygenase have also been isolated from many WRF strains (Wesenberg et al., 2003; Doddapaneni et al., 2005; Aguiar et al., 2006). Out of all of these enzymes, aryl-alcohol oxidase (AAO) and glyoxal oxidase (GOx) are supposed to be the main enzymes responsible for the

production of H_2O_2, extra-cellularly (Shah and Nerud, 2002). The generation of oxygen radicals and activity of peroxidases requires the presence of extra-cellular H_2O_2. Different white-rot fungi have different mechanisms to generate hydrogen peroxide with the aid of specific enzyme/group of enzymes (Fig. 3.3). Further role of each of these enzymes in lignin degradation remains to be elucidated.

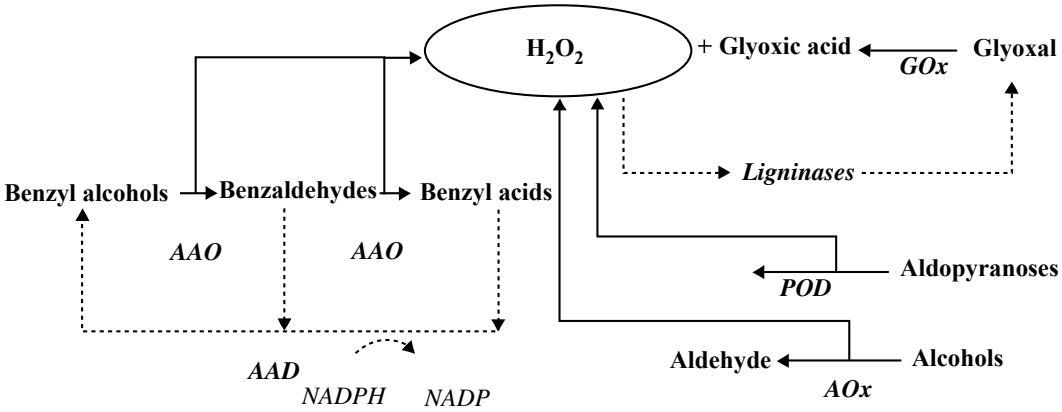


Fig. 3.3: Various pathways leading to the formation of hydrogen peroxide in white-rot fungi. GOx, glyoxal oxides; POD, pyranose oxidase; AOx, alcohol oxidase; AAD aryl alcohol dehydrogenase; AAO, aryl alcohol oxidase. Adapted from: Shah and Nerud, (2002)

There are many constraints and issues still needed to be addressed to utilize these organisms and their enzymes for the remediation of effluents at industrial scale. Also, a great deal is remaining to be explored and discussed about the fundamentals of how a fungus mineralizes pollutants/effluents and even less is known about the degradation mechanisms used by fungi in general. These studies are essential as they will perpetually increase the efficiency of the bioremediation process.

3.1.4 *Effect of various parameters on production of lignin degrading enzymes*

It is evident that the potential applications in industrial and environmental technologies require huge amounts of enzymes at low cost. Therefore, it is important to develop strategies for their over-production. The basic aspect of

ligninolytic enzymes production on a large scale is still lacking in the literature. Recently the culture conditions for the production of LDEs by specific white-rot fungi have been overviewed (Singh and Chen, 2008; Elisashvili and Kachlishvili, 2009).

The secretion of extra-cellular lignin-degrading enzymes by white-rot fungi are greatly influenced by carbon or nitrogen source supplied (Fu et al., 1997; Kapdan et al., 2000). On the basis of culture studies of lignin peroxidase expression by white-rot basidiomycetes, Fog, (1988) proposed that decreased rates of decomposition for litter of high lignin content under conditions of high nitrogen availability was the result of reduced oxidative enzyme expression. The lignin degrading enzymes are produced during secondary metabolism under conditions of limited nitrogen (Buswell et al., 1995; Reddy, 1995). Industrial effluents varying in their nitrogen content and source may sometimes inhibit or enhance the activity of fungal growth or their enzymes. Thus, for effective bioremediation of such colored effluents by white-rot fungi it is imperative to study these interactions. Differential regulation of ligninolytic enzyme-encoding genes in response to culture conditions has been documented (Dittmer et al., 1997). Effect of inducers of specific laccase isozymes has been demonstrated in some of the basidiomycetes (Muńoz et al., 1997; Palmieri et al., 2000).

Onset of lignin degrading enzymes is triggered by nitrogen depletion in the medium (Keyser et al., 1978). An adverse effect of nitrogen on delignification is proposed to be due to:

(1) High nitrogen content, which promotes rapid depletion of energy sources known to be essential for lignin metabolism (Kirk et al., 1976).

(2) Nitrogen metabolism that competes with lignin metabolism through requirements for the same co-factors

(3) The fact that it regulates the synthesis of one or more components of the lignin-degrading system

(4) The increased formation of biomass, which in turn speeds up the rate of respiration (Reid, 1979).

In nature, nitrogen limitation has been a major factor in enhancing ligninolytic enzyme production. In general, the increased carbohydrate (carbon) supply stimulates, while increased nitrogen inhibits lignin degradation. The concentration of nitrogen, optimal for laccase production seems to be more ambiguous than that of carbon. Moreover, nitrogen source and concentration in the culture medium are known to influence laccase production (Gianfreda et al., 1999). Effect of nitrogen sources and vitamins on the production of ligninolytic enzymes and decolorization has been reported (Levin et al., 2010). N-limited medium (2–3 mM) has been shown to favor laccase production in most of the species of fungi, for example, *Phanerochete sanguineus* (Pointing et al., 2000), *Pleurotus ostreatus* (Hou et al., 2004) and *Cerrena unicolor* MTCC 5159 (D'Souza et al., 2006).

Peroxidase production by white-rot fungi is typically induced by nitrogen starvation (Hammel, 1997). 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 as low nitrogen medium (LNM). In contrast, several white-rot fungi were also reported to produce LDEs in the presence of high nitrogen (Collins and Dobson, 1997; Kuhad et al., 1997; D'Souza et al., 1999; Hatvani and Mecs, 2002; Dong and Zhang, 2004). In general, the above studies strongly support the idea that the presence of nitrogen usually represses LDE expression, but expression of some enzymes is less sensitive to this repression (Kachlishvilli et al., 2006).

It is evident from the foregoing that white-rot fungi cannot degrade lignin unless the co-substrate (carbon source) is supplemented simultaneously in the growth medium. The basidiomycetes display a wide diversity in their response to carbon sources and their concentration in nutrient medium (Elisashvili and Kachlishvili,

2009). Wang et al. (2008) reported that the gene expression for ligninolytic enzymes in *P. chrysosporium* is triggered by the depletion of carbon. A combination of low nitrogen and high carbon concentration favored both biomass and laccase production (D'Souza-Ticlo et al., 2009). However, laccase production was delayed in the presence of high concentrations of glucose in another study (Monteiro and de Carvalho, 1998). Easily assimilable components such as glucose allow for constitutive as well as inducible laccase production but repress its induction in several fungi (Bollag and Leonowicz, 1984). D'Souza-Ticlo et al. (2009) proposed the use of slow assimilable carbon sources to avoid the delay in laccase production. It had been experimentally proven that laccase production is highly dependent on the conditions of cultivation of the fungus (Heinzkill et al., 1998) and media supporting high biomass does not necessarily support high laccase yields (Xavier et al., 2001). Excessive concentrations of glucose as a carbon source in the cultivation of laccase producing fungi had an inhibitory effect on laccase titer (Eggert et al., 1996). These observations suggest that the fungus-specific carbon source at the suitable concentration should be provided to enhance the enzyme production. MnP, LiP and laccase were detected when these marine-derived fungi were cultured in malt extract, however when grown on basal medium containing glucose and wheat bran LiP was not detected and yet an increase in MnP and laccase was observed (Bonugli-Santos et al., 2010)

Ions are known activators of many oxidases that play a role in delignification of effluents by ligninases. Kirk et al. (1986) reported a 1.7–fold increase in ligninolytic enzyme activity following an incorporation of a six-fold excess of a trace metal solution containing Mn, Mg, Fe, Co, Ca, Zn, Cu, Mo, Al, in the culture medium of *P. chrysosporium.* The promoter regions of laccase genes have been shown to contain various recognition sites that are specific for heavy metals which when bound to, induce laccase production (Sannia et al., 2001).

Copper is required especially to enhance the production of laccases. Copper atoms serve as cofactors in the catalytic core of laccase; thus, a minimum concentration (millimolar range) of copper ions is necessary for production of the active enzyme (Tetsch et al., 2005). In *Trametes versicolor*, copper regulates laccase at the level of gene transcription (Collins and Dobson, 1997). Effect of copper on induction of laccase isoenzymes was demonstrated in the white-rot fungus *Pleurotus ostreatus* (Palmieri et al., 2000). Excess copper may have a toxic effect on fungal biomass and thus decrease laccase production. Addition of copper during the exponential phase of fungal growth gives optimal laccase activity while minimizing the inhibitory effect of copper on fungal growth (Galhaup and Haltrich, 2001; Galhaup et al., 2002; D'Souza et al, 2006; Revankar and Lele, 2006a; Fonesca et al., 2010).

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 (Boominathan and Reddy, 1992; Gill and Arora, 2003). Several natural substrates like wood chips and shavings from soft and hard wood have been used to induce production of both of these peroxidases (Niku-Paavola et al., 1990).

With increasing interest in laccase from fungi for bioremediation applications, efforts have been made to enhance the laccase titer. Laccases may be constitutive or inducible enzymes. Addition of various aromatic compounds analogous to lignin or lignin derivatives, have induced laccase production (Gianfreda et al., 1999). Several workers have demonstrated the production of LDEs especially laccase, in terrestrial fungi was 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 a laccase gene *cglcc*1 in the presence of tannic acid in the white-rot basidiomycetous fungus *Coriolopsis gallica* and *laccase 3* in the ascomycete *Cryphonectria parasitica* (Carbajo et al.,

2002; Chung et al., 2008).The expression as confirmed by Northern hybridization, suggests that laccases in *Heterobasidium annosum* are constitutively expressed with enhanced production in the presence of an inducer such as ferulic acid or oxalic acid (Asiegbu et al., 2004). 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 and Kuhad, 2002; Dhawan et al., 2003; Dhawan et al., 2005).

Kirk et al. (1978) have also shown that lignin degradation is quite sensitive to pH and that adequate buffering is essentially required to control pH during lignin decomposition. The control of pH was problematic at high concentrations of nutrient nitrogen and when salts of carboxylic acids served as growth substrate. Facultative marine fungi have been demonstrated to grow and produce various extra-cellular enzymes at pH 7-8 (Raghukumar, 2008). The pH and temperature activity profiles of LiPs from different sources vary significantly with optimum activities shown between pH 2-5 and 33-55 ºC respectively (Yang et al., 2004; Asgher et al., 2007). For MnPs, optimum pH of 4-7 and optimum temperature of 40-60 ºC is reported (Ürek and Pazarlioglu, 2004; Barborová et al., 2006). Whereas, the pH and temperature optima of laccases from different white-rot fungi vary from 2 to 10 and 40 – 65 ºC respectively (Lu et al., 2005; Ullrich et al., 2005; Murugesan et al., 2006; Zouari-Mechchi et al., 2006; D'Souza et al., 2006; Quaratino et al., 2007). In general, room temperature (25-35 ºC) and slightly acidic media are frequently used.

The oxygen partial pressure has a profound effect on the rate and extent of lignin degradation, but not on the growth of the organism. In the secondary phase, the process of lignin degradation is strictly an oxidative process and needs the presence of oxygen at a partial pressure equal to that in the natural atmosphere; increasing the O_2 levels in culture has a strong activating effect on the rate of lignin degradation (Buswell and Odier, 1987; Kirk and Farrell, 1987). Lignin is degraded much faster in the presence of oxygen than air and that ligninolysis is

not observed in sub-atmospheric (5%) partial pressure of oxygen (Kirk et al., 1978). Increasing the oxygen level in the medium has been postulated to lead to increased LDE production and increased production of the components of the H_2O_2-producing systems (Kirk and Farrell, 1987; Boominathan and Reddy, 1992). Oxygenation had a marked positive influence on laccase production by *P. Chrysosporium* (Srinivasan, 1995). The fastest rate of enzyme dependent olive-mill wastewater decolorization occurred in cultures of *P. chrysosporium* flushed with 100% oxygen; under an air atmosphere, only 25% color removal could be attained (Sayadi and Ellouz, 1992). Raghukumar and Rivonkar, (2001) achieved best decolorization of molasses spent wash in oxygenated cultures. Production of LDEs may be increased if studies on optimal aeration rate or oxygen concentration during fermentation are undertaken.

Agitation is generally used to increase the rate of gas exchange between the atmosphere and culture medium. Kirk et al., (1978) reported that an initial period without agitation is needed to avoid severe inhibition of lignin degradation. Production of LDEs was reported to take place only in shallow undisturbed stationary cultures (Boominathan and Reddy, 1992). It is a well known fact that laccase is best produced under stationary conditions where the fungal mycelium is in maximum contact with the atmospheric surface (Butt et al., 2001). The extent of growth is good in both agitated as well as static fungal cultures. However, culture agitation results in pellet formation and strongly suppresses ligninolytic activity. Further, if the pre-grown mycelial mat is agitated, it does not seem to affect lignin degradation (Kirk et al., 1978; Yang et al., 1980). Agitation of cultures to increase the oxygen supply prevents optimal degradation, which is perhaps due to a disturbance of the physiological state of cells on the pellet surface that prevents the formation of cleavage catalysts.

Presence of Tween 80 caused the increase in the LDE activity in *P. chrysosporium* (Asther et al., 1987; Venkatadri and Irvine, 1990). Saturated and unsaturated fatty acids liberated by the degradation of Tween 80 might be the

means that trigger the production of LDEs by the organism and it may also protect LDEs from being mechanically inactivated (Singh and Chen, 2008). Further, the surfactant property of Tween 80 emulsifies the fungal membrane aiding in the release of cell membrane-associated laccases (Cserháti, 1995). Addition of surfactants such as Tween 20 or Tween 80 to the culture media helped in overcoming the inhibition in production of LDEs in bioreactors and agitated cultures (Gomez-Alarcon et al., 1989; Svobodová et al., 2006).

In conclusion to the above factors, type and quantity of carbon and nitrogen sources in the growth medium play an important role in the production of lignin-degrading enzymes in the white-rot basidiomycetous fungi. Besides this, production of lignin degrading enzymes (LDE's) is affected by several culture conditions such as medium composition, carbon and nitrogen ratio, inducers, surfactants, pH, temperature, and aeration.

3.1.5 *Various parameters to be monitored during bioremediation*

Successful bioremediation of phenolic wastewater relies on many factors, including fungal growth, growth medium composition, culture age and activity, enzyme production and time of addition of the pollutant to the culture (Ryan et al., 2007). Decontamination of some dyes occurs in part through adsorption onto fungal membranes prior to complete oxidation (Baldrian and Snajdr, 2006). Some wastewaters may not support adequate growth of the fungus as they may be pre-loaded with inhibiting and toxic pollutants. For example, bioremediation of real dye-laden wastewater continues to be a challenging venture, as these effluents also contain some pesticides, heavy metals and pigments (Zouari-Mechichi et al., 2006) that might inhibit the treatment process. Measurement of these contents is essential before subjecting them for biological methods of treatment. The difficulty of growing organisms in a hostile medium may be by passed by using higher loads of purified enzyme.

Another aspect that must be considered is the effluent toxicity and its evolution during wastewater treatment, as required by the Integrated Pollution Prevention and Control regulations introduced in various countries. It has been reported that effluents from paper and pulp mills and textile dye waste waters are toxic and mutagenic (Reddy, 1995). In fact, in some cases, the decolorization results in the formation of colorless but toxic and mutagenic compounds resulting in an increase of the waste-water toxicity (Pearce et al., 2003; Keenan et al., 2007; Sharma et al., 2007). In some cases, enzymatic oxidation of phenolic pollutants can generate by-products that are more toxic than the parent molecules. Many of the transformation products generated through environmental photo-modification exhibit greater toxicity than the parent dyes (Bizani et al., 2006). Electrochemical treatment of textile dyes and dye-house effluents resulted into sharp increase in toxicity suggesting the formation of persistent by-products (Chatzisymeon et al., 2006). Thus besides decolorization, detoxification of wastewaters is an important parameter to be monitored. There is a need to test the toxicity of the end products formed. Many studies have been conducted on studying toxicity of the reactive dyes. 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).

There is a definite gap in our current knowledge of decolorization and, even more so, of mineralization mechanisms. With a lack of insight concerning potentially toxic albeit colorless accumulating intermediates, our capacity to

evaluate the true technical potential of WRF and their LDE's remains incomplete. However, these difficulties are even greater if one considers that complex mixed effluents are extremely variable in composition in one and the same factory, as is often the case in the textile industry. Thus, the decolorization of real effluents requires an appropriate choice of fungal strain as well as of reactor environment. Several studies aimed at determining the chemical mechanism of azo and anthraquinonic dye decolorization using either fungus or their enzymes in the last decade (Soares et al., 2002; López et al., 2004; Zille et al., 2005; Svobodová et al., 2007; Vanhulle et al., 2008; Casas et al., 2009; Osma et al., 2010; Różalska et al., 2010). The inherent complexity of both the dyes structures and the enzymatic transformation mechanisms makes the elucidation of the degradation pathways a difficult task.

Judicious combination of chemical and physical parameters with biological schemes is essential for bioremediation. 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; D'Souza-Ticlo et al., 2009b).

3.1.6 *Combination of various techniques for remediation of effluents*

Presently available biological, physical and chemical methods do not appear to be the ultimate solution to waste-water treatment problems but rather to transform the waste to another form. The need of textile industries is the economical, simple and environment friendly method for the treatment of effluents. A majority of previous studies have focused on treatment of simulated effluents (Prigione et al., 2008b) with one to several dyes added to defined media. The raw effluents

contain not only dyes but also high concentrations of various inorganic chemicals such as sulfides, sulfates, chlorides and carbonates. Therefore, these are required to be diluted several fold (Wesenberg et al., 2002). Textile effluents differ widely in their chemical characteristics and pH (Hai et al., 2007). Small structural differences in dye mixtures can markedly affect decolorization, and this may be due to electron distribution and charge density, although stearic factors may also contribute. Thus, in spite of the high decolorization efficiency of some strains, decolorizing a real industrial effluent is quite troublesome. Therefore, no single organism can detoxify and decolorize them.

Virtually all the known physicochemical and biological techniques have been explored for decolorization, none has emerged to solve all the issues. Each and every technique has their limitations (Table 3.1).

Table 3.1 Principal existing and emerging processes for remediation.
Modified from: **(Crini, 2006)**

Technology		Advantages	Disadvantages
Conventional treatment processes	Coagulation and Floculation	Simple, economically feasible	High sludge production and disposal problems
	Biodegradation	Economically attractive, publicaly acceptable treatment	Slow process, necessary to create an optimal favorable environment, maintenance and nutrition requirements
	Adsorption on activated carbons	The most effective adsorbant, great, capacity, produce a high-quality treated effluent	Ineffective against disperse and vat dyes, the regeneration is expensive and results in loss of the adsorbent, non destructive process
Established recovery processes	Membrane separations	Removes all dye types, produce a high-quality treated effluent	High pressures, expensive, incapable of treating large volumes
	Ion-exchange Oxidation	No loss of sorbent on regeneration, effective	Economic constraints, not effective for disperse dyes
	Oxidation	Rapid and efficient process	High energy cost, chemicals required
Emerging removal processes	Advanced oxidation process	No sludge production, little or no consumption of chemicals, efficiency for recalcitrant dyes	Economically unfeasible, formation of toxic by-products, technical constraints
	Selective bioadsorbants	Economically attractive, regeneration is not necessary, high selectivity	Requires chemical ,modification, non-destructive process
	Biomass	Low operating cost, good efficiency and selectivity, no toxic effect on microorganisms	Slow process, performance depends on some external factors (pH, salts)

It appears that a single, universally applicable end-of-pipe solution is unrealistic, and combination of different techniques is required to devise a technically and economically feasible option. In light of this, researchers have put forward a wide range of hybrid decolorization techniques (Fig. 3.4).

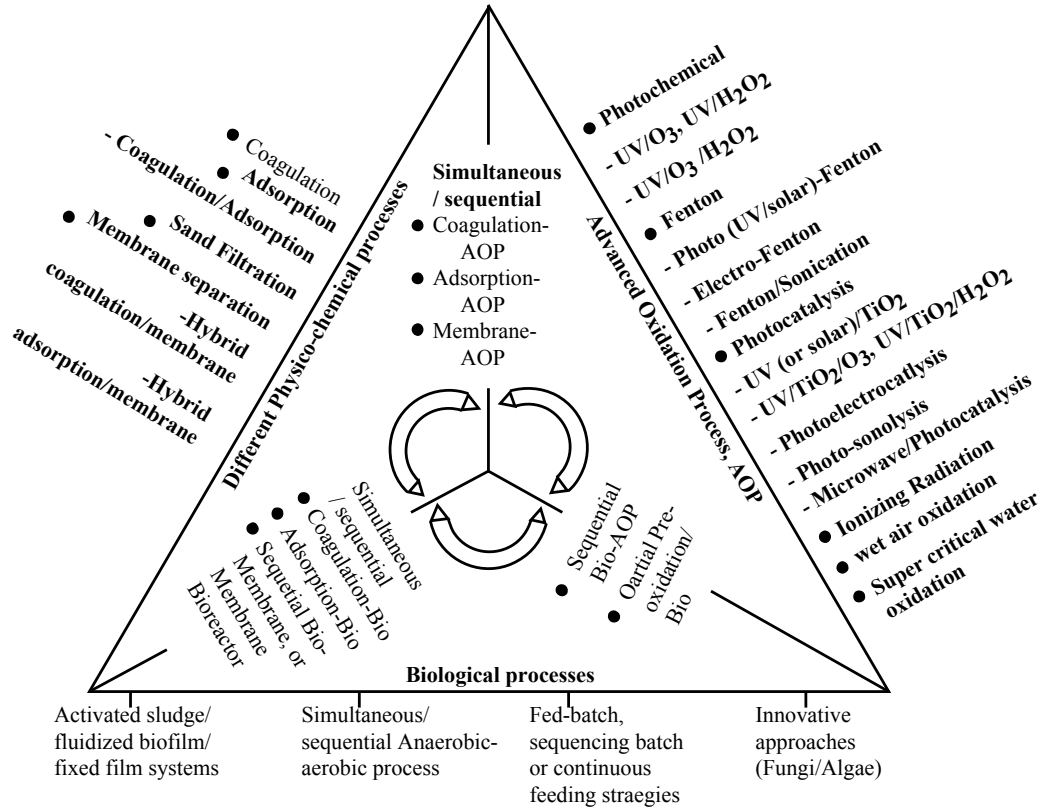


Fig. 3.4: Broad spectrum of combinations proposed for treatment of waste water. Adapted from: (Hai et al., 2007).

The combination of chemical oxidation with a biological treatment reduces the operating cost (Oller et al., 2010). Kusvuran et al., (2004) suggested optimization of efficient but economically less feasible advanced oxidation processes (AOP's) by adjusting process conditions and/or coupling them with other economically feasible methods such as biological treatment. Few studies have been carried out using several combinations for the treatment of raw effluents. Sequential treatment to reduce the toxicity of olive oil mill waste-water using fungi and photo-Fenton reaction has been attempted (Justino et al., 2010). Bioremediation followed by post-photooxidation and coagulation for black liquor effluent treatment was attempted (Helmy et al., 2003). Advanced oxidation process

(AOPs) as a primary and aerobic biological process as a secondary step was applied to decolorize an azo dye (Lucas et al., 2007). These processes are known to have inhibitory effect on the microbial growth but a combination of these processes is beneficial to achieve the desirable goal. For example, the inhibitory effect of the AOPs as a pretreatment on the microbial growth during subsequent biodegradation of textile wastewater accounted for only 10%, while untreated wastewater exhibited 47% of inhibitory action (Ledakowicz and Gonera, 1999). Integration of ozonation with aerobic biodegradation process is effective in achieving considerable enhancement of mineralization of the refractory model compounds such as gallic acid, tannin and lignin (Saroj et al., 2006). A method based on UV-irradiation followed by biodegradation was used for the treatment of various mixtures of PAHs (Guieysse and Viklund, 2005).

3.1.7 *Marine fungi and their advantages in bioremediation*

A vast literature is available on the involvement of lignin-degrading fungi from terrestrial sources in treatment of such effluents (Garg and Modi, 1999), but in a few instances non-ligninolytic fungi have also been used for this purpose (Nagarathnamma and Bajpai, 1999; Sumathi and Phatak, 1999). The biotechnological potential of marine and mangrove fungi have been documented by few authors (Raghukumar et al., 1996; Pointing et al., 1998; Raghukumar, 2008). Efficiency of marine-derived fungi in treatments of such effluents has largely remained unexplored. Marine-derived fungi grow and produce degradative enzymes in sea-water media and thus may be useful in treating wastewaters with high salt content. A basidiomycete *Phlebia* sp., strain MG-60 isolated from mangrove stands was reported as a hyper-saline tolerant lignin-degrading fungus which participated in bio-bleaching of pulp and decolorization of dyes (Li et al., 2002a, b) 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). Besides, it decolorized several synthetic dyes in the presence of sea-water. Three marine-derived fungi showed high values of MnP and laccase activities in the presence of 12.5% and 23% (w/v) salinity highlighting the potential of these fungi for industrial applications and in bioremediation (Bonugli-Santos et al., 2010).

3.2 *Objectives*

The aim of this study was to investigate:

1) The effect of various nitrogen sources incorporated in the growth medium on enzyme production and decolorization of industrial effluents by the marine-derived fungus NIOCC #2a. Decolorization of these effluents by the culture filtrate (*ex situ*) containing lignin-degrading enzymes obtained from media with different N sources was also compared.
2) Decolorization and detoxification of two raw, dye containing textile mill effluents varying in their pH, chemical and dye composition, added at high concentrations in media prepared with sea-water was addressed using four marine-derived fungi belonging to different classes of phylum ascomycetes and basidiomycetes.
3) Bioremediation of four molasses-based effluents using sequential scheme Wherein an attempt was made to develop an efficient bioprocess for industrial application involving use of these fungi and their enzymes in combination.
4) Finally, remediation of an anthraquinonic dye Reactive Blue 4 was attempted using enzymatic degradation followed by biosorption.

3.3 *Material and Methods*

3.3.1 *Effect of nutrient nitrogen on LDE production and effluent decolorization*

This work was done to investigate the effects of various nitrogen sources incorporated in the growth medium on LDE production and decolorization of industrial effluents. Marine-derived basidiomycetous NIOCC #2a was used for this study. The fungus could not be identified by classical morphological taxonomy since no reproductive structures were observed. Based on partial 18S rRNA gene sequence alignment with GenBank database, it was shown to have 99% homology to *Cerrena unicolor*. However, Internal Transcribed Spacer (ITS) sequence analysis of #2a showed 100% homology to an unknown basidiomycete, but closest positively identified match was *C. unicolor*. The culture has been deposited at the Microbial Type Culture Collection (MTCC) Chandigarh, India under the accession number MTCC 5159 as per the Budapest treaty for patent culture deposition (D'Souza et al., 2006).

The Isolate NIOCC #2a was grown in malt extract broth for seven days. The fungal biomass after rinsing to remove the residual medium was mechanically homogenized using glass beads under sterile conditions and the resulting mycelial suspension was used at 10% (v/v) concentration for inoculating low nitrogen (LN) medium (Appendix 6.1.1) having the same composition except varying in the type of nitrogen sources namely, 1) KNO_3, 2) glutamic acid, 3) glycine, 4) beef extract and 5) corn steep liquor at a final concentration of 0.1% nitrogen. The pH was adjusted to 7 with citrate phosphate buffer. The cultures were raised in 250 ml capacity Erlenmeyer flasks, under stationary conditions. The cultures were oxygenated every third day with pure oxygen for 1 min using tygon tubing and Pasteur pipettes, under sterile conditions. Cupric sulphate ($CuSO_4$) at a final concentration of 2mM was added to the 4-day old cultures.

The effect of various colored industrial effluents on the production of lignin-degrading enzymes as well as the ability of the fungus to decolorize these

effluents *in situ* (Whole-fungal culture), was studied by adding different effluents individually to the 6-day old fungus grown in each nitrogen source. The final concentration of each effluent namely, textile effluent A and B (TEA and TEB), Molasses spent wash (MSW) and Black liquor (BL) was 10%. The controls for the experiment were the cultures grown in varying nitrogen sources without any added effluent.

Textile effluent A (TEA) and Textile effluent B (TEB) were supplied by Atul Pvt. Ltd, Gujrat, India. The black liquor (BL) was obtained from Seshasayee Paper Mills, Erode, Tamil Nadu, India. It is a baggase and wood chip-based newsprint unit. Raw untreated molasses spent wash (MSW) was obtained from Rhea Distilleries Ltd., Goa, India.

The cultures were allowed to grow for another 6 days after the addition of the effluents. The biomass of each of the 12 day-old cultures was obtained by filtering the contents through oven-dried, pre-weighed Whatman No. 1 filter paper discs. The dry weight of the fungus was determined as the difference in weight after drying the filter papers at 60 ºC until a constant weight.

The lignin-degrading enzymes, lignin peroxidase (LiP), Manganese peroxidase (MnP) and laccase as well as the amount of decolorization that had occurred in the filtrate obtained from these cultures were estimated (Appendix 6.4.1, 6.4.2, 6.4.3).

***Decolorization of effluents with whole fungal culture* (*in situ*)**

Decolorization of the effluents on day 12 was determined by monitoring the absorbance maxima. The absorbance obtained immediately upon addition of the effluent was considered to be 100%. The extent of decolorization was recorded as percentile residual color or percentile decolorization. Decolorization of TEA and TEB were monitored at their absorbance maxima of 505 and 667 nm respectively and MSW and BL were monitored at 475 and 317 nm respectively. All experiments were carried out in triplicates and the average values are presented.

***Decolorization of effluents with concentrated culture supernatant* (*ex situ*)**

The enzyme source for the decolorization of effluents, *ex situ* was the five fold concentrated culture filtrate obtained from the culture grown for 12 days in various nitrogen sources without the added effluent. Thus, five different enzyme sources corresponding to the different nitrogen sources were used for the *ex situ* decolorization of each of the effluents. The culture filtrates were concentrated by ultra-filtration using Centricon tube with a 10 KDa cut-off membrane (Millipore, USA) at 5,000 rpm at 4 ºC. Each of the effluents mentioned above having final concentration of 1000 color units was incubated at 30 ºC with concentrated culture filtrates possessing 10 U of laccase activity. Color units of the various effluents were determined by measuring its absorbance maximum in UV/Visible spectrophotometer (Shimadzu, Japan). One color unit is defined as the amount of colored material in 1 ml giving an optical density of 1.0 in a path length of 1.0 cm at its absorbance maximum (Eaton et al., 1980). Decolorization of various effluents *ex situ* was monitored at 6, 12, 24, 36 and 48 hours after addition of the crude enzyme to the effluent. Decolorization achieved was calculated with reference to the zero hour reading.

3.3.2 *Remediation of raw textile mill effluents*

Qualitative assay for effluent decolorization

The LDE-positive fungal isolates as described in chapter 2 and Fig.2.8 belonging to both ascomycetes and basidiomycetes were grown in the plates containing B&K medium incorporated with 10% of textile effluent A or B (TEA and TEB). The decolorization of these effluents under and around the fungal colony indicated potential of these isolates for the degradation of textile effluents.

Culture conditions

Four laccase-producing fungi namely #16V and #C3 belonging to the phylum ascomycetes and #2a and #15V to basidiomycetes were short listed after the preliminary qualitative plate assay as described above for further study. They were grown in B & K broth (20 ml in 100 ml Erlenmeyer flasks) for 7 days, homogenized in sterile sea-water in Omni Macro-homogenizer (model No. 17505, Marietta, GA, USA) for 5 s. The mycelial suspension (10%, v/v) was used for inoculating 20 ml of B & K broth in 250 ml Erlenmeyer flasks. The fungi were incubated at room temperature (30 ºC) under static conditions.

Decolorization of textile mill effluents by whole cultures (in situ)

Textile effluent A (TEA) and textile effluent B (TEB) were each added separately at 20, 50 and 90% final concentration (equivalent to 5, 2 and 1.1-fold dilutions, respectively) to 4 day-old cultures raised in B & K broth as described above. The day of addition of effluents to the pre-grown cultures was considered as day zero for all the color measurements. Decolorization of these two effluents was monitored by changes in the absorbance scanned from 360 to 800 nm wavelengths. Percentage decolorization was calculated as the extent of decrease of the spectrum area with respect to that of the control (0 day sample). Triplicate cultures were maintained for each treatment.

Laccase, LiP, MnP and Glucose oxidase activity were assayed in the culture supernatants (Appendix 6.4). Total phenolics and chemical oxygen demand were measured in the culture supernatants of fungi grown in the presence of effluents on day 0 and 6 and expressed as percentage increase or decrease (Appendix 6.5, 6.6).

Residual color from the fungal biomass was extracted in 10 ml of methanol:water (1:1) by homogenization for 1 min in a Macro-homogenizer. Mycelial fragments were removed by filtering the content over Whatman No. 1 filters. The filtrate was lyophilized and the residue was re-suspended in 1 ml of

water and the percentage color adsorbed was calculated spectrophotometrically as described above.

As the two effluents differed in their pH (as shown in Table 3.2), this parameter was normalized in one of the experiments by changing the pH of TEA from 8.9 to 5.0 with glacial acetic acid before adding it to the pre-grown cultures. Decolorization was measured as described above and compared with that obtained by using pH unaltered TEA.

Toxicity test

Detoxification of culture supernatants of different fungi grown in the presence of effluents (at 20, 50 and 90%) was assayed on day 6 using nauplii of *Artemia salina* (Barahona-Gomariz et al., 1994). Fungal treated and untreated effluents (as control) were diluted to different concentrations with 0.22 μm-filtered sea-water and larval mortality was assayed in these. The nauplii (15–25 organisms) were incubated in the diluted effluents at room temperature and mortality was estimated after 24 h. The organisms incubated in the 0.22 μm-filtered sea-water was used as a control. Multiple dilutions were used to obtain linearity in concentration against mortality. Lethal concentration that resulted in 50% mortality (LC_{50} value) by 24 h was calculated by plotting dilutions of effluent versus number of dead organisms. Average values of triplicate treatments were recorded.

Mass spectrometric analyses of treated effluents

Electrospray ionization mass spectrometry (ESI-MS) analysis was performed on a quadrapole-time of flight mass spectrometer (Model Qstar XL, Applied Biosystems, Rotkrenz-Switzerland). Culture supernatants from fungi with TEA and TEB (added at 20%), respective control cultures without effluents and uninoculated B & K broth were diluted with methanol:water (1:1) and directly analyzed by ESI-MS. The samples were introduced at a constant flow rate into the

electrospray source using an integrated syringe pump. The mass/charge (m/z) MS-survey range was 0–1,000 in positive mode.

Decolorization of effluents by culture supernatants (ex situ)

As all the four fungi showed laccase activity in the culture supernatants, their efficiency in decolorization of the two effluents was tested *ex situ*. For this purpose the fungi were grown in low nitrogen medium because it is reported to support high laccase production (D'Souza et al., 2006). Concentrated culture supernatants from 12-day old cultures (when maximum laccase activity was recorded) were used for decolorization of TEA and TEB. The effluents were diluted to 50% with sodium acetate buffer 0.1 M (pH 5.0) and incubated with culture supernatants for varying time period and reduction in color was monitored. The percentage decolorization was calculated as described above.

Low molecular weight phenolic compounds are known to enhance laccase activity (Majeau et al., 2010). These laccase mediators such as ABTS, vanillic acid, veratryl alcohol, p-coumaric acid, 1-hydroxy benzotriazole (HBT), and acetosyringone (Wong and Yu, 1999) were added at 50 and 500 µM concentrations to enhance the decolorization of TEA and TEB obtained through laccasem activity. Appropriately diluted effluents were incubated with culture supernatants of these fungi along with mediators. These were scanned from 360 to 800 nm wavelengths at 0, 6, 12, 24, 48 and 72 h. The reduction in color was calculated and was expressed in percentage as described above. The results were compared with control treatment that did not receive any mediators.

As NIOCC #2a produced highest laccase titer among the four test fungi, its efficiency was compared with a commercial laccase preparation from *Trametes versicolor* (Sigma Chemicals, USA) for decolorization of TEA and TEB. For this purpose multiple concentrations of these two laccases were incubated with 20% TEA and TEB and reduction in color was measured at 12 and 36 h.

Reusing fungal biomass for decolorization

The fungi were tested for repeated use in decolorization of these effluents. Briefly, to 4-day old cultures, the effluents were added at 20% final concentration and the reduction in color in the culture supernatants was measured after 6 days. After draining the culture supernatants, fresh B & K broth with 20% effluent was added to the fungal biomass and the reduction in color was measured once again after 6 days. This procedure was repeated for two more cycles with each of the four fungi.

Decolorization of bioadsorbed effluent

The possibility of decolorization of the adsorbed color from the fungal biomass by culture supernatant containing high laccase activity was tested. Mycelial biomass of the ascomycete #C3 after adsorption was homogenized and incubated with 100 U of laccase from the basidiomycete #2a for 48 h at 120 rpm and the residual color in the fungal biomass was extracted and measured as described above. In the control treatment the mycelial biomass was incubated with distilled water for 48 h and the color removal was compared with laccase-treated samples.

Developing a process for enhanced decolorization

To enhance the decolorization process, the possibility of using a combination of fungal biomass and laccase from different fungi was tested. To achieve this, 4-day old ascomycetous fungal biomass from #C3 and laccase from #2a were incubated with 20% TEA or TEB for 48 h and the residual color, both in the supernatant and the mycelial biomass was measured.

Statistical analyses

All comparisons between treatments or cultures were analysed by student T-test and correlation coefficient in Excel (Microsoft, USA) program for statistical significance.

3.3.3 *Remediation of Molasses-based raw effluents*

Waste water

Four molasses-based raw effluents were used for this study. Reverse osmosis feed (ROF) and Reverse Osmosis Reject (ROR) were provided by Jeypore Sugar Co. Ltd., Chagallu, Andhra Pradesh, India. Conventional aeration tank inlet (CAT I) and conventional aeration tank outlet (CAT O) were provided by the Emmellen Biotech Pharmaceuticals Ltd., Mahad, Maharashtra, India. See Table 3.9 for details.

Physico-chemical analyses of waste waters

All the four effluents were centrifuged at 8000 rpm for 15 min before further analysis. The analysis for different physico-chemical parameters of waste waters was accomplished as described in standard methods for examination of water and wastewater (APHA, AWWA, WEF, 2005). The effluents were stored at 4 ºC in the dark. The working concentration of these effluents was adjusted to A_{475} = 3.5.

3.3.3.1 *Hybrid technology for the treatment of molasses-based raw effluents*

A three-step sequential treatment of four molasses-based effluents was carried out using a combination of **Step-1)** ultrasound-induced acoustic cavitation, **Step-2)** whole-fungal culture treatment using the marine-derived ligninolytic fungus, NIOCC #2a followed by **Step-3)** biosorption of the residual color with heat-inactivated wet biomass of the same fungus.

Sonication and analysis of the effluents

Sonication was carried out using ultrasonic horn (Labsonic M, Sartorius, Germany) with an operational frequency of 30 kHz and calorimetric energy

efficiency of 600 M cm^{-2}. Sonication of the effluents (40 ml in 100 ml Schott Duran bottle) was carried out for 30 min at 100% amplitude using a 2 mm titanium probe. Sonicated effluents were analysed for the reduction in turbidity, color, COD, total phenolics and toxicity. Absorbance spectra (200 – 800 nm) of the effluents before and after sonication were compared.

Decolorization by the whole-fungal culture and partially purified laccase

The white-rot fungus NIOCC #2a was grown in B & K broth (20 ml in 100 ml Erlenmeyer flasks) for 7 days. The fungal biomass after rinsing to remove the residual medium was homogenized in sterile sea water in Omni Macrohomogenizer (No. 17505, Marietta, GA, USA) for 5 s. The mycelial suspension (10%, v/v) was used for inoculating 20 ml of low nitrogen (LN) medium (D'Souza et al., 2006). After 6 days of growth under stationary condition, unsonicated and sonicated effluents as specified above were added to the culture broth under aseptic condition. Before addition the pH of the effluents was adjusted to 5.0 with 0.1 M sodium acetate buffer. Decolorization of the effluents in the culture supernatants was monitored at 475 nm after appropriately diluting with 0.1 M sodium acetate buffer at pH 5.0 (Ohmomo et al., 1988) on day 0, 3, 6 and 9. Decrease in absorbance with respect to that of abiotic control (effluent without the culture) was used for calculating % decolorization. Triplicate cultures were maintained for each treatment. The fungal biomass was collected on day 9 after centrifugation of the culture at 5000 rpm for 10 min and was washed twice with distilled water to remove the salts. It was lyophilized and the dry weight was estimated.

ex situ decolorization was performed using partially purified laccase. Partially purified laccase was obtained by concentrating culture filtrate (500 ml) from 12-day old culture of NIOCC #2a with YM3 membrane (Millipore, USA). The concentrate after filtering through a 0.22 μm filter was applied to High Load 16/60 Superdex 75 preparative grade column and eluted with 0.2 M Na acetate

buffer (pH 4.5), containing 1.0 M KCl at a flow rate of 1 ml min^{-1} using a fast protein liquid chromatography system (Amersham Biosciences, Sweden). The fraction showing maximum absorbance at 280 nm and laccase activity was collected and concentrated using Amicon ultra-centrifugal filter tubes with 3 kDa cut off membrane. Thus partially purified laccase with 50 U of activity and effluents diluted appropriately with 0.1M sodium acetate buffer (pH 5.0) were mixed together and incubated at 30 ºC and 100 rpm. Change in the color was monitored periodically as described above.

Estimation of lignin-degrading enzymes in the whole-fungal culture

The activity of the lignin-degrading enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Appendix 6.4) was measured in the culture supernatants of NIOCC #2a grown in the absence and presence of sonicated and unsonicated effluents on day 0, 3, 6 and 9.

Fungal biomass preparation for biosorption

The residual color in the culture supernatants containing effluent was further removed by biosorption using the fungal biomass of NIOCC #2a as follows. The culture was grown in LN medium. After 6 days, the growth medium was decanted and the biomass was rinsed several times with distilled water to remove the residual medium and inactivated in NaCl solution (9 g L^{-1}) by autoclaving at 121 ºC for 15 min. The biosorption studies were carried out with; (1) wet biomass (equivalent to 0.5 g dry weight), after squeezing through cheese cloth to remove water, (2) 0.5 g lyophilized biomass and (3) 0.5 g of lyophilized powdered biomass (100–200 μm). These were separately introduced in 100 ml Erlenmeyer flask containing 20 ml of whole culture-treated supernatant (Step-2). Effluents without fungal biomass served as control. The flasks were incubated at 30 ºC and 100 rpm. Change in color, COD, total phenolics and toxicity were estimated at regular intervals after centrifugation at 5000 rpm for 10 min.

Toxicity test

Toxicity test of the effluents, after each step of treatment [(1) ultrasound-induced acoustic cavitation, (2) whole-fungal culture treatment using the marine-derived ligninolytic fungus, NIOCC #2a followed by (3) biosorption of the residual color with heat-inactivated wet biomass of the same fungus] was carried out. Treated and untreated effluents were serially diluted with 0.22 μm-filtered sea-water. Bioassay was carried out in disposable multiwell test plate with 24 (6 × 4) test wells. The nauplii (10 organisms) of *Artemia salina* were incubated in the suitably diluted effluents at room temperature in the dark and mortality was estimated after 24 h. Lethal concentration that resulted in 50% mortality (LC_{50} value) by 24 h was calculated with 95% confidence limits with the aid of computer program EPA Probit analysis, version 1.5 (Finney, 1971).

Analytical methods

Total phenolics and chemical oxygen demand (COD) were measured and the changes were expressed as percentage.

Effluents after each stage of treatment were freeze-dried. The lyophilized samples were dissolved in methanol:water (1:1) and directly analysed by Electrospray ionization mass spectrometry (ESI-MS) in positive mode as describe above.

Proton nuclear magnetic resonance (NMR) spectra were recorded with Bruker Avance 300 spectrometer (300 MHz) in deuterated water (D_2O). Effluents after each stage of treatment were freeze-dried. The lyophilized samples were dissolved in D_2O and the chemical shifts were recorded in ppm.

Fourier transform infra-red spectra (FT-IR) of lyophilized fungal mycelium of NIOCC #2a, before and after various steps of treatment, were recorded between 4000 and 700 cm^{-1} using FT-IR (model 8201PC, Shimadzu, Japan) with 4 cm^{-1} resolution. Pellets were prepared by mixing 5 mg of

lyophilized mycelia with 50 mg KBr (dried at 105 ºC for 72 h) using DRS (diffused reflectance spectroscopy) accessory.

Statistical analyses

The significance of the results obtained was evaluated by one way analysis of variance (ANOVA) and Tukey post hoc using the software Prism Pad 5 for Windows (version 5.03).

3.3.3.2 *Effect of laccase and its mediator on different fractions of molasses-based effluents*

The physio-chemical characteristics and quantity of various melanoidin pigments present in the effluents depend on the type of amino-acid(s) and sugar moieties reacting at different temperature. A study was conducted to determine the pattern of distribution of these pigments in the four molasses-based effluents and the effect of laccase and a laccase mediator on decolorization of these fractions.

Known amount of each molasses-based effluent was passed through the Sephadex G-50 size exclusion column (1.0×65 cm^{-2}). Column was previously equilibrated with sodium acetate 0.1M buffer (pH 5.2) and effluents were eluted with the same buffer. The flow rate was maintained at 1ml min^{-1} and 3ml fractions were collected. The molecular markers used were bovine serum albumin (66 KDa), chiken egg albumin (45 KDa), carbonic anhydrase (29 KDa) and α-lactoalbumin (14.2 KDa). These fractions were incubated with 30 units of partially purified laccase from NIOCC #2a at 100 rpm and 30 ºC. In another set of the same fractions, 50 µM of 1-hydroxy benzotriazole (HBT), a known laccase mediator was also added along with the enzyme before incubation. Decolorisation of these fractions was estimated at regular time intervals spectrophotometricaly as mentioned above.

3.3.4 *Remediation of Reactive Blue 4 by sequential treatment*

Chemicals

Commercial C.I. Reactive Blue 4 (RB4), an anthraquinone dye was obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA and was used without any further purification. All other chemicals were of analytical grade.

Organism and Culture conditions

The white-rot fungus NIOCC #2a was grown in B & K broth (20 ml in 100 ml Erlenmeyer flasks) for 7 days. The fungal biomass after rinsing was homogenized in sterile sea water in Omni Macro-homogenizer (No.17505, Marietta, GA, USA) for 5 s. The mycelial suspension (10 %, v/v) was used for inoculating 20 ml of low nitrogen (LN) medium with 10 % fructose as the carbon source. The fungus was incubated at 30° C under static conditions. On day 6, $CuSO_4$ at 2 mM concentration was added to the fungus under aseptic conditions to stimulate the laccase production.

Partial Purification of laccase

After twelve days, when laccase activity reached its maximum, the culture filtrate was obtained by filtering through Whatman GF/C filter paper and subsequently through 0.22 μm filter paper. It was frozen at -20°C to precipitate out the exopolymeric substance produced by the fungus. The precipitated exopolymeric substances were removed from the thawed culture filtrate by centrifugation at 14,000 rpm for 15 min. The culture supernatant was then concentrated by ultra-filtration using YM3 membrane (Millipore, USA). The concentrate after filtering through 0.22 μm sterile filter was mixed appropriately with Bio-Lyte (3/10) ampholyte (Bio-Rad, USA) and 10% (v/v) glycerol. The proteins in the concentrated culture filtrate were separated on the basis of respective pI by

loading to the focusing chamber of a mini-Rotofor system (Bio-Rad, USA). The typical initial voltage and current were 300 V and 25 mA with the voltage stabilizing in 3.0-4.0 h at approximately 1500 V and 8 mA. The temperature of the chamber was maintained at ~ 4°C with the aid of refrigerated circulating water bath. After focusing was complete, 20 separate fractions were rapidly collected by vacuum aspiration. Aliquots (20 ml) containing laccase activity were pooled and applied to High Load 16/60 Superdex 75 preparative grade column and eluted with 0.2 M Na acetate buffer (pH 4.5) containing 1 M KCl at a flow rate of 1 ml min^{-1} using a fast protein liquid chromatography system (Amersham Biosciences, Sweden) (D'Souza-Ticlo et al., 2009a). The chromatographic fractions with laccase activity were pooled and concentrated using 10 KDa Amicon Ultra centrifugal filter devices (Milipore Corporation, USA). This whole procedure was repeated several times to get a sufficient amount of enzyme. The partially purified enzyme was used for degradation studies.

Enzymatic degradation of RB4

Degradation reactions were carried out in 100ml Erlenmeyer flasks containing 10ml of RB4 at a concentration of 1000 mg L^{-1}, dissolved with 0.1M Na acetate buffer (pH 5.0). Partially purified laccase solution (10ul) having 100 U of activity was added and incubated on rotary shaker at 100 rpm and 30°C. Analytical studies were carried out regularly during incubation.

Biomass preparation and Biosorption studies

After 12h of enzymatic treatment, RB4 solution was further subjected to biosorption using the fresh fungal biomass of NIOCC #2a which was prepared as follows. The culture was grown in LN medium. After six days, the growth medium was decanted and the biomass was rinsed several times with distilled water to remove the residual medium and inactivated in NaCl solution (9g L^{-1}) by autoclaving at 121°C for 15 min (Prigione et. al., 2008a). After washing

repeatedly with distilled water to remove the salts and other precipitated components, it was lyophilized and powdered. Powdered mycelium was sieved to collect the particle size of 100-200 μm. 0.2 g of powdered biomass was introduced in 100 ml Erlenmeyer flask containing 10 ml of untreated or enzymatically treated RB4. The flasks were incubated at 100 rpm at 30^{o}C. Color was estimated regularly after centrifugation at 8000 rpm for 10 min until equilibrium was established.

Analysis

Removal of color was determined by calculating the total area under the plot by integration of the absorbance between 400 and 800 nm of the spectrum (Shimadzu UV-2450, double beam spectrophotometer, Japan). Percentage decolorization was calculated with respect to the 0h sample. The dye solution without any enzyme was maintained as a control during the incubation period.

The aromaticity of RB 4 and its reaction products formed due to enzymatic activity were analyzed by the UV Spectrum and by Ultra performance liquid chromatography (UPLC) (Waters Corporation, MA, USA). The mixture of Acetonitrile and H_2O was used as mobile phase and scan was taken for the retention time of 0 to 7 min.

Untreated and enzymatically treated samples were freeze-dried. The lyophilized samples were dissolved in methanol:water (1:1) and directly analyzed by Electrospray ionization mass spectrometry (ESI-MS) as described above in a negative mode. On the basis of this study, probable transformation products formed during the different time intervals of enzymatic treatment are proposed.

Toxicity test with Artemia larvae

Toxicity of the untreated RB4 and after each step of the treatment was assessed following the method as described above under the section 3.3.3.

Phytotoxicity test

The phytotoxicity of the original and the treated dye was assessed by the seed germination of green gram (*Vigna radiata*). Three replicates, each with 10 seeds were used for each test. After 40h of incubation at 30^{o}C, the percentage of seed germination and plumule length were determined. The values obtained from the seeds incubated in de-ionised water were used as a control. Germination index was calculated according to Osma et al., 2010. For more precision, the root was excised from the cotyledons and lyophilized until constant weight was obtained. The total dry biomass of each replicate was determined.

3.4 *Results*

3.4.1 *Effect of nutrient nitrogen on LDE production and effluent decolorization*

The effects of various nitrogen sources on the LDE production and decolorization of effluents by #2a was assessed.

The two textile effluents differed in their dye composition, chemical constituents, pH, and salt content (Table 3.2). As per the data provided by the distillery, MSW was of a pH of 4.3, BOD of 42,000 mg L^{-1} and COD of 80,000 mg L^{-1}. The BL was reported to have COD of 416 mg L^{-1} and BOD of 190 mg L^{-1}.

Table 3.2: Characteristics of textile effluent A (TEA) and textile effluent B (TEB)

Parameters	TEA	TEB
pH	8.9	2.5
Color (Pt-Co units)	1,44,180	52,500
Absorbance maxima (nm)	505	667
COD (mg L^{-1})	30,000	20,000
Total phenolics (g L^{-1})	0.1	0.02
Dye components	Azo dye-20	Reactive blue 4, reactive blue 140 base, reactive blue 140, reactive blue 160 base, reactive blue 163, reactive red 11, reactive yellow 145, reactive green 19
Total solids (g L^{-1})	0.254	0.51
Carbonates (g L^{-1})	30.0	36.0
Na^+ (g L^{-1})	0.043	0.013
Ca^+ (g L^{-1})	0.03	0.009
SO_4 (g L^{-1})	7.23	1.23
Cl (g L^{-1})	150.0	191.7
PO_4 (g L^{-1})	0.021	0.02

The structures of component dyes (wherever available), ColorIndex (C.I.) names and λmax are listed in Table 3.3.

Effect of N source on growth of NIOCC #2a in media supplemented with various effluents

Fungal growth was best in the presence of glutamic acid as the nitrogen (N) source when no effluent supplements were added. The fungus showed enhanced growth in all the nitrogen sources except glutamic acid in the presence of black liquor (BL). Fungal biomass was more with molasses spent wash (MSW) supplemented with KNO_3 and beef extract (BE) than in other N sources. No significant difference in the biomass was observed in the presence or absence of MSW when supplemented with glutamic acid. On the other hand, growth was inhibited in the presence of MSW when corn steep liquor (CSL) and glycine were supplemented. The fungus showed enhanced growth with all of the N

Table 3.3. Effect of nitrogen source and effluents on the production of biomass and lignin-degrading enzymes by NIOCC # 2a.

Nitrogen Source	Effluents				
	Control (without effluent)	TEA[#]	TEB[#]	MSW[#]	BL[#]
	Biomass (mg/ 20 ml)				
KNO_3	106.5	141.6	80.4	149.7	118.2
Glycine	81.2	104.8	111.5	53.9	154.2
Glutamic acid	144.7	150.5	96.5	142	106.2
Beef extract	77.4	89.3	74.9	190.4	138.5
Corn steep liquor	95.4	99.1	94.6	61.7	146.1
	Laccase Activity (U L^{-1})				
KNO_3	16,687	2,224	10,385	6,652	2,622
Glycine	34,659	3,702	49,628	5,644	12,210
Glutamic acid	47,567	9,756	20,471	26,552	2,287
Beef extract	10,346	1,979	8,254	8,320	1,445
Corn steep liquor	12,973	620	12,172	2,553	2,058
	MnP Activity (U L^{-1})				
KNO_3	0	353	0	0	2421
Glycine	12	508	30	0	1621
Glutamic acid	0	0	38	394	1679
Beef extract	25	493	0	450	2372
Corn steep liquor	46	1760	222	604	2178
	LiP Activity (U L^{-1})				
KNO_3	26	266	150	85	913
Glycine	2	343	65	98	1114
Glutamic acid	17	75	103	180	2591
Beef extract	25	473	141	0	671
Corn steep liquor	26	29	0	75	0
	Decolorization (%)				
KNO_3	-	64	78	30	0
Glycine	-	56	88	61	5
Glutamic acid	-	64	70	36	1
Beef extract	-	28	77	49	0
Corn steep liquor	-	53	92	71	0

TEA =Textile effluent A; TEB =Textile effluent B; MSW =Molasses Spent Wash; BL = Black Liquor

supplements in the presence of TEA whereas; growth was inhibited in media containing TEB except in the presence of glycine (Table 3.4).

Overall, the best growth in the presence of TEA was when supplemented with glutamic acid; in TEB when supplemented with glycine; in MSW when supplemented with beef extract and in BL when supplemented with glycine (Table 3.4).

Effect of N source and effluent on the production of lignin-degrading enzymes

Among all the nitrogen sources, glutamic acid supported maximum laccase production. This trend was maintained even in the presence of TEA and MSW. On the other hand, TEB and BL supported maximum laccase production when glycine was the N source. Irrespective of the nitrogen source used, laccase production was inhibited when supplemented with BL and TEA (Table 3.4).

Production of manganese peroxidase (MnP) was enhanced by several folds in the presence of BL in all of the N sources. To a certain extent, TEA and MSW also enhanced the production of MnP in some of the nitrogen sources. Similarly, production of LiP was enhanced by several folds in the presence of BL and to a certain extent in the presence of MSW, followed by TEA and TEB (Table 3.4). Fungal biomass and lignin-degrading enzyme production did not show any correlation.

***Effect of N source on decolorization* of *colored effluents* (*in situ*)**

Among the effluents, BL was least decolorized, irrespective of the N source used; whereas, TEB was decolorized equally well in the presence of all the N sources. Decolorization of TEA and MSW was achieved to a moderate extent in media with different N sources (Table 3.4). As the volume and not the color units of the effluent added (10% v/v) was kept constant, decolorization of the individual pollutants varied vastly. The black liquor at the same volume gave intense color with much higher color units than the other effluents.

Table 3.4: Effect of nitrogen source and effluents on the production of biomass and lignin-degrading enzymes by NIOCC # 2a.

Nitrogen Source	Effluents				
	Control (without effluent)	TEA#	TEB#	MSW#	BL#
	Biomass (g L^{-1})				
KNO_3	5.3	7.1	4.0	7.5	5.9
Glycine	4.1	5.2	5.6	2.7	7.7
Glutamic acid	7.2	7.5	4.8	7.1	5.3
Beef extract	3.9	4.5	3.7	9.5	6.9
Corn steep liquor	4.8	4.5	4.7	3.1	7.3
Laccase Activity (U L^{-1})					
KNO_3	16,687	2,224	10,385	6,652	2,622
Glycine	34,659	3,702	49,628	5,644	12,210
Glutamic acid	47,567	9,756	20,471	26,552	2,287
Beef extract	10,346	1,979	8,254	8,320	1,445
Corn steep liquor	12,973	620	12,172	2,553	2,058
MnP Activity (U L^{-1})					
KNO_3	0	353	0	0	2421
Glycine	12	508	30	0	1621
Glutamic acid	0	0	38	394	1679
Beef extract	25	493	0	450	2372
Corn steep liquor	46	1760	222	604	2178
LiP Activity (U L^{-1})					
KNO_3	26	266	150	85	913
Glycine	2	343	65	98	1114
Glutamic acid	17	75	103	180	2591
Beef extract	25	473	141	0	671
Corn steep liquor	26	29	0	75	0
Decolorization (%)					
KNO_3	-	64	78	30	0
Glycine	-	56	88	61	5
Glutamic acid	-	64	70	36	1
Beef extract	-	28	77	49	0
Corn steep liquor	-	53	92	71	0

TEA =Textile effluent A; TEB =Textile effluent B; MSW =Molasses Spent Wash; BL = Black Liquor. Standard deviation values were less than 10%.

***Decolorization of the effluents* (*ex situ*)**

Equal color units (1000 CU) of all the effluents and the concentrated culture supernatant having the same amount of laccase (10U) were used for this experiment. All the effluents were decolorized to the maximum by 48 hours *ex situ*. The process might have continued, but the experiment was terminated at 48h in the present study (Fig. 3.5). Enzymes from the medium containing, KNO_3 as N source performed continous decolorization of TEA (Fig. 3.5). On the other hand, enzymes from all the N sources decolorized TEB continuously (Fig. 3.5) without any repolymerization. A similar phenomenon was observed in the case of MSW where continuous decolorization occurred only when enzyme from beef extract as N source was used (Fig. 3.5). Continuous depolymerization of black liquor occurred, when the enzyme source from KNO_3 was used (Fig. 3.5). In the presence of enzymes obtained from media containing other N sources, an initial decolorization of BL was followed by increase in color, which is probably due to its repolymerization (Fig. 3.5). The decolorization was not the effect of pH, as there was no change in pH at the end of 48h in any of the reaction mixtures.

3.4.2 *Remediation of raw textile mill effluents*

Qualitative decolorization of textile mill effluents

All the LDE positive isolates mentioned in chapter 2 (Fig. 2.8) were tested for the qualitative decolorization of the two textile mill effluents namely, textile effluent A (TEA) and textile effluent B (TEB). The fungal isolate, NIOCC #2a registered maximum removal of color from both the textile effluents, during the qualitative plate assay. The effluents were partially decolorized by few other isolates also as shown in Fig. 3.6.

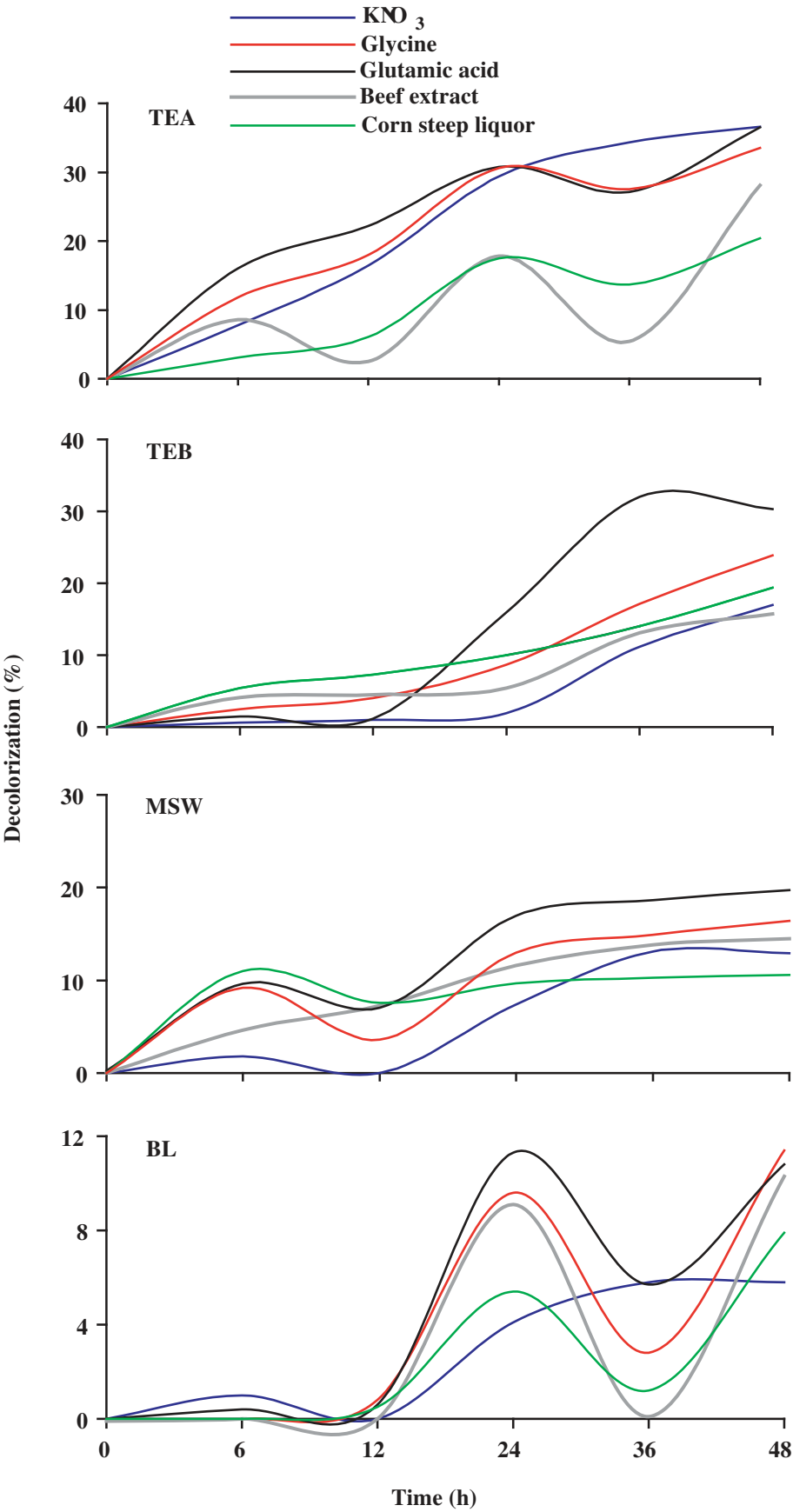


Fig. 3.5: *In vitro* **decolorization of effluents with 1000 color units each using 10U of crude laccase obtained from different nitrogen sources namely, KNO 3, Glycine, Glutamic acid, Beef extract and Corn steep liuor. Decolorization was monitored from 0 to 48 h. The standard deviation values were less than 10%.**

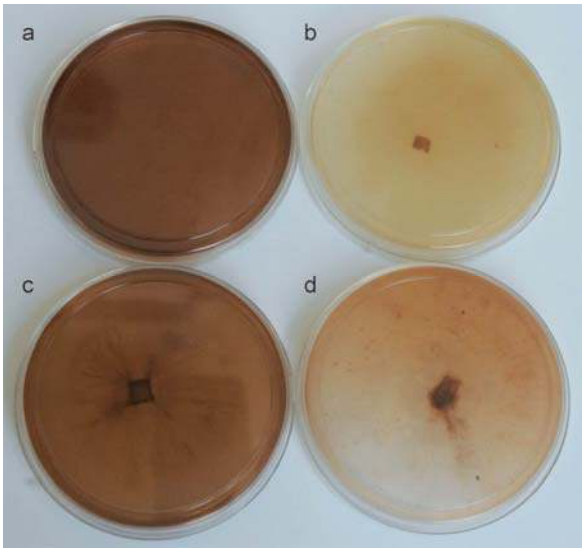


Fig. 3.6(A): Qualitative decolorization of textile effluent A; a) Uninoculated Plate (Control), b) NIOCC #2a, c) NIOCC #16V, d) NIOCC #15V

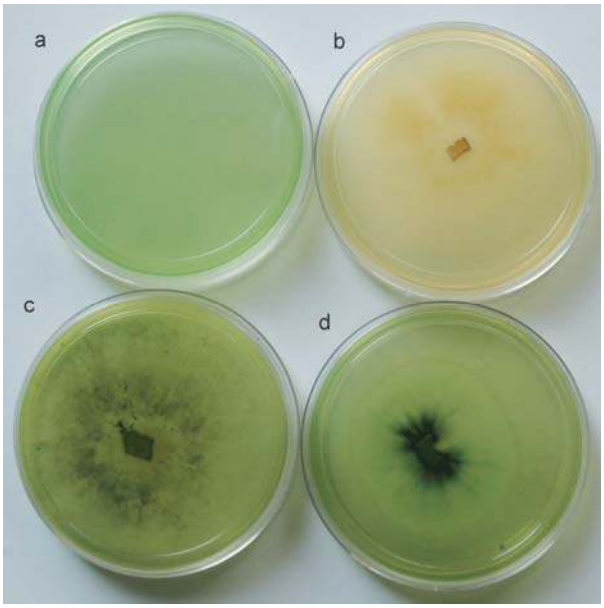


Fig. 3.6(B): Qualitative decolorization of textile effluent B; a) Uninoculated Plate (Control), b) NIOCC #2a, c) NIOCC #16V, d) NIOCC #13V

Laccase production and decolorization by whole cultures (in situ)

Besides the basidiomycete NIOCC #2a, three other marine-derived fungi were used for the remediation of TEA and TEB. These isolates were NIOCC #15V, #16V and #C3. Sequence analysis of 18S of #15V identified it to be *Coriolopsis byrsina*. However, ITS sequence analyses of this isolate showed 97% homology to an uncultured fungus clone. The fungal isolates #16V and #C3 were identified as *Diaporthe* sp. and *Pestalotiopsis* sp. respectively (Chapter 2, Table 2.3). The isolate #15V belonged to the phylum Basidiomycota whereas, #16V and #C3 clustered with Ascomycota (Chapter 2, Fig. 2.6, 2.7). The cultures #15V, #16V and #C3 were deposited at American Type Culture Collection, USA under the accession No. ATCC MYA-4557, ATCC MYA-4558 and ATCC MYA-4556, respectively.

The two isolates #16V and #C3 belonging to ascomycetes produced lower titer of laccase than basidiomycetes (#2a and #15V) in the presence of both the effluents (Fig. 3.7 a-d). The basidiomycetes showed 20 to 60-fold (Fig. 3.7 a, c) and the ascomycetes two to ten-fold (Fig. 3.7 b, d) higher laccase production in the presence of TEB than in the presence of TEA. Decolorization of 20% TEA and TEB by the two ascomycetes reached plateau by day 3, irrespective of the laccase titer produced by them (Fig. 3.7 b, d).

Among the two effluents TEB was decolorized to the higher extent by all the four fungi than TEA (Fig. 3.7 a-d). Most of the decolorization of TEB by ascomycetes was achieved by day 2 (Fig. 3.7 d) whereas this was not the case with TEA. In general, about 40–60% color reduction of TEA and 60–80% color removal of TEB was obtained by day 9. No correlation was observed between decolorization of TEA or TEB with laccase production. Color removal of both the effluents by adsorption on the fungal biomass was greater in ascomycetes than in the basidiomycetes (Table 3.5).

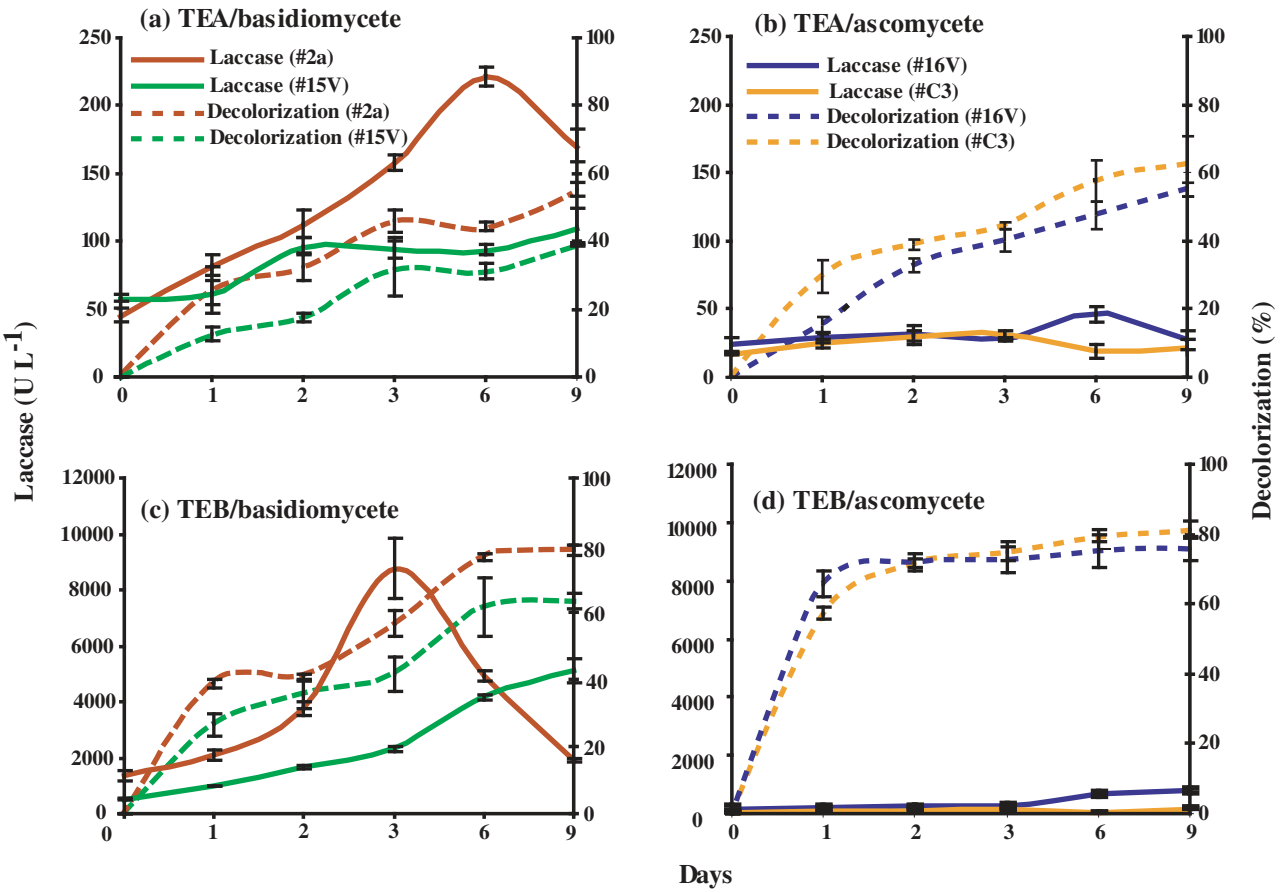


Fig. 3.7: Laccase production and decolorization of effluents (in situ) with four marine-derived fungi. The textile effluents were added to 6-day old culture and this was considered day 0 for the decolorization measurements.

Table 3.5: Percentage of color adsorbed on the live fungal biomass after growing them in the presence of 20 % TEA and TEB for 6 days.

Cultures (Phylum)	TEA	TEB
# 2a (Basidiomycete)	32.3 ± 5.4	6.8 ± 1.9
# 15V (Basidiomycete)	27.7 ± 2.3	8.2 ± 0.6
# 16V (Ascomycete)	38.9 ± 4.8	41.3 ± 4.6
# C3 (Ascomycete)	43.0 ± 5.7	29.8 ± 2.3

Fungal biomass grown in the presence of TEA and TEB was collected by centrifugation, homogenized in methanol:water (1:1) and filtered. The filtrate was lyophilized and resuspended in water and the color was determined spectrophotometrically as described under Material and methods.

The cultures were tested for the presence of other lignin-degrading enzymes. The culture # 2a alone produced a maximum of 70 U L^{-1} of MnP but not LiP in the B & K medium. Addition of TEA to this medium did not inhibit MnP production but TEB inhibited its production by five to six folds. The basidiomycetes produced about 200–300 U L^{-1} glucose oxidase in this medium supplemented with TEB, whereas the ascomycetes produced about 50–100 U L^{-1}. The production of this enzyme was reduced by four to five folds in the presence of TEA in the medium. In the ascomycetes, its production was largely inhibited.

In order to verify whether the low decolorization of TEA was due to alkaline pH, decolorization experiment was also performed after lowering the pH of TEA from 8.9 to 5. No increase in percentage decolorization was noticed after altering the pH, ruling out the possibility that alkaline pH was the limiting factor for decolorization.

Spectral scans of culture supernatants from fungi grown in the presence of TEA and TEB showed a decrease in absorbance throughout the visible range (Fig. 3.8 a, b). Spectral scans of #C3, #2a and #16V-treated TEB showed total disappearance of absorbance maximum peaks in the region 560–700 nm. The absorbance between 400 and 450 nm reduced considerably without altering the

pattern of the peak. The λ_{max} of reactive yellow 145 fell into this spectral range (Table 3.3).

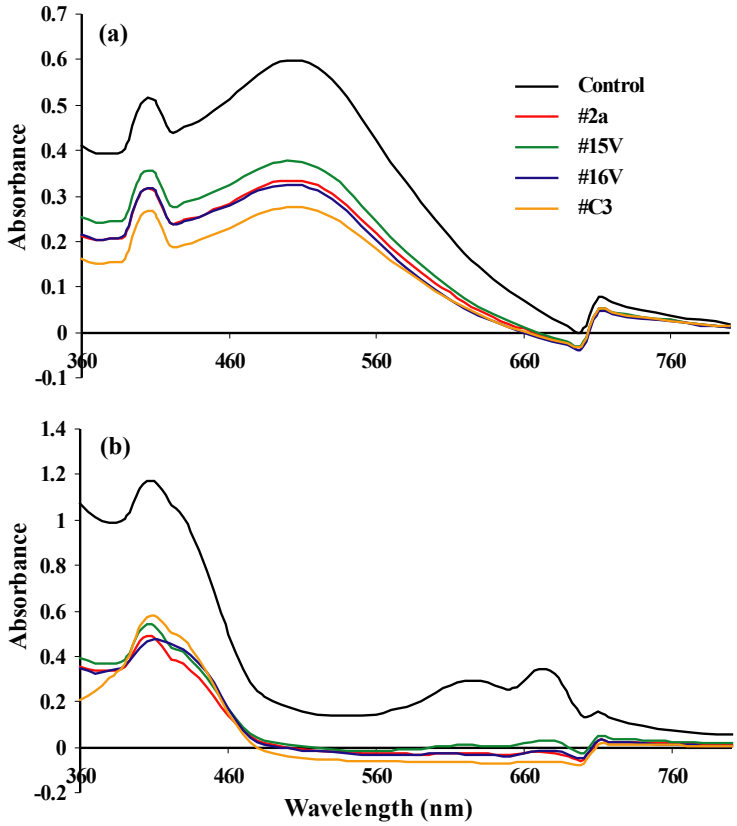


Fig. 3.8: Absorbance spectra of a) TEA and b) TEB in the visible regions after growth of four fungi for six days and abiotic control. The cultures contained 20% of the effluents in B & K medium prepared with half strength sea water.

As all the four fungi decolorized both TEA and TEB incorporated at 20% (five-fold diluted) in the medium, efficiency of these fungi to decolorize higher concentrations of these effluents was tested by adding them at 50 and 90% concentration (two-fold and 1.1- fold diluted). All the four fungi showed biomass and laccase production in the presence of TEA and TEB at these concentrations (Tables 3.6, 3.7; Fig. 3.9, 3.10). The growth of isolates in the presence of these effluents was better than the control in most of the fungi (Tables 3.6, 3.7; Fig. 3.9,

3.10). Decolorization of TEA in the range of 27–57% (Table 3.6; Fig. 3.9) and 34–68% of TEB (Table 3.7; Fig. 3.10) added at 50 and 90% concentrations was possible with these fungi.

Table 3.6: Response of the fungi in the presence of TEA added at 20 and 50 % to 6-day old cultures (*in situ*) . All the parameters were estimated on day 6 after addition of the effluent.

Parameters measured	Concen of the effluent (%)	Basidiomycete		Ascomycete		Control[b]
		NIOCC #2a *Cerrena unicolor*	NIOCC #15V *Coriolopsis byrsina*	NIOCC #16V *Endothia* sp.	NIOCC #C3 *Pestalotiopsis* sp.	
Biomass (g L^{-1})	Control[a]	3.6 ± 0.2	4.1 ± 0.5	3.7 ± 0.9	5.1 ± 0.3	-
	20	4.0 ± 0.3	4.6 ± 0.5	4.3 ± 0.8	6.3 ± 0.3	
	50	4.9 ± 0.7	5.3 ± 0.9	4.8 ± 0.6	6.2 ± 1.1	
	90	4.2 ± 1.7	4.4 ± 0.8	5.3 ± 1.3	5.9 ± 0.8	
Laccase (U L^{-1})	Control[a]	2,015 ± 615.8	1,951 ± 74.5	52 ± 12.9	39 ± 8.1	-
	20	141.9 ± 42.6	82.4 ± 16.8	32.8 ± 7.1	22.3 ± 4.1	
	50	46.3 ± 4.5	39.8 ± 11.6	41.5 ± 12.1	41.8 ± 28.6	
	90	38.4 ± 7.4	40.9 ± 5.9	47.8 ± 3.6	23.6 ± 2.4	
% Decolorization	20	44.4 ± 1.5	31.2 ± 2.1	48.0 ± 3.7	58.0 ± 4.7	-
	50	55.6 ± 1.6	29.9 ± 0.3	27.3 ± 0.7	56.6 ± 0.3	
	90	23.9 ± 5.8	26.7 ± 7.2	31.9 ± 4.3	30.7 ± 7.9	
24 h-LC_{50}	20	14.2 ± 1.4	28.0 ± 14.1	18.1 ± 1.8	16.7 ± 2.2	6.5 ± 1.4
	50	4.3 ± 0.1	5.0 ± 1.1	5.7 ± 1.3	4.7 ± 0.5	1.9 ± 0.0
% reduction in total phenolics	20	19.3 ± 0.9	28.1 ± 1.1	21.1 ± 0.6	22.8 ± 2.4	-
	50	22.6 ± 2.5	24.9 ± 6.6	21.7 ± 5.4	34.2 ± 2.8	
% reduction in COD	20	48.3 ± 3.0	62.9 ± 11.3	90.0 ± 0.6	69.1 ± 5.5	-
	50	20.6 ± 11.7	28.5 ± 4.4	24.1 ± 6.8	40.4 ± 1.2	

All the parameters were estimated on day 6 after addition of the effluent

[a] without any effluent

[b] Only TEA in the medium without any culture

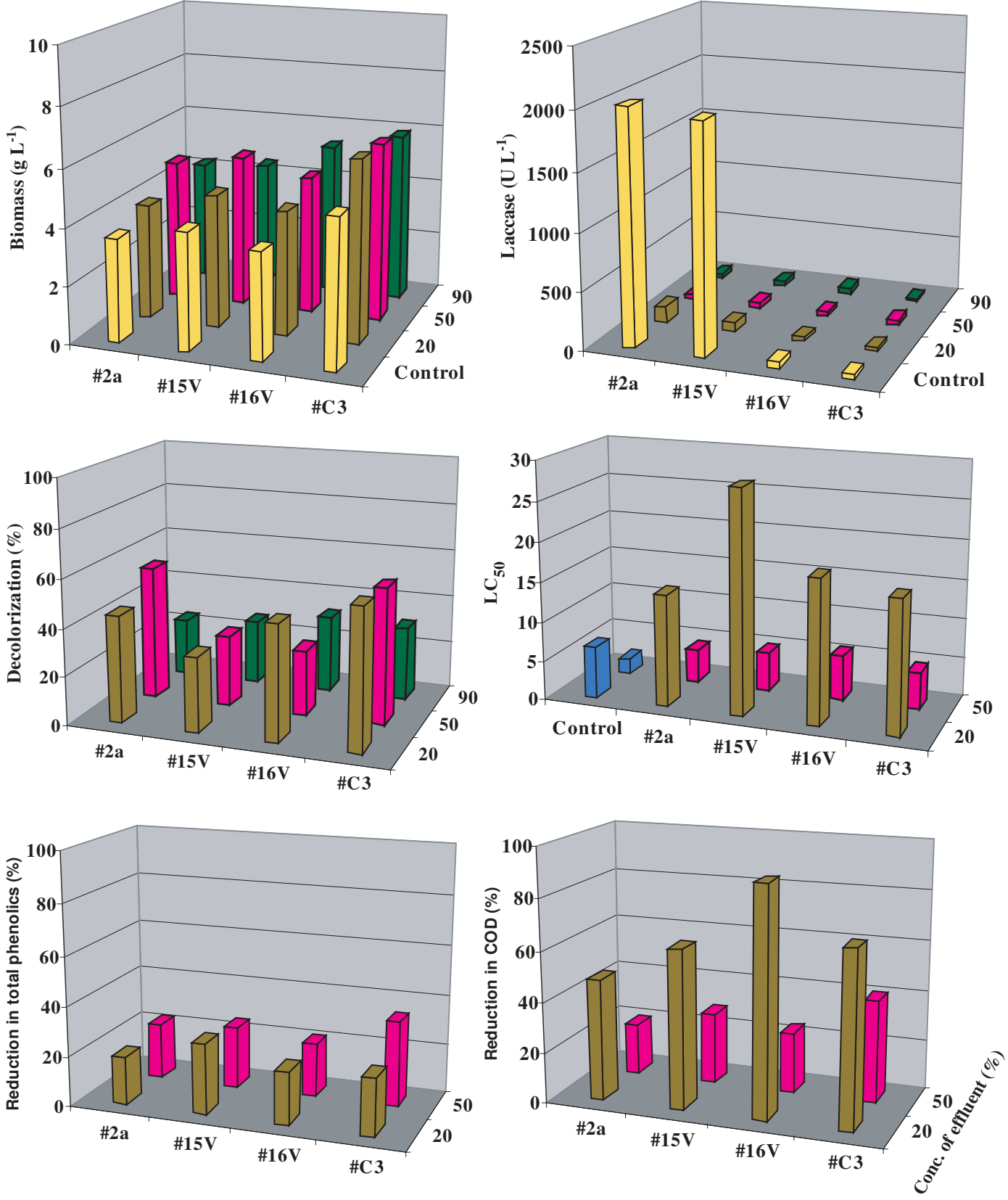


Fig. 3.9: Response of fungi in the presence of TEA added at 20% , 50% and 90% to 6-day old cultures (in situ)

Controls: Without any effluent, Only TEA in the medium without any culture.

Table 3.7: Response of fungi in the presence of TEB added at 20 and 50 % to 6-day old cultures (*in situ*). All the parameters were measured on day 6 after addition of the effluent.

Parameters measured	Concen of the effluent (%)	Basidiomycete		Ascomycete		Control[b]
		NIOCC #2a *Cerrena unicolor*	NIOCC #15V *Coriolopsis byrsina*	NIOCC #16V *Endothia* sp.	NIOCC #C 3 *Pestalotiopsis* sp	
Biomass (g L^{-1})	Control[a]	3.6 ±0.2	4.1 ± 0.5	3.7 ±0.9	5.1 ± 0.3	-
	20	3.9 ± 0.2	3.7 ± 0.1	6.1 ± 0.9	4.4 ± 0.1	
	50	6.9 ± 1.3	4.3 ± 0.5	9.0 ± 1.6	7.5 ±0.8	
	90	3.1 ± 0.1	4.2 ± 0.2	9.4 ±2.4	6.7 ±1.6	
Laccase (U L^{-1})	Control[a]	2,015 ± 615.8	1,951 ±74.5	52 ±12.9	39 ± 8.1	-
	20	5,015 ± 519.7	4,165 ± 95.1	406 ± 69.8	105 ± 13.0	
	50	534 ± 24.9	145 ± 20.3	159 ± 24.7	30 ±3.0	
	90	855 ± 61.9	284 ± 85.7	146 ±20.5	191 ± 69.5	
% Decolorization	20	76.4 ± 0.9	61.5 ± 8.7	75.1 ± 4.6	79.3 ± 1.7	-
	50	42.8 ± 1.6	32.4 ± 3.7	53.5 ± 4.8	58.1 ± 1.3	
	90	38.6 ± 3.0	38.8 ± 4.7	59.7 ± 5.3	67.9 ±2.5	
24 h-LC_{50}	20	57.9 ± 14.4	38.2 ± 9.9	39.6 ± 9.5	53.3 ± 3.7	19.8 ±
	50	13.5 ± 3.4	13.7 ± 2.9	8.4± 0.8	8.5 ± 1.0	6.5 ±
	90	7.9 ± 1.3	9.5 ± 2.7	10.6 ±2.5	7.5 ± 0.9	3.8 ± 0.4
% reduction in total phenolics	20	68.5 ± 5.3	52.9 ± 6.6	70.6 ± 4.8	76.5 ± 9.2	-
	50	82 ± 4.1	70 ± 15.3	87 ± 5.9	82 ± 2.3	
	90	83 ± 2.4	67 ± 11.2	79 ± 9.4	71 ± 5.9	
% reduction in COD	20	43.7 ± 7.0	52.3 ± 7.6	73.3 ± 0.6	98 ± 2.1	-
	50	46 ± 1.6	37 ± 8.8	90 ± 1.3	84 ± 2.3	
	90	48 ± 1.2	55 ± 15.2	72 ± 6.9	86 ±10.3	

All the parameters were estimated on day 6 after addition of the effluent
[a] without any effluent
[b] Only TEB in the medium without any culture

Detoxification of textile mill effluents in whole cultures (*in situ*)

Detoxification of TEA as measured by a decrease in percentage mortality of Artemia larvae with reference to untreated control was best by NIOCC #15V followed by #16V, #C3 and #2a (Fig. 3.11a) whereas in TEB, lowest mortality was noticed in the presence of #2a followed by #C3, #16V and #15V (Fig. 3.11b). The four fungi brought about three to five-fold reduction in toxicity of TEA (Table 3.6; Fig. 3.9) whereas toxicity of TEB was reduced two to three-fold (Table 3.7; Fig. 3.10). In general TEA was more toxic than TEB (Tables 3.6, 3.7; Fig. 3.9, 3.10). About 19–34% reduction in total phenolics and 50–90% reduction

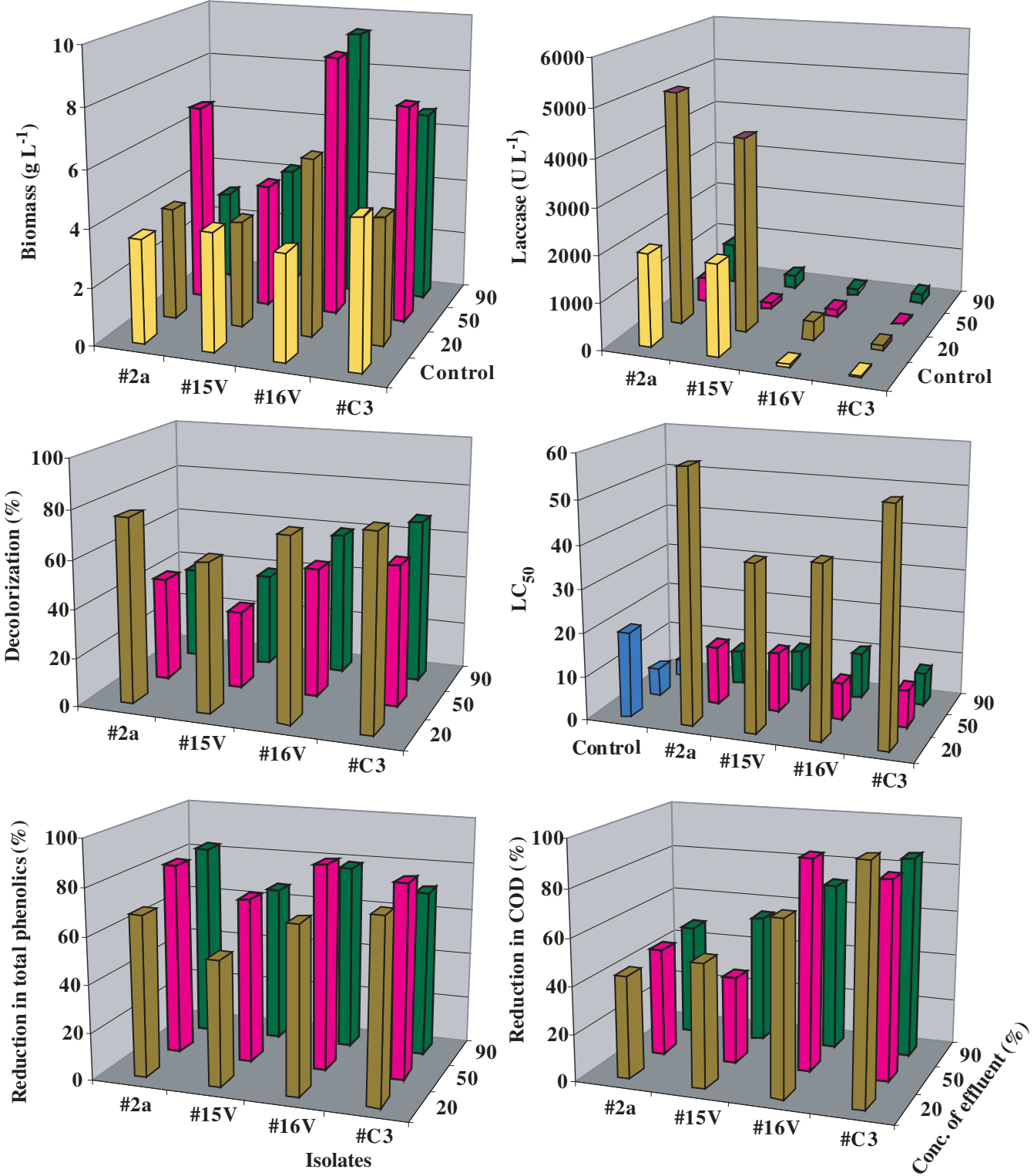


Fig. 3.10: Response of fungi in the presence of TEB added at 20% , 50% and 90% to 6-day old cultures (in situ)

Controls: Without any effluent, Only TEB in the medium without any culture.

in COD of TEA were brought about by the four fungi (Table 3.6; Fig. 3.9). Treatment of TEB with the four test fungi resulted in a reduction in total phenolics by 50–90% and 44– 98% reduction in COD (Table 3.7; Fig. 3.10). Toxicity of TEB was reduced better by basidiomycetes whereas ascomycetes proved better in COD reduction (Table 3.7; Fig. 3.10).

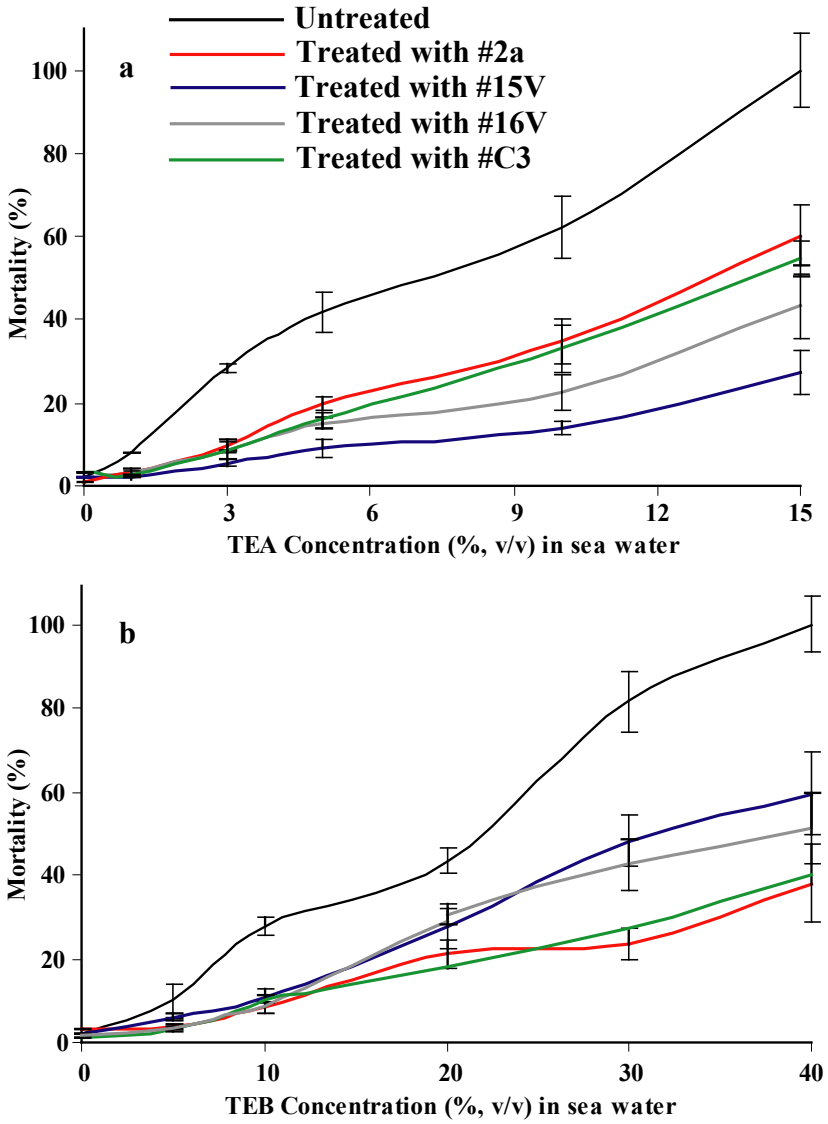


Fig. 3.11: Mortality percentage of *Artemia salina* growing in the presence untreated and treated TEA (a) and TEB (b).

Mass spectrometry analysis of the textile effluents

Mass spectrometric scans of culture supernatants of fungi grown in the presence of TEA and TEB showed distinct changes indicating fragmentation and degradation of the components of these effluents (Figs. 3.12, 3.13). The spectra shown in the figures are after subtracting common peaks found in the un-inoculated and inoculated B & K broth. These modifications appear to reflect in percentage decolorization. Chromatograms of TEA showed decrease in intensity and disappearance of most of the peaks after the treatment with all the fungi (Fig. 3.12). Scan of TEB after treatment with #15V showed the maximum number of degradation products whereas after treatment with the rest of the fungi, most of the peaks disappeared (Fig. 3.13).

Decolorization of textile effluents by culture supernatants **(ex situ)**

The efficiency of culture supernatants (*ex situ*) of the four fungi in decolorization of TEA and TEB at 50% concentration was tested. No color reduction of TEA was observed up to 72 h. Culture supernatants of NIOCC # 2a, #15V, #16V and #C3 with the laccase titer of 64, 29, 0.06 and 0.03 U ml^{-1} respectively, brought about a color reduction of TEB by 23, 17, 9 and 5%, respectively within 72 h. As TEA was not significantly decolorized by the culture supernatants alone of all the fungi, effect of low molecular weight mediators to enhance decolorization was tested. Although decolorization of TEA used at 10% concentration did occur, no clear effect of mediators was observed. Therefore, TEA was finally used at 1% in combination with two different concentrations of various mediators. The mediators HBT, vanillic acid and acetosyringone were effective in enhancing the decolorization efficiency of the basidiomycetes #2a and #15V (Fig. 3.14). Decolorization by the ascomycete #16V was comparatively lesser than basidiomycetes. However, it was enhanced by all the three mediators whereas none of the mediators were effective in enhancing the decolorization efficiency of the ascomycete #C3. These studies indicated that the mediators were more

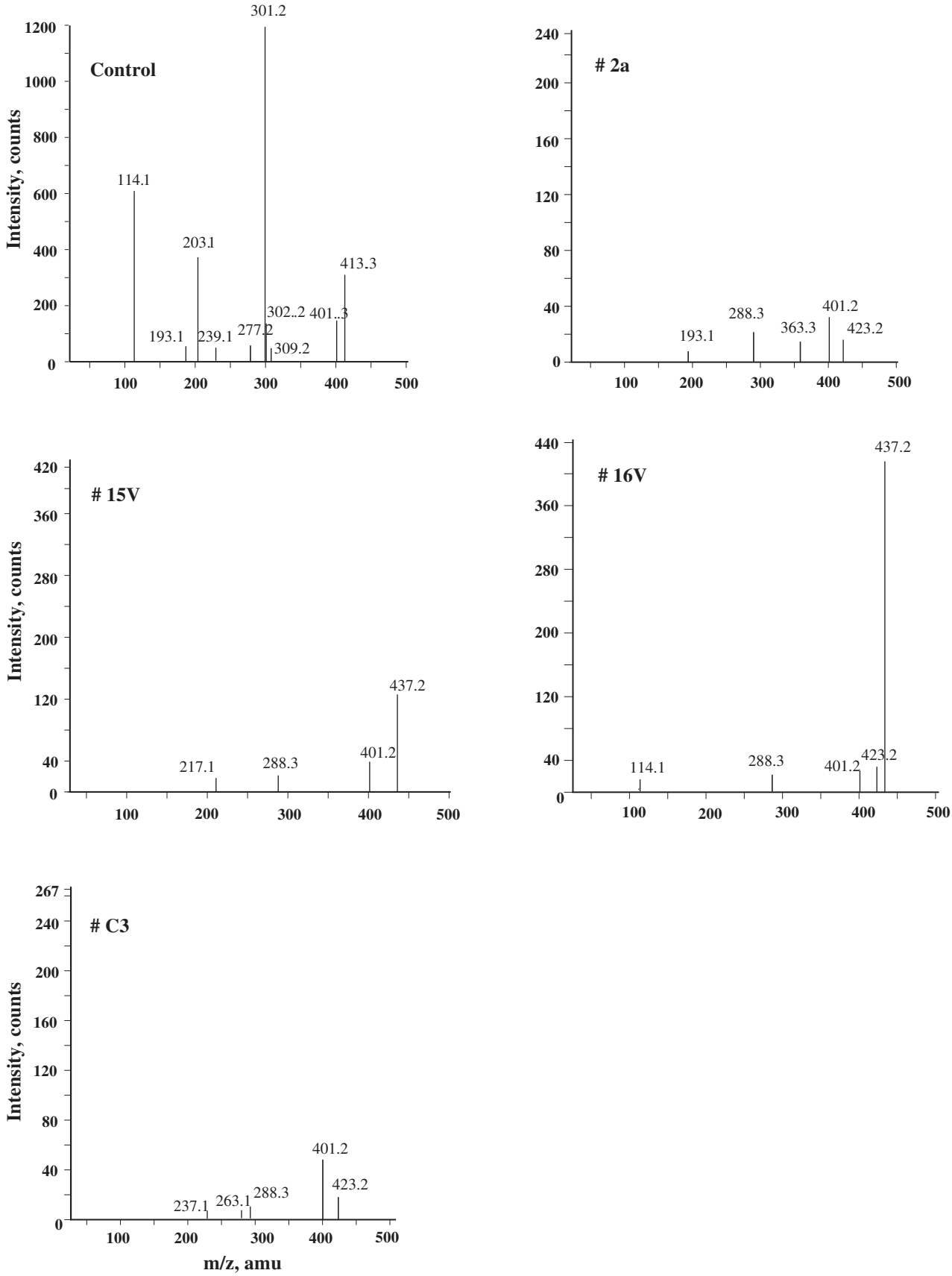


Fig. 3.12: Electrospray ionozation mass spectra with characteristic ions related to specific low mass ions were recorded at 0-1,000 m/z in positive mode. Mass spectra of untreated (control) and fungal-treated (in situ) TEA. Note the difference in intensity counts (y axis) in various cultures and the control

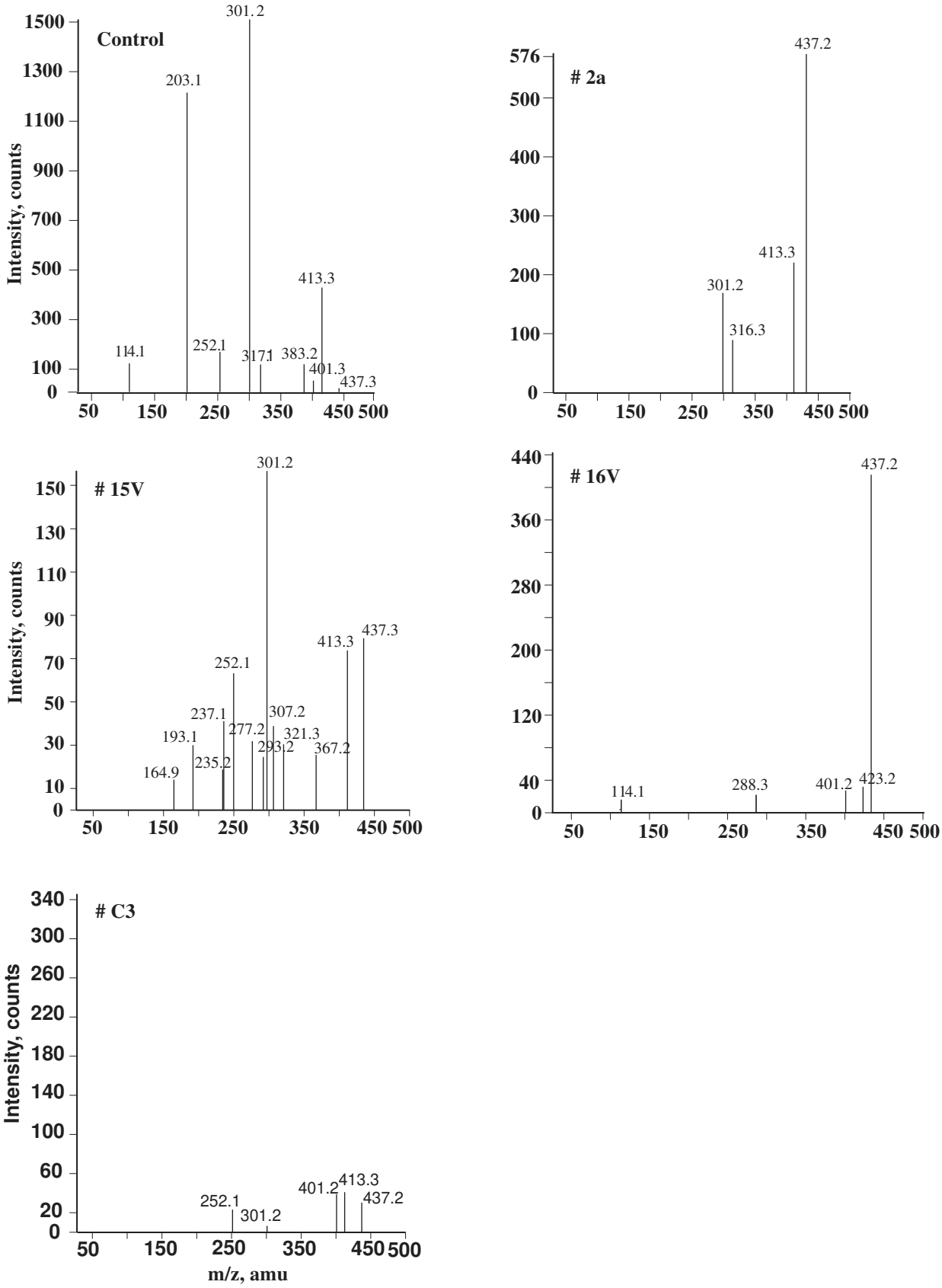


Fig. 3.13: Electrospray ionization mass spectra with characteristic ions related to specific low mass ions were recorded at 0-1,000 m/z in positive mode. Mass spectra of untreated (control) and fungal-treated (in situ) TEB. Note the difference in intensity counts (Y axis) in various cultures and the control

efficient in decolorization of both the effluents in the presence of culture supernatants from basidiomycetes than the ascomycetes.

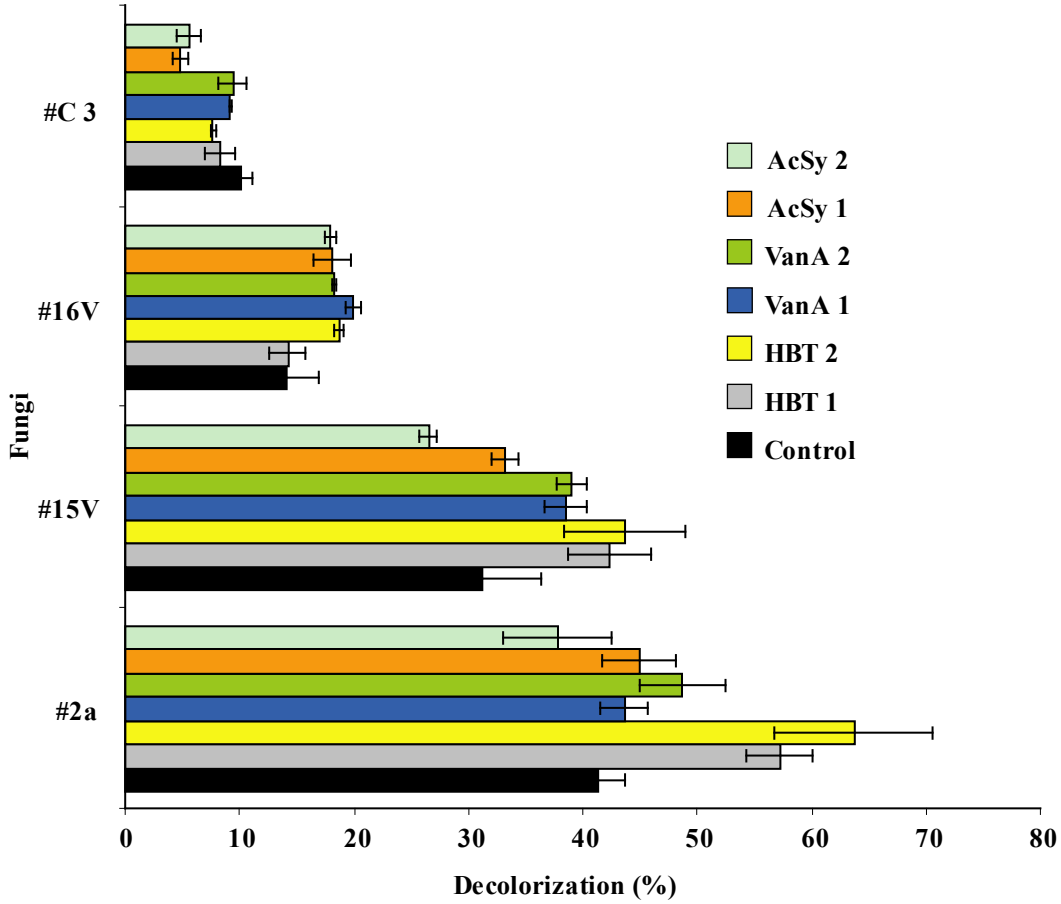


Fig. 3.14: Effect of mediators on decolorization of TEA (1% concentration) after 72h incubation with culture supernatants of four fungi at 30 ºC. Control is without mediators, 1 = 50 µM and 2 = 500 µM. AcSy acetosyringone, VanA vanillic acid, HBT 1-hydroxy benzotriazole

Culture supernatants from laccase-hyper-producing isolate #2a and commercial laccase preparation of *Tremetes versicolor*, at varying concentrations of laccase (0.5–20 U ml^{-1}) were effective in decolorization of 20% TEA and TEB. Decolorization correlated with laccase concentrations in these studies (Table 3.8). Polymerization, as indicated by reduction in percentage decolorization with

longer incubation period was noticed to occur occasionally in TEA treated with both the laccase preparations.

Table 3.8: Decolorization (*ex situ*) of TEA and TEB (20%) by crude laccase from NIOCC #2a and commercial laccase from *Trametes versicolor.*

Laccase ($U\ ml^{-1}$)	Decolorization (%)							
	TEA (20 %)				TEB (20 %)			
	NIOCC #2a (*C. unicolor*)		*T. versicolor*		NIOCC #2a (*C. unicolor*)		*T. versicolor*	
	Hours							
	12	36	12	36	12	36	12	36
0.5	0	0	3 ± 0.9	4 ± 1.1	10 ± 0.9	13 ± 1.4	3 ± 0.2	7 ± 2.1
2.5	14 ± 2.3	18 ± 3.8	13 ± 1.6	11 ± 0.8	12 ± 3.8	16 ± 3.3	13 ± 2.8	19 ± 0.7
5.0	26 ± 1.9	21 ± 4.9	12 ± 2.2	16 ± 2.9	15 ± 0.8	28 ± 2.7	15 ± 1.9	27 ± 0.8
10.0	33 ± 3.7	29 ± 5.6	30 ±5.7	29 ± 2.8	39 ± 3.6	42 ± 2.7	17 ± 5.5	51 ± 2.2
20.0	39 ± 7.3	39 ± 4.7	25 ± 6.5	22 ± 6.6	43 ± 6.4	52 ± 5.9	29 ± 3.3	60 ± 9.7

Effluents were diluted with 0.1 M sodium acetate buffer (pH 5.0) to 20 % final concentration and incubated with different concentrations of laccase from NIOCC #2a (*Cerrena unicolor*) or *Trametes versicolor* (Sigma Chemicals, USA). Absorbance spectra of TEA and TEB from 360 to 800 nm were acquired and % decolorization was calculated by the difference in spectral area from those of 0 h samples.

Reusing fungal biomass for decolorization

Reuse of fungal biomass for decolorization of TEA was not effective whereas decolorization of TEB up to 3 cycles was effective with the basidiomycetes #2a and #15V. In the ascomycetes #16V and #C3, a 50% reduction in decolorization efficiency was observed in the second cycle itself (Fig. 3.15). However, color removal in the first cycle was comparable with that of basidiomycetes.

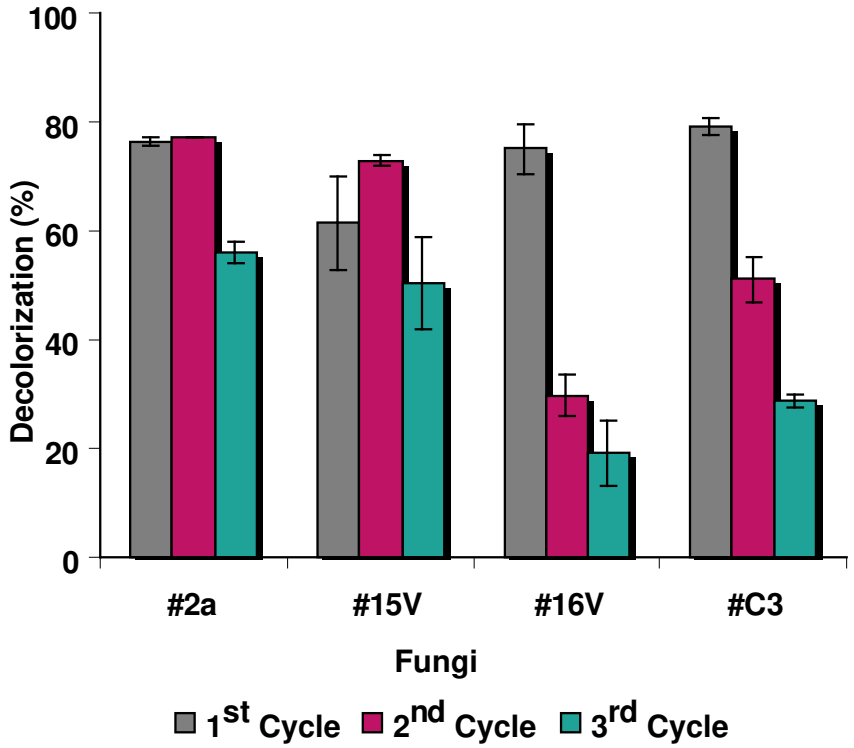


Fig. 3.15: Decolorization of TEB by reusing the fungal biomass in three cycles.

Decolorization of the residual color from the fungal biomass

The culture supernatants of #C3, an ascomycete grown in the presence of TEA or TEB were removed and in the second step the fungal biomass was incubated with 100 U of laccase from #2a for 48 h. The residual color of TEA and TEB from the fungal biomass was removed by 49 and 84%, respectively. The respective controls, incubated with distilled water did not show any decolorization. These results indicated that bioadsorbed effluent can also be decolorized using laccase from a basidiomycete.

A process for enhanced decolorization

Based on the above results, sorption capacity of the ascomycete #C3 and efficiency of laccase from the basidiomycete #2a were coupled together to

enhance decolorization process. This was carried out by incubating pre-grown fungal biomass of #C3 with 100 U laccase from #2a and 20% TEA or TEB. No decolorization was noticed in the control treatment where the fungal biomass was incubated with distilled water. A total of 52% decolorization of TEA and 93% of TEB occurred from the biomass and the supernatant together within 48 h. It was noticed that by this process, decolorization of the effluent in the liquid phase and the solid phase occurred simultaneously.

3.4.3 *Remediation of molasses-based raw effluents*

The four molasses-based raw effluents differed significantly in their COD content, color units and turbidity (Table 3.9). As per the information furnished by the concerned industries, ROF and ROR were reverse osmosis feed and rejects, respectively. CAT I and CAT O were anaerobically digested, while CAT O was further subjected to aerobic digestion.

Table 3.9: Physico-chemical characteristics of the molasses-based effluents.

Source of the effluents	Effluents Analysed			
	Sugar Mill Effluent		Biotech Pharmaceutical Company	
Parameters analysed	**ROF (Reverse Osmosis Feed)**	**ROR (Reverse Osmosis Reject)**	**CAT I (Conventional aeration tank Inlet)**	**CAT O (Conventional aeration tank Outlet)**
pH	7.5	7.4	7.7	7.6
COD (g L^{-1})	30.8	52.8	23.2	18.0
Total Phenolics (g L^{-1})	1.9	2.0	1.9	1.7
Color Units (Pt-Co Units)	72,500	49,000	52,000	35,000
Total Reducing Sugars (g L^{-1})	3.8	1.7	1.7	1.2
Total Solids (g L^{-1})	0.04	0.061	0.025	0.028
Sulphates (SO_4^{--}) (g L^{-1})	0.12	0.21	0.82	0.78
Turbidity (NTU)	96.0	98.9	338	185

3.4.3.1 *Hybrid technology for the treatment of molasses-based raw effluents*

A three step sequential method was employed for the remediation of above mentioned four molasses-based raw effluents. The three steps in a sequence were (1) ultrasound-induced acoustic cavitation, (2) whole-fungal treatment using the marine-derived ligninolytic fungus #2a followed by (3) biosorption of the residual color with heat-inactivated wet biomass of the same fungus.

***Effect of sonication on the effluents* – step 1**

Acoustic irradiation (sonication) removed the foul odor of the effluents significantly and reduced the turbidity by 10–40%. However, there was no reduction in COD, color, total phenolics and toxicity. A reduction in the absorbance in UV region was observed but the pattern of the spectra remained unchanged. There were negligible changes in the absorbance or spectral pattern in the visible region (Fig. 3.16).

***Bioremediation of the effluents with the whole-fungal culture* – step 2**

Experiments were conducted by addition of unsonicated and sonicated effluents separately to the 6-day old culture of the marine-derived fungus NIOCC #2a. No significant change in the pH was observed during the incubation period. Dry biomass of the fungus increased by 5– 10% in the presence of various effluents. Reduction in color, COD and phenolic content were significantly greater in the culture supplemented with sonicated effluents (see the P values in Table 3.10). The reduction in color in the culture supernatant supplemented with unsonicated effluents ranged from 20% to 30% whereas it was 40–60% in sonicated effluents. Similarly, COD reduction in unsonicated and sonicated effluents was in the range of 30–50% and 50–70%, respectively. Total phenolics were reduced in the range of 25–45% in unsonicated effluents whereas in sonicated effluents it ranged from 50 to 65% (Table 3.10).

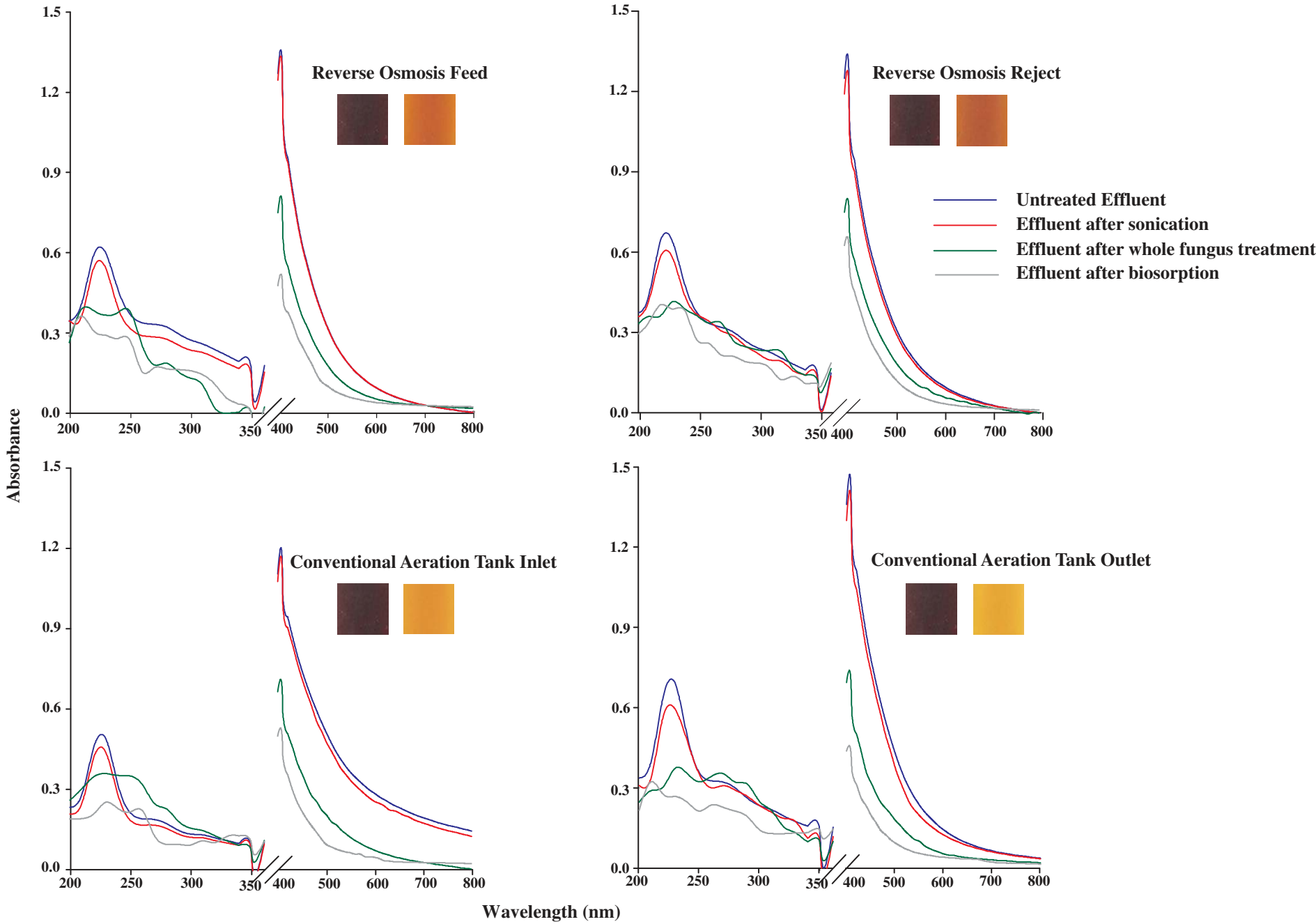


Fig. 3.16: Visible and UV spectra of the four molasses-based effluents. The UV visible spectra were taken at different dilutions The inset indicates initial and final color of the effluents

Toxicity as estimated by LC_{50} values against *Artemia* larvae was reduced 2–3 folds. In particular, sonicated ROF and CAT I showed greater reduction in toxicity than the unsonicated ones (Table 3.11).

Production of MnP and laccase were initially inhibited in the effluent-supplemented cultures but laccase steadily increased to overcome this inhibition by day 3–6 (Fig. 3.17). There was a positive correlation between decolorization and laccase production (Fig. 3.17) in the presence of all the effluents (the P value being 0.001) whereas it was negatively correlated with MnP production (P = (-) 0.001). Production of LiP was negligible in this fungus and was totally inhibited in the presence of the effluents. An overall 16-18% decolorization was achieved in an *ex situ* study using partially purified laccase.

The FT-IR spectra of the fungal biomass before and after treatment were recorded (Fig. 3.18) and the band positions of the main functional groups are listed in Table 3.12. Unloaded fungal biomass (NIOCC #2a) had intense peaks at a frequency level of 3500–3200 and 1533.3 cm^{-1} representing amino groups stretching vibrations. Mycelia in the step 2 treatment at 0 h (immediately after addition of the effluent) showed no change in this peak (Fig. 3.18, Table 3.12). The lowering of band to 3271 cm^{-1} at 24 h suggested slow adsorption of the effluent. The shift in the peak to 3290.3 cm^{-1} by day 9 may be attributed to desorption/degradation of the adsorbed effluent.

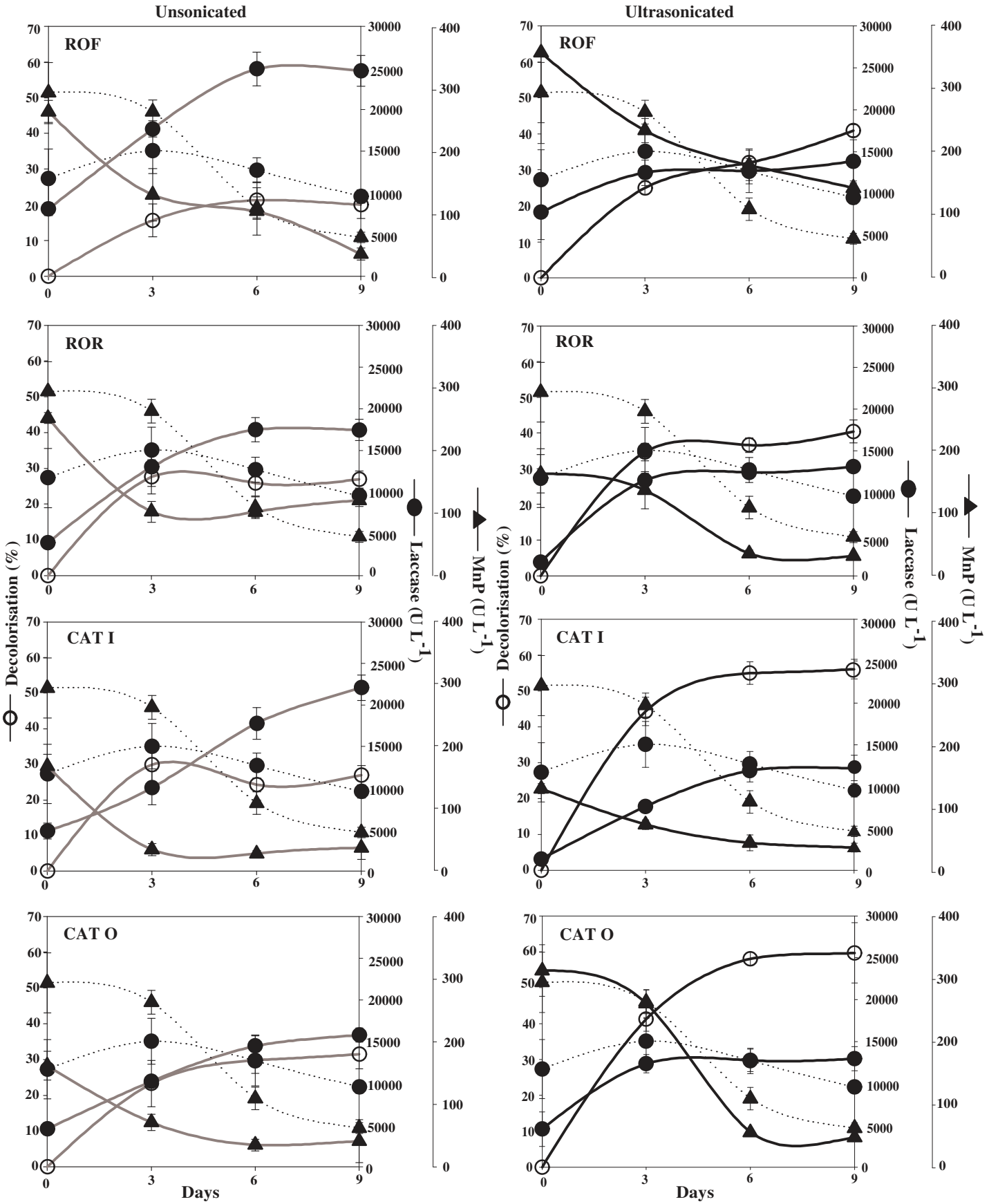


Fig. 3.17: Decolorization, laccase and MnP production by NOCC #2a in LN medium during whole-fungal culture treatment (Step 2). Four graphs on the left are with unsonicated effluents (——), and the four graphs on the right are with sonicated effluents (——). Laccase and MnP production in the control culture, without any effluents are shown as dotted lines (·····) in all the graphs.

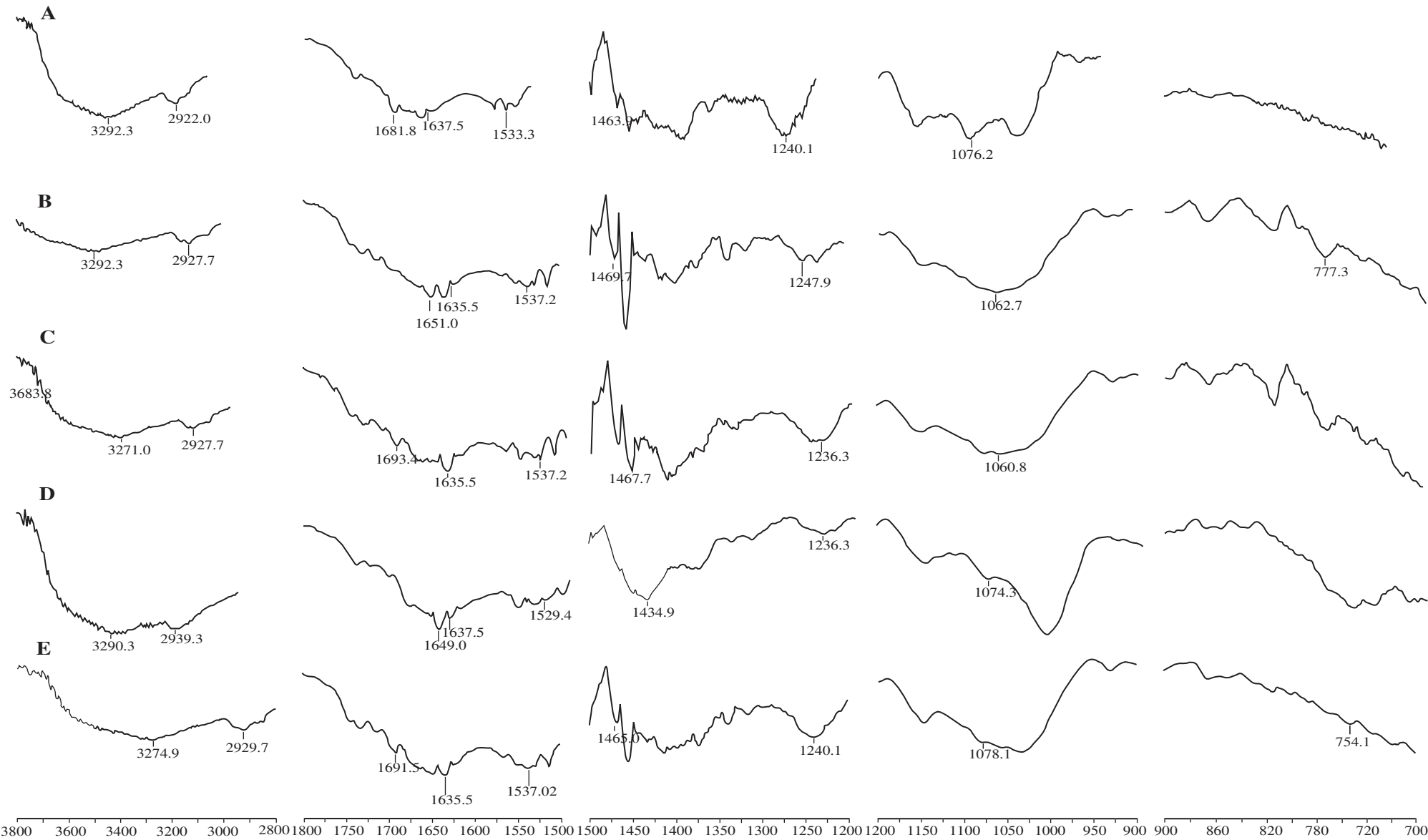


Fig. 3.18: FTIR spectra of A) unloaded fungal biomass and loaded with CAT O, B) Step 2, day 0; C) Step 2, day 1; D) Step 2, day 9; E) Step 3 (biosorption), 2 h.

Table 3.12: Band positions at different steps of the treatment by FTIR technique.

Assignment	Band Positions (cm^{-1})				
	Unloaded biomass	Step 2			Step 3
		At 0 h	After 48 h	9^{th} day	After 2 h
-OH and/or –NH stretching (3500-3000 cm^{-1})	3292.3	3292.3	3271.0	3290.3	3274.9
-CH stretching (3000-2800 cm^{-1})	2922.0	2927.7	2927.7	2939.3	2929.7
Amide-I / Amide-II band (1800-1500 cm^{-1})	1681.8 1637.5 1533.3	1651.0 1635.5 1537.2	1693.4 1635.5 1537.2	1649.0 1637.5 1529.4	1691.5 1635.5 1537.2
Mixed region (1500-1200 cm^{-1})	1463.9 1240.1	1469.7 1247.9	1467.7 1236.3	1460.0 1236.3	1465.0 1240.1
Polysaccharide region (1200-900 cm^{-1})	1076.2	1062.7	1072.0	1074.3	1078.1
Finger print region (900-700 cm^{-1})	-	777.3	774.3	757.0	754.1

Unloaded biomass is the fungal mycelium without any effluent and the remaining steps were after the addition of CAT O.

***Further bioremediation by biosorption* – step 3**

The culture supernatants containing effluents from step 2 were subjected to sorption for removal of the residual color using a fresh batch of biomass of the same fungus. Sorption ability of heat-inactivated (1) wet biomass, (2) lyophilized biomass and (3) lyophilized powdered biomass was compared. The first method yielded maximum removal of both color and COD whereas; the other two methods resulted in increase in COD without decreasing the color.

Adsorption by the wet biomass reached its equilibrium by 2 h. The reduction in color in unsonicated effluents after biosorption increased up to 50–55% whereas in sonicated effluents the increase was even greater, being up to 60–80% (Table 3.10). The percentage COD and total phenolics reduction also increased significantly. Toxicity of the unsonicated as well as sonicated effluents after biosorption was not significantly reduced from step 2 except in CAT O

(Table 3.10). Over all reduction in toxicity was comparatively higher in the sonicated effluents than in the unsonicated effluents except in CAT O.

The FT-IR spectrum of the effluent-loaded biomass during step-3 treatment showed lowering of the band corresponding to the amino group up to 3247.9 cm^{-1} due to –CH stretching vibrations (Table 3.12). The carboxyl chelate stretching vibrations of amide I band was observed at 1681.8 and 1691.5 cm^{-1} for unloaded and effluent-loaded biomass, respectively. An adsorption band at 1533.3 cm^{-1} of the unloaded biomass can be attributed to amide II band. This band slightly shifted to 1537.2 cm^{-1} in the effluent-loaded biomass. No shift in the absorption band at 1240.1 cm^{-1} was noticed. An intensity decrease and a slight band shifting from 1076.2 to 1078.1 cm^{-1} relates to P=O stretching and P–OH stretching vibrations. New absorption bands appearing between 700 and 900 cm^{-1} for the effluent-loaded biomass may be attributed to the aromatic –CH– bending vibrations. An over all shift and change in the intensity of several functional groups indicate their relevance in biosorption.

Mass spectrometry and NMR analysis

Comparative mass finger print values recorded (ESI-MS) of untreated CAT O and after each step of treatment showed extensive variations (Fig 3.19). Peak with higher molecular mass present in the untreated effluent disappeared after sonication and several peaks clustered in the range of 250–350 m/z (Fig. 3.19A, B). After step 2, five distinct clusters of peaks appeared in the range of 50–500 m/z. The peaks in the lower (100-400 m/z) region increased and several new peaks were observed in higher molecular mass range (600–850 m/z) (Fig. 3.19C). Maximum number of peaks disappeared after the step 3 treatment. (Fig. 3.19D).

The NMR spectra after each stage of treatment showed reduction in the intensity of chemical shifts in the region of 7–9 ppm (Fig. 3.20). This region attributes to the aromatic nature of the effluent. After sonication, a change in aliphatic components occurred as was evident by appearance of additional

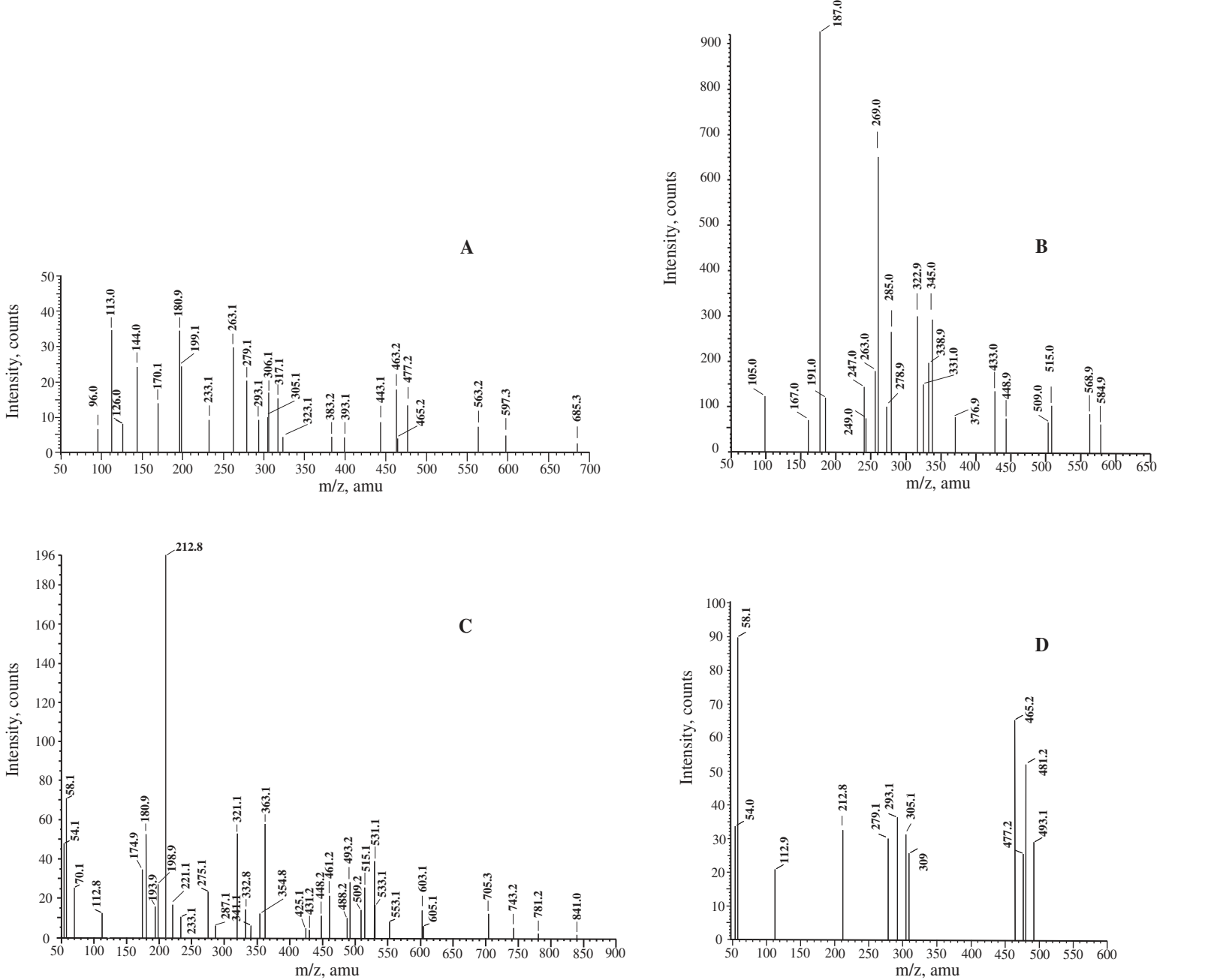


Fig. 3.19: Mass spectra finger printing of A) untreated CAT O, B) after Step 1-sonication, C) after Step 2- whole fungus treatment, D) after Step 3- biosorption.

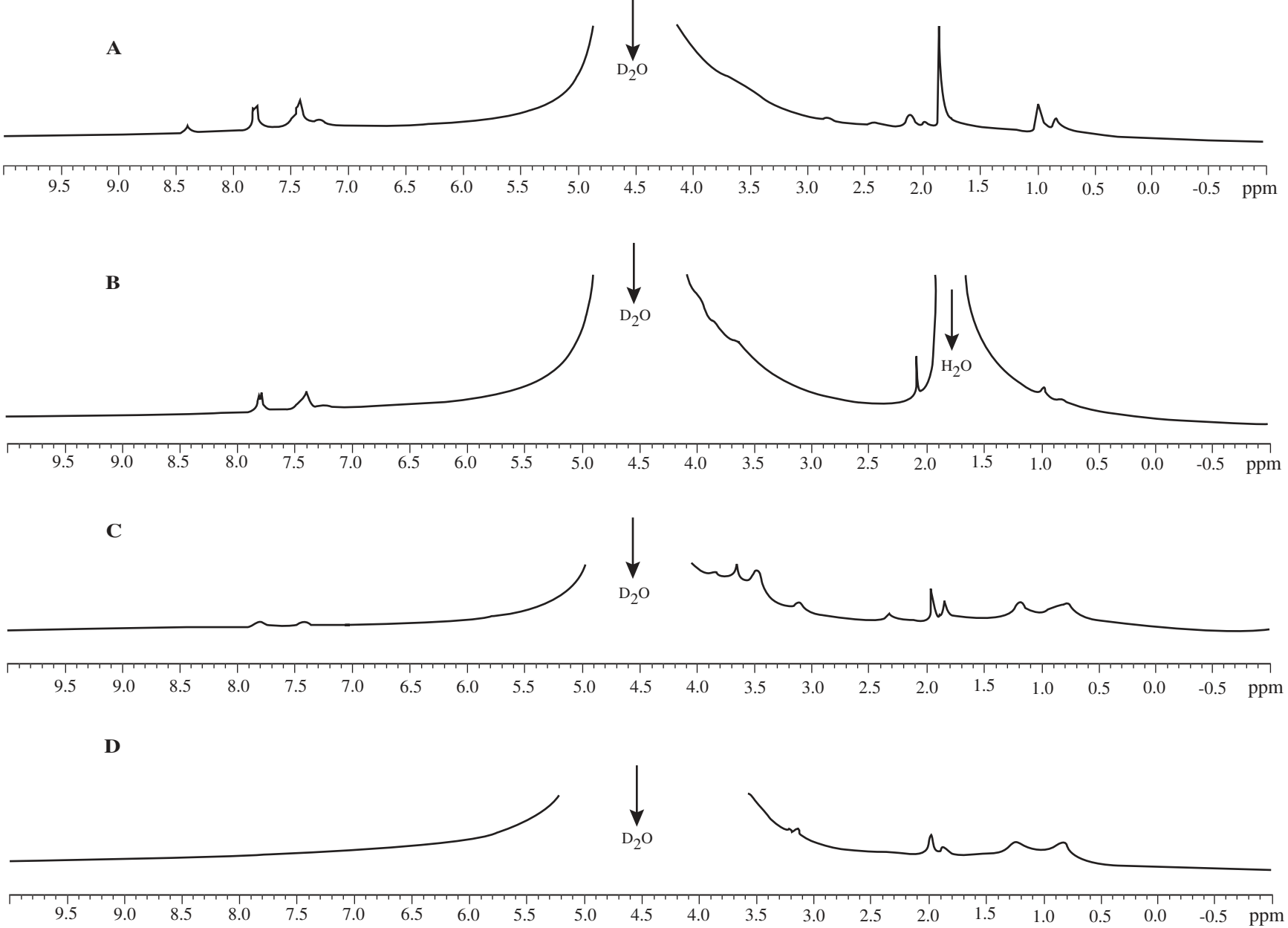


Fig. 3.20: H1 NMR spectra A) untreated CAT O; B) after Step 1-sonication; C) after Step 2- whole fungus treatment; D) after Step 3- biosorption.

chemical shifts in the range of 0–3.7 ppm. Besides, the chemical shift δ 8.4 disappeared completely (Fig. 3.20B). After step 2, the number of peaks in the range of 0–3.7 ppm increased. Of these peaks, δ 0.7 and 1.2 were contributed by the fungal metabolites as confirmed by H^1 NMR of the culture supernatant of NIOCC #2a (Fig. 3.20C). The intensity of these peaks decreased and chemical shifts at δ 7.8, 7.4 and 7.2 disappeared completely after the step 3 treatment (Fig. 3.20D).

3.4.3.2 *Fractionation of molasses-based effluents and effect of laccase*

The four molasses-based effluents were fractionated using size exclusion chromatography to estimate the molecular weight distribution of melanoidin pigments present in these effluents. Also, the effect of partially purified laccase and its mediator on the decolorization (*ex situ*) of these fractions was determined (Fig. 3.21).

The fractions of all four effluents equivalent to molecular weight 50-30 K Da were more colored than the remaining fractions. The fractions of CAT I and CAT O were decolorized better than ROF and ROR with partially purified laccase (Fig. 3.21).

In the presence of laccase mediator (HBT) the four effluents showed variable results for the different fractions of each effluent. The laccase mediator was able to enhance the decolorization up to 10-40% of the fractions of ROF and ROR ranging from molecular weight equivalent to 60-36 K Da. The molecular weight fractions equivalent to 30-45 K Da of CAT I and CAT O were decolorized by 10-60%. Along with decolorization, polymerization was also noticed in certain fractions of CAT I and CAT O (Fig. 3.21).

3.4.4 *Degradation studies and sequential remediation of Reactive Blue 4*

The aim of this study was to determine the ability of partially purified laccase from #2a to decolorize and degrade a model dye, Reactive Blue 4 (RB4). An

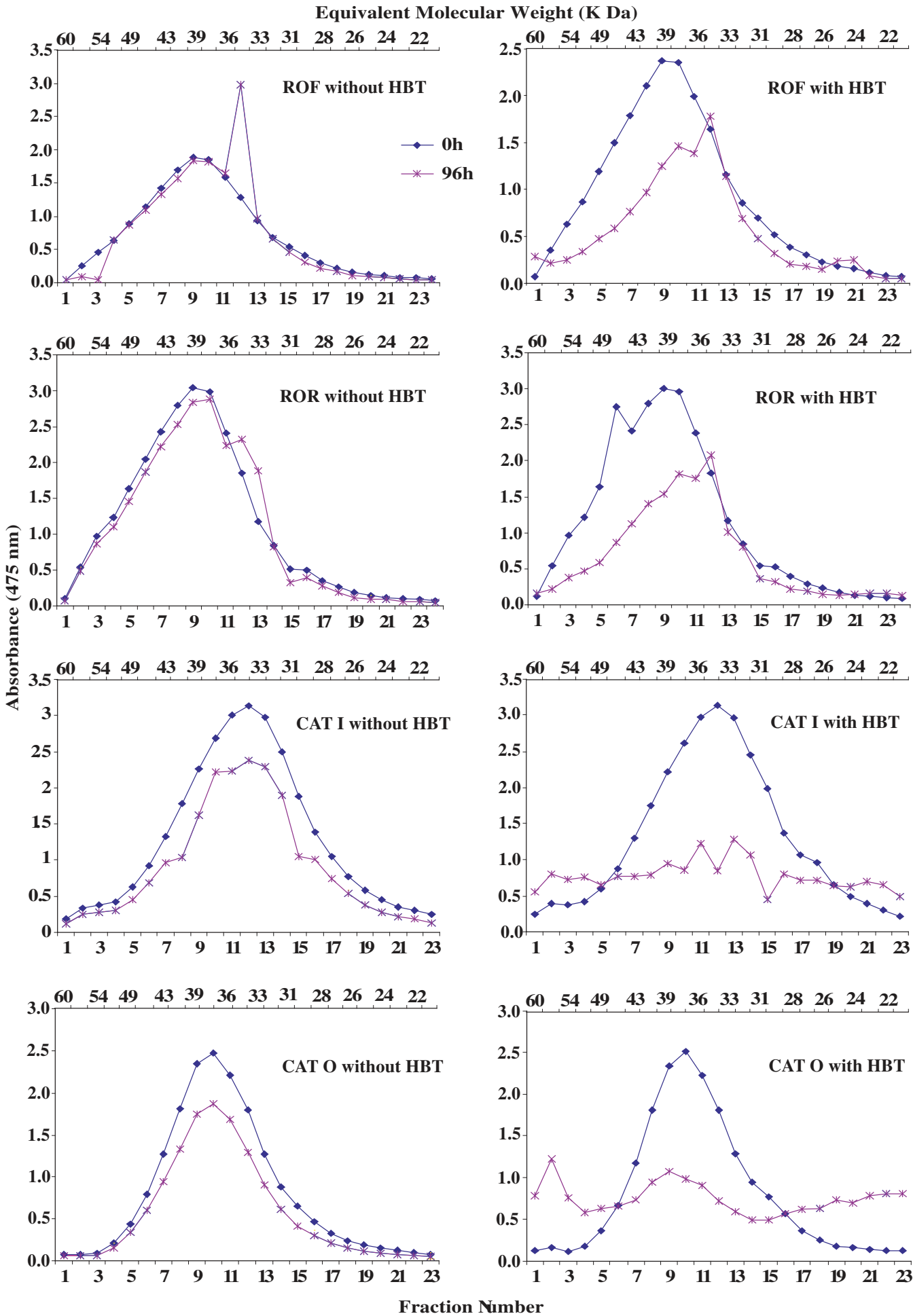


Fig. 3.21: Effect of Laccase and HBT on the Fractions of Molasses-based effluents.

attempt was made to describe the degradation products resulting due to enzymatic activity. Further, aim of this study was to develop a quicker and non-conventional approach for the decolorization and detoxification using a combination of (1) enzymatic degradation followed by (2) biosorption of the degradation products.

The molecular structure of the Reactive Blue 4 ($C_{22}O_8H_{22}N_6S_2C_{l2}$) is given in the Table 3.3. It is an anthraquinonic dye of molar mass of 637.4 and λ_{max} at 596nm.

The hyper-laccase producing isolate NIOCC #2a efficiently decolorized Reactive Blue 4 (RB4) at the concentration of 300 mg L^{-1} during qualitative plate assay (Fig. 3.22).

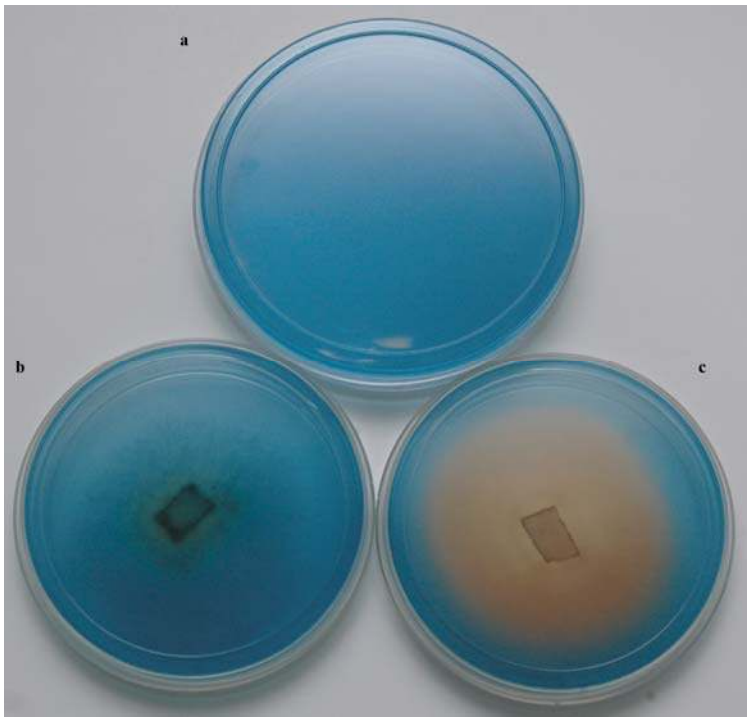


Fig. 3.22: Decolorization of Reactive Blue 4. (a) Uninoculated Plate [Control]; (b) *Pestalotiopsis* sp. (No Decolorization); (c) NIOCC #2a (positive for decolorization)

Degradation of RB4 using partially purified laccase

Visible region spectral analysis of RB4 and its degradation metabolites showed continuous decrease in the absorbance up to 12h of incubation. Although, more than 60% enzyme activity was still there, no significant spectral change was observed afterwards. About 61% of color was reduced during this period (Fig. 3.23) and the color was changed to pale brown (see the inset in Fig. 3.23). An increase of absorbance in the Ultra-violet spectrum was observed after enzymatic treatment (Fig. 3.23).

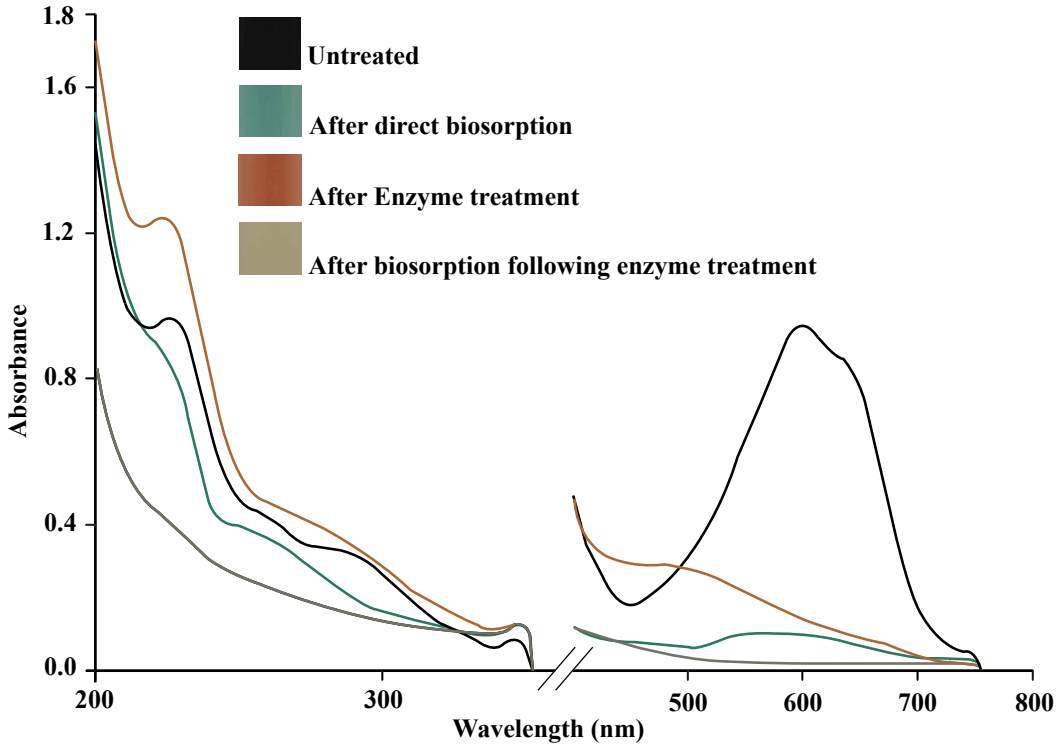


Fig. 3.23: Visible and UV spectra of the Reactive Blue 4; Untreated (———), after direct biosorption (———), after enzymatic treatment(———) and after enzymatic treatment followed by biosorption(———). The insets indicate colors of untreated dye and after each step of treatment.

The aromatic character of the dye and solution under incubation was also analyzed by the Ultra performance liquid chromatography (UPLC). The peaks at retention time 4.9 and 3.7 represented the dye content. Several new peaks appeared after the powdered dye was mixed with buffer and enzyme solution

(Fig. 3.24). These new peaks were contributed by the buffer and enzyme mixture. The peaks corresponding to the dye content were reduced considerably and new peaks appeared at the considerably lower retention times. The number of peaks at lower retention times increased gradually as the incubation period progressed (Fig. 3.24).

Characterization of degraded compounds

Electron-spray ionization mass spectra were recorded after 0h, 2h, 4h, 6h, and 12h of laccase treatment, followed by Collision induced dissociation of major molecular ion (CID) in tandem mass spectrometry to give characteristic fragment ions (Fig. 3.25). The dichloro dye, RB4 showed doubly charged characteristic quartate at m/z 354.9457, 356.9457, 358.9434 and 360.9353, in negative ion mass spectrum, indicating the presence of three halogen atoms therefore; it exists as an amine hydrochloride salt. Two hours of treatment showed the presence of quartate with reduced intensity of starting dye and additional peaks at m/z 657.2351, 521.1845, 443.1280, 385.1382, 302.1701, 249.0867, 202.0786, 205.0766, 199.2710, 69.0305 and 29.0432. However, no characteristic isotopic halogenated fragment ions appeared. Gradual disintegration of the dye solution into smaller and simpler molecules occurred as indicated by spectra at 4, 6 and 12h (Fig. 3.25 B, C, and D). The probable degradation products were identified and listed in Fig. 3.26. No spectral changes of RB4 were detected in the control samples.

Biosorption of the untreated and laccase treated dye solution

Laccase treated dye solution was further subjected to sorption using powdered biomass of NIOCC #2a. The sorption equilibrium was reached within 10min and this resulted in further decrease in color up to 93%. Also, there was a significant decrease in the absorbance in the UV region (Fig. 3.23).

In the same manner, initial dye solution (without any enzymatic treatment) was also subjected to biosorption. In this case, equilibrium was established in

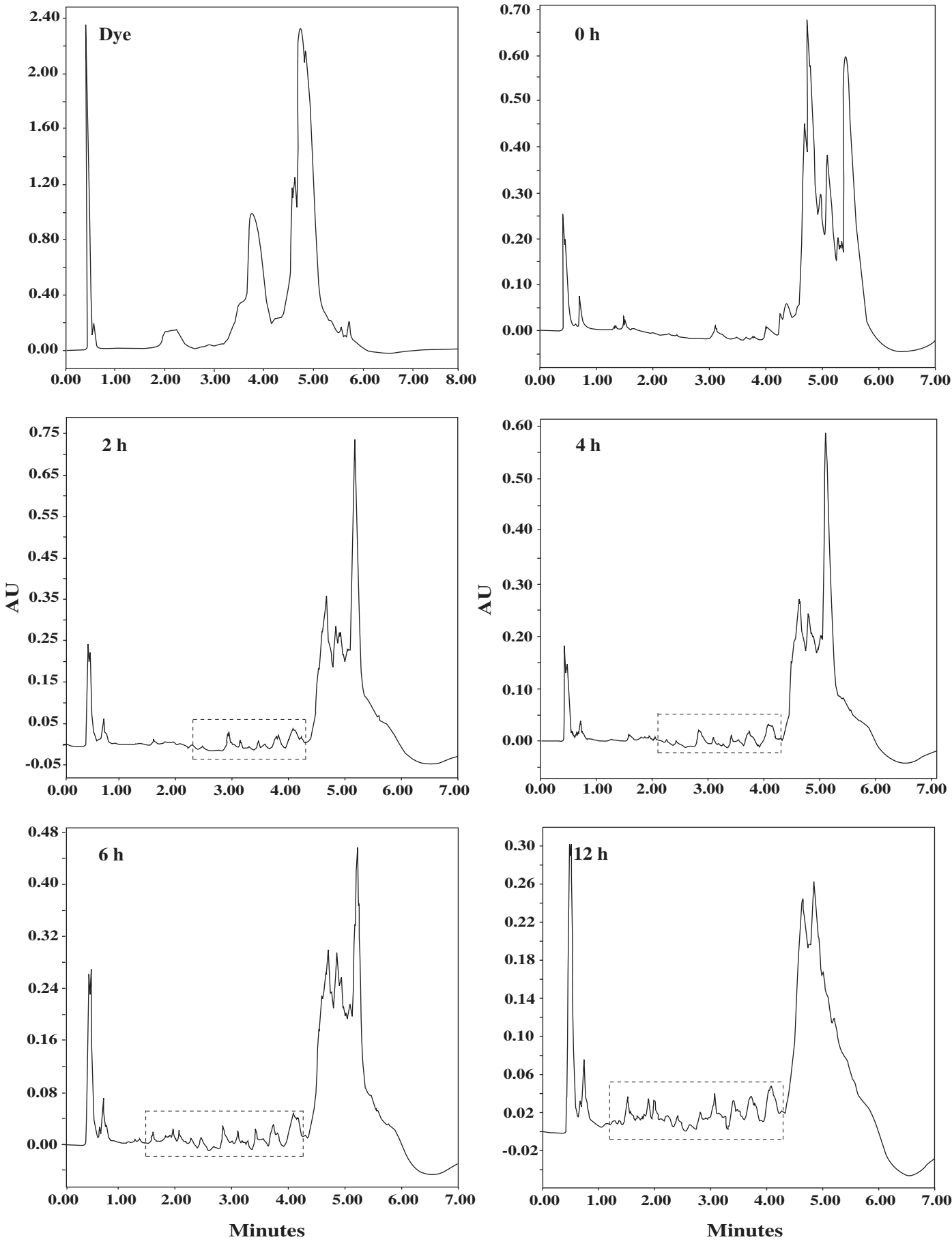


Fig. 3.24: The ultra performance liquid chromatogram of untreated Reactive blue 4 and during different time periods of treatment. Increasing nos. of peaks at lower retention time are marked with dotted box.

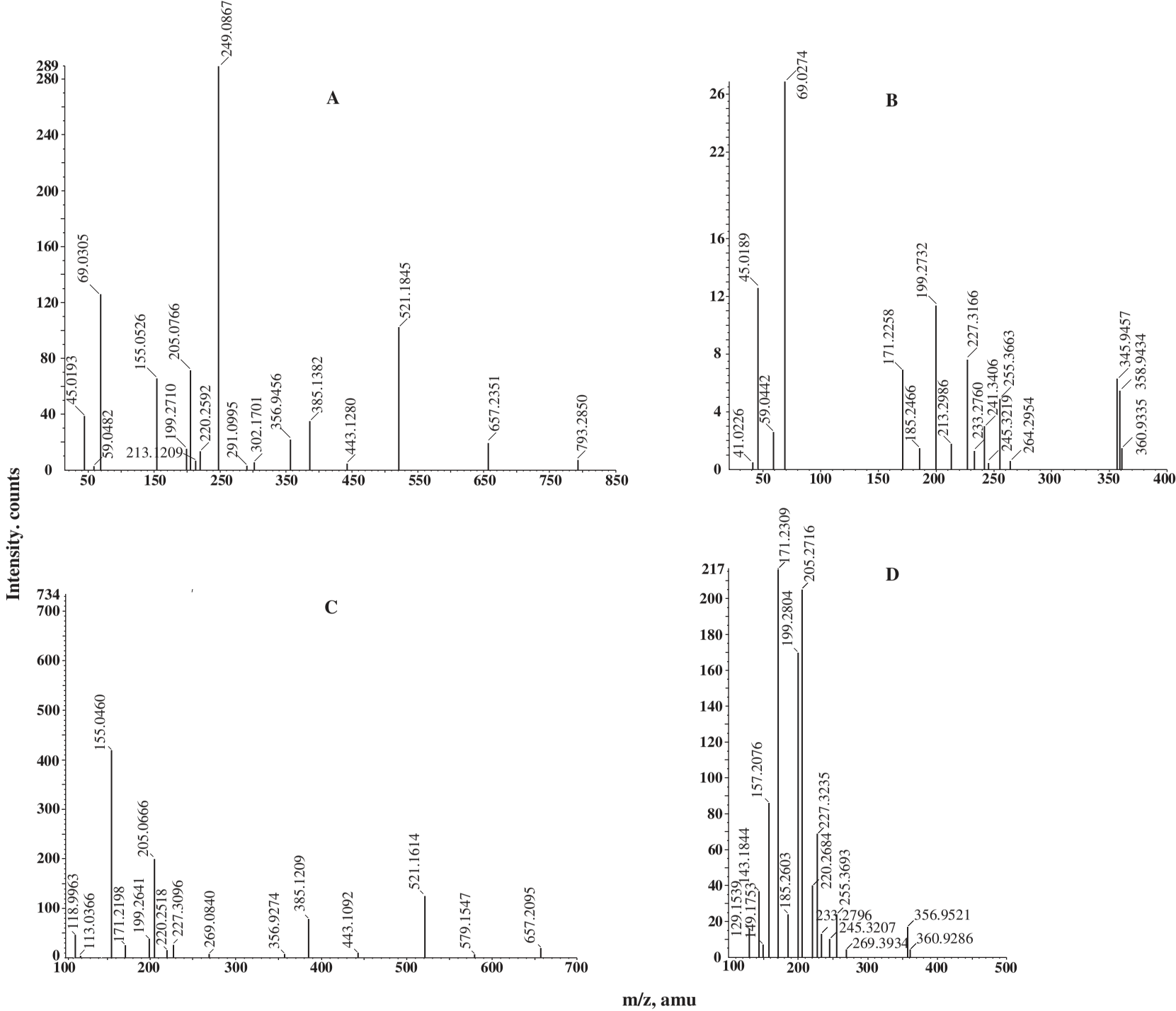


Fig. 3.25: Mass spectra finger printing of laccase treated RB4 after time intervals A) 2 h, B) 4h, C) 6h, D) 12h.

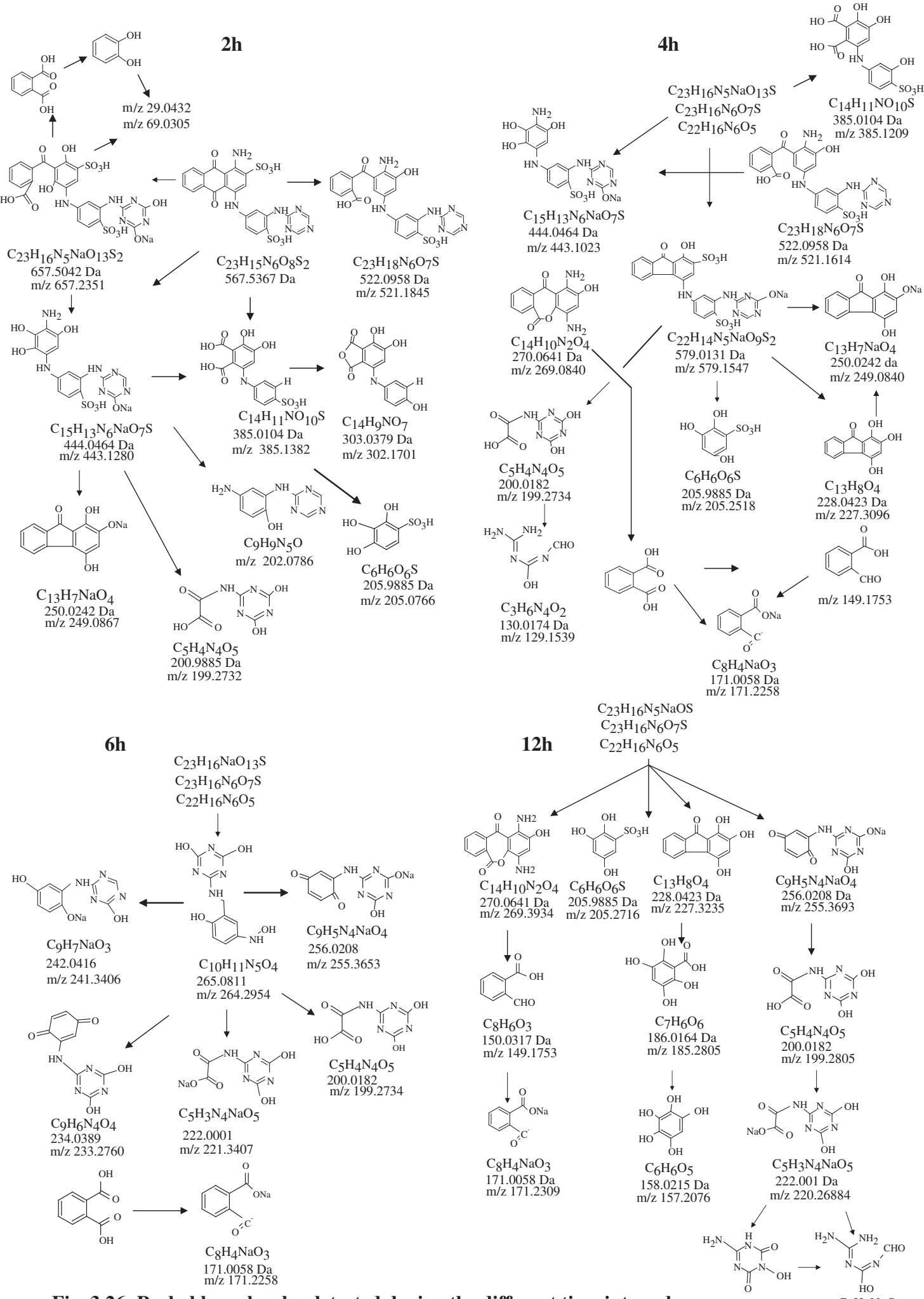


Fig. 3.26: Probable molecules detected during the different time intervals of enzymatic treatment

about 20min and 84% reduction in color occurred, but there was not much change in the pattern of UV-spectrum (Fig. 3.23).

Toxicity of RB4 before and after treatment

To evaluate the toxicity changes the viability of *Artemia* napulli was assessed. LC_{50} values for untreated and treated samples as given in the Table 3.13, indicated more than two-fold decrease in toxicity after the two step treatment of the dye. There was no significant change in toxicity against *Artemia* larvae in either of the single step treatments.

The toxicity to plants was assessed by the seed germination experiment. The germination index for the dye solution directly subjected for sorption was not significantly improved. There was decrease in phyto-toxicity in the enzyme treated dye solution which improved considerably after it was followed by mycelial sorption (Table 3.13).

Table 3.13: Toxicity analysis of untreated and treated Reactive blue 4

Toxicity tests		Control (D/W)	Untreated	Direct Sorption	Enzyme treated	Enzyme treated followed by Sorption
Artemia test (24h-LC_{50})[#]		--	48(43-95)[a]	57(48-68)[a]	51(45-59)[a]	99(79-135)[b]
Phytotoxicity (48h)[$]	**Germination index (%)**	--	57.6(0.4)[a]	63.9(1.7)[ab]	66.9(1.7)[b]	91.4(4.5)[c]
	Dry weght (root)	2.6(0.1)[a]	0.6(0.03)[b]	1.0(0.02)[c]	1.0(0.0)[c]	1.8(0.04)[d]

[#] LC_{50} values are in ul ml^{-1}. Upper and lower confidence limits are within brackets.
[$] Standard deviation values are within brackets.
Different letters indicate significant differences between the methods of treatment for a particular test (Tukey's test at the level ≤0.05%).

3.5 Discussion

3.5.1 *Effect of nutrient nitrogen on LDE production and effluent decolorization*

In the present work, role of nutrient nitrogen sources present in growth media on production of lignin-degrading enzymes namely, laccase, lignin peroxidase and manganese peroxidase as well as on the decolorization of industrial effluents like black liquor, molasses spent wash and textile mill effluents was studied using the basidiomyceyous fungus NIOCC #2a. The results suggested that well defined organic N sources such as glutamic acid and glycine were better than beef extract and corn steep liquor for laccase production (Table 3.4). Glutamic acid and glycine being amino acids not only serve as a nitrogen source but also as a readily available source of carbon. This carbon in addition to the carbon supplied by the fructose probably aided in biomass build-up which in turn positively affected laccase production. Among, the two amino acids, glutamic acid supplies more carbon than glycine for a constant amount of nitrogen. This is reflected in the difference in the amount of biomass obtained.

Mansur et al. (1997) showed that fructose induced 1100-fold increase in laccase production in the basidimycetous fungus CECT 20197. Stajic et al. (2006) demonstrated the effect of inorganic and organic nitrogen sources on laccase production in different species of *Pleurotus*. Elisashvilli et al. (2001) observed highest laccase activity in the medium with ammonium sulfate as the N source in *Cerrena unicolor* IBB 62.

Textile effluent B was less turbid than the other effluents and had a pH of 2.5, the pH at which most of the lignin-degrading enzymes show their optimum activity (Baldrian, 2006). Also, it had lesser color units than TEA (Table 3.2) Textile mill effluent A with a pH of 8.9 and more color units showed less decolorization since the lignin-degrading enzymes show negligible activity at alkaline pH (Baldrin, 2006). Although MnP and LiP production was enhanced in the presence of black liquor, this did not result in its decolorization (Table 3.4).

On the other hand, mutants of *Phanerochaete chrysosporium*, a well known lignin-degrading white-rot fungus that lacked the ability to produce LiP but produced MnP, showed about 80% decolorization of bleach plant effluent, suggesting that MnPs play an important role in decolorization of bleach plant effluent (Michel et al., 1991). Black liquor enhanced the growth of the fungus, whereas, TEB inhibited its growth. In spite of this, TEB was decolorized up to 70-90%, whereas, black liquor was decolorized only up to 5%. Maximum laccase was produced in the presence of TEB and similarly TEB was decolorized to the maximum extent (Table 3.4), Suggesting that laccase play a key role in decolorization of this particular textile effluent. The important role of laccases in textile dye decolorization has been reported (Wong and Yu, 1999).

Since, the color units of the effluents varied greatly, this might have resulted in variable decolorization percentages. However, this factor was corrected in an *ex situ* experiment but results were still variable (Fig. 3.5).

These results led us to hypothesize that the N source in the medium regulated the production of lignin-degrading enzymes and this in turn affected its decolorization ability. However, no direct correlation between enzyme units and percentage declorization was observed in any of the N sources. Therefore, it appears that besides the lignin-degrading enzymes and the source of N, the composition of the effluents plays an equally important role in decolorization.

Differential regulation of laccase-encoding genes in response to culture conditions has been documented in the terrestrial fungus *Phanerochaete chrysosporium* (Dittmer et al., 1997). The effect of the type of N source as well as type of effluent on LDE production and decolorization has been demonstrated in the present study.

The results indicated that the type of nitrogen source used, not only influence the amount and type of lignin-degrading enzymes produced but also has an effect on the decolorization of these effluents. The amount of extracellular peroxidases increased by several fold in the presence of effluents whereas in their

absence they were of negligible quantity. Some of the effluents had an inhibitory effect on laccase production. Decolorization of these effluents by the concentrated culture filtrate obtained from media containing different nitrogen sources further proved the importance of the type of nitrogen source in decolorization of colored industrial effluents.

3.5.2 *Marine-derived fungi for remediation of textile mill effluents*

The decolorization and detoxification of two raw textile mill effluents with extreme variations in their pH and dye compositions (Table. 3.2, 3.3) was attempted. The four marine-derived fungi, two each belonging to ascomycetes and basidiomycetes decolorized textile mill effluent A (TEA) by 30-60% (Table 3.6) and TEB by 33-80% (Table 3.7) used at 20-90% concentrations. This was accompanied by two to three-fold reduction in toxicity (Table 3.6, 3.7; Fig. 3.9, 3.10, 3.11) and 70-80% reduction in chemical oxygen demand and total phenolics (Table 3.6, 3.7; Fig. 3.0, 3.10).

Most of the industrial effluents contain various inorganic chemicals such as sulfides, sulfates, chlorides and carbonates (Bartlett, 1971) and such effluents with high salt contents are required to be diluted several fold for any biotreatment. In the present study, high concentrations of effluents (20–90%) could be decolorized (Table 3.6, 3.7; Fig. 3.9, 3.10), thus minimizing dilutions. The fungi used in this study showed growth, laccase production and decolorization in media prepared with seawater of 15–17 psu salinity. Decolorization and detoxification in seawater medium indicate that they can be used for effluents containing high salt content and varying pH. A few fungi belonging to the class zygomycetes have been demonstrated to decolorize and detoxify simulated textile wastewaters of varying composition characterized by high concentrations of salts and dyes by bioadsorption (Prigione et al. 2008a, b). Bioremediation by dye sorption has been reported in *Aspergillus* sp. (Fu and Viraraghavan, 2002; Corso and de Almeida,

2009) and by laccase and other lignin-degrading enzymes in basidiomycetes (Wesenberg et al., 2002; Faraco et al., 2009).

Dye decolorization is dependent on their structure, pH, concentration of dyes and enzyme (Wong and Yu, 1999). Anthraquinonic dyes that are substrates for laccase are easily degraded whereas non-substrates like azo and indigo dyes are removed to a lesser extent. In the present studies, decolorization of TEB which contained a mixture of reactive dyes (Table 3.2) was much higher than TEA which contained only one azo dye. Azo dyes are usually difficult to remove in wastewater (Riu et al., 1997). However, a number of white-rot fungi have been reported to breakdown individual azo dyes (Nyanhongo et al., 2002). Knapp and Newby, (1999) were the first to report the decolorization of an effluent of the chemical industry containing an azo-chromophore by white-rot fungi. Wesenberg et al., (2002) have reported about 22% color removal of textile effluent (used at 25% concentration) containing azo dyes by day 9 with a white-rot fungus, *Clitocybula dusenii*. Isolates in the present study, removed 27–57% of color from 50% TEA containing azo dye-20 after 6 days (Table 3.6; Fig. 3.9). Altering the pH of TEA from alkaline to acidic did not increase decolorization indicating that pH alone was not the limiting factor.

Generally each kind of effluent is decolorized by a specific fungus (Faraco et al., 2009) or consortium (Senan and Abraham, 2004). Physical adsorption and enzymatic degradation are the mechanisms for color removal by fungi (Ali et al., 2008). In many cases, adsorption of dye to the fungal surface is the primary mechanism of decolorization (Zümriye and Karabayir, 2008; Prigione et al. 2008a, b). In the present study, adsorption of color from TEA and TEB by fungal biomass was two to threefold higher in the ascomycetes than in the basidiomycetes (Table 3.5). Initially higher color removal by fungal biomass was noticed in ascomycetes (NIOCC # 16V and # C3) than in basidiomycetes (see Figs. 3.7 d, 3.15) indicating adsorption as the primary mechanism of color removal in ascomycetes.

Enzymatic degradation plays a primary role in biodegradation of colored effluents in basidiomycetes (Wesenberg et al., 2003; Faraco et al., 2009). The following points suggest that decolorization of dye containing effluents in the basidiomycetes appeared to be primarily laccase-mediated. (1) Decolorization of TEA by the culture supernatants (ex situ) increased in the presence of laccase mediators in the basidiomycetes NIOCC #2a and #15V whereas in the ascomycetes #16V and #C3 with lower laccase activity, this effect was not evident (see Fig. 3.14). (2) Ex situ experiment further demonstrated that laccase from NIOCC #2a was as efficient as the commercial laccase from *Trametes versicolor* in decolorization of TEA and TEB (see Table 3.8). (3) Increased amounts of laccase from NIOCC #2a and commercial laccase from *Trametes versicolor*, showed corresponding increase in decolorization of both the effluents (see Table 3.8). (4) Reusing the fungal biomass from NIOCC #2a and #15V (basidiomycetes) showed decolorization in three subsequent cycles indicating involvement of laccase whereas with ascomycetes with much lower laccase titer showed a substantial reduction in decolorizing capacity in the second cycle itself (see Fig. 3.15). (5) Laccase production in the presence of TEB was high and it also got decolorized to a greater extent than TEA. Faraco et al. (2009) reported laccase to be solely responsible for decolorization of model dye-containing industrial wastewaters by *Pleurotus ostreatus*. Present studies also indicate that laccase appears to be involved in decolorization of textile effluents by basidiomycetes.

A direct correlation between lignin-degrading enzyme production and industrial effluent decolorization was reported by Wesenberg et al., (2002). By *ex situ* studies Wong and Yu, (1999) demonstrated that increased decolorization capacity of laccase from *Trametes versicolor* involved decolorization of non-substrate dyes in effluents via substrate dyes that act as mediators. *Ex situ* experiments established a linear relationship (r value $\geq$ 0.8) between the laccase titer and decolorization in the present studies. On the other hand, studies involving

the whole cultures (*in situ*) did not show direct correlation between laccase titer and extent of decolorization consistently due to the involvement of several other factors such as nature of effluent, pH, adsorption capacity of the fungi, presence of natural mediators in the culture supernatants and presence of other oxidative enzymes which may trigger cascade reactions. Only NIOCC #2a produced MnP in low quantity. However, all the cultures showed glucose oxidase activity to a varying degree in the culture medium. Glucose oxidase may play role in generating H_2O_2 which by Fenton type of reaction can produce highly reactive hydroxyl radicals (Henriksson et al., 2000). These hydroxyl radicals can participate in methoxylation/hydroxylation of many aromatic compounds in converting non-phenolic compounds to phenolic ones. These in turn are easily oxidized by laccases or peroxidases (Hilden et al., 2000). *In situ* decolorization by my cultures appears to be a collective action of laccase and other cascading reactions besides adsorption.

Reports of decolorization of dye-containing textile effluents using ascomycetes or hyphomycetes are very few. *Aspergillus fumigatus* XC6 is one such fungus reported to decolorize dye industry effluent although laccase production in this fungus was not reported (Jin et al., 2007). A laccase-producing ascomycete, *Pestalotiopsis* sp. (Hao et al., 2007) was reported to decolorize an azo dye and another ascomycete *Myceliophthora thermophila* was reported to decolorize several synthetic dyes by the action of laccase (Kunamneni et al., 2008) but these were not tested for decolorization of dye-containing raw effluents. The present study demonstrates decolorization and detoxification of dye-containing raw textile effluents by marine ascomycetes.

Reduction in toxicity is one of the important criteria to be considered while developing a process for decolorization of dye wastewaters. A substantial reduction in toxicity was observed as evidenced by LC_{50} dosage values, total phenolics and COD in treated effluents (Table 3.6, 3.7; Fig. 3.9, 310, 3.11). There was a reduction in toxicity, COD and total phenolics by different fungi when

grown in the presence of TEB as high as 90% (Table. 3.7; Fig. 3.10). Mass spectrometric analyses also indicated a distinct change in the spectra of untreated and fungus-treated effluents suggesting degradation of effluent components. Laccases were shown to be responsible for reduction in toxicity and COD of model textile effluents by *Pleurotus ostreatus* (Faraco et al., 2009). In the present study, both ascomycetes and basidiomycetes were able to reduce toxicity irrespective of laccase titer (Fig 3.11).

Although bioadsorption is an efficient method of color removal from effluents (Prigione et al., 2008a, b), as observed in the present study also, it only transfers color from liquid phase to the solid phase. Therefore, the problem of final color removal persists. Based on the current studies a bioremediation process is suggested involving these two groups of fungi which include instant color removal by adsorption using ascomycetes followed by treatment with laccase from basidiomycetes to remove the adsorbed color from the fungal biomass. An added advantage in growing the ascomycetes in effluents is the reduction in COD, total phenolics and toxicity in contrast to use of inert material for adsorption (Rodríguez-Couto et al., 2009). In the present study also it has been demonstrated that pre-grown ascomycetous biomass and laccase from a basidiomycete can be used simultaneously to enhance and speed up decolorization of raw textile effluents. The fungal biomass is able to decolorize and detoxify highly concentrated effluent (50–90%) and therefore the proposed method has high applicability at industrial scale. Although the cultures did not grow in plain effluents without added nitrogen and carbon source, they showed better growth in the medium containing 50 and 90% effluent than in the control medium. One of the laccases of the culture #2a has been characterized in detail and was found to be highly thermo- and alkaline-stable besides being halo- and metal tolerant (D'souza-Ticlo et al., 2009). These points further favor their use on an industrial scale.

3.5.3 *Sequential remediation of molasses-based raw effluents*

Decolorization and detoxification of recalcitrant molasses-based raw effluents was attempted using a three step combinatorial technology. Sonication in the first step removed the foul odor and turbidity and increased their accessibility to enzymatic degradation by ligninolytic fungus. Biosorption using heat killed wet biomass of the same fungus decolorized the effluent further (Fig. 3.16).

Low-frequency ultrasound treatment (sonication) alone cannot mineralize the pollutants and the time-scale and energy requirement makes it cost prohibitive and unfeasible. However, wastewater treatment using sonication in combination with other conventional oxidation methods is recommended (Sangave et al., 2007; Sangave and Pandit, 2006). Pre-treatment of distillery waste water with sonication has been shown to increase the biodegradability during conventional aerobic oxidation (Sangave and Pandit, 2006) and reduce the toxicity of the original effluent (Gonze et al., 1999). In the present study, sonication alone was not effective in reducing color, COD, total phenolics and toxicity from molasses-based effluents.

Whole culture of the ligninolytic fungus NIOCC #2a was chosen in the step 2 treatment for the following reasons; (1) molasses waste waters contain reducing sugars which can easily be utilized by the live fungus for its growth and enzyme production, (2) incubation period of 9 days in the step 2 was to provide maximum allowance for enzymatic degradation of the effluents. Although decolorization and detoxification occurred with unsonicated effluents, the process of remediation improved significantly when sonicated effluents were introduced during whole culture treatment (Table 3.10, 3.11).

Of the three LDEs, only laccase correlated with decolorization (Fig. 3.17) during the step-2. However, these results do not confirm the involvement of laccase in *in situ* decolorization, as stress conditions such as pollutants are known to induce laccase production. Therefore, an *ex situ* study was conducted using partially purified laccase for decolorization of these effluents. An overall 16–18%

decolorization was achieved in 12 h. Involvement of several other factors besides laccase in *in situ* decolorization cannot be ruled out.

González et al. (2008) reported a direct correlation between decolorization of melanoidin fractions and molasses waste water with that of laccase production in *Trametes* sp. I-62. In an earlier study using another marine-derived fungus NIOCC #312, decolorization of molasses spent wash was reported to be directly correlated with glucose oxidase production (Raghukumar et al., 2004). Intracellular sugar oxidase enzymes were considered to play a major role in decolorization of molasses spent wash in *Coriolus* sp. No. 20 (Mohana et al., 2009). Thus, it appears that the enzyme system responsible for decolorization of specific effluent varies from fungus to fungus.

Although laccase production was repressed immediately after addition of the effluents (day 0) the fungus overcame this inhibitory effect and its production increased by several folds by day 3 itself (Fig. 3.17). These nutrient-containing effluents might prolong the primary phase of the fungal growth which would ultimately delay laccase production. On the contrary, induction of laccase by molasses spent wash and melanoidin fractions in several white-rot fungi has been reported (D'Souza et al., 2006; González et al., 2008). As hypothesized by González et al., (2008) copper is released during breakdown of melanoidins which can induce laccase production. D'Souza et al. (2006) have shown induction of laccase by several thousand folds in NIOCC #2a by copper. Aromatic monomers, which are some of the breakdown products of molasses, were also shown to induce laccase production in NIOCC #2a (D'Souza et al., 2006) and in *Trametes* sp. 1–62 (Terrón et al., 2004). In one of my preliminary studies, activity of partially purified laccase of NIOCC #2a increased in the presence of synthetic melanoidin pigments. Thus besides increased production, induction in laccase activity per se was observed in this fungus in the presence of the molasses-based effluents. The above results confirm that laccases are induced in fungi under stress conditions. Further, sonication also might exert a positive influence on the

oxygen-dependent laccase activity as it helps in degassing and removal of H_2S from the effluents. Fungal enzymes that are not inhibited in the presence of industrial effluents may make them good candidates for bioremediation.

In the present study it was noticed that after addition of the effluents, the fungal mycelia turned dark brown in color. In the whole-fungal culture treatment, biosorption by the live mycelia would also be playing a role in removal of color from the culture broth to a certain extent. Fourier transform infra-red analysis of all the biological sorbent materials show intense absorption bands around 3500–3000 cm^{-1}, representing stretching vibrations of hydroxyl and/or amino groups (Bayramoğlu and Arica, 2007). The shift in these bands in our study may be attributed to sorption of the effluent contents (Table 3.12). The extra-cellular ligninolytic enzymes would be simultaneously degrading and mineralizing the colored compounds (Park et al., 2007). The changes observed in the spectra of unloaded biomass and different stages of loaded biomass may indicate that several other functional groups are also responsible for biosorption of the effluent components (Fig. 3.18, Table 3.12).

In the step-3, sorption of residual color occurred only when heat-inactivated wet biomass was used. Use of lyophilized and powdered biomass may have several advantages for the sorption such as increase of surface area and reduced volume to handle, but it was not effective in this particular case. This may have happened due to release of cellular contents during the drying process of the mycelia. Tigini et al. (2010) also observed an increase in COD in several textile effluents using similar methods.

The use of dead biomass for sorption has several advantages, as they do not require nutrients for growth, will not be inhibited by the toxic effluents and there is no fear of their pathogenicity or toxins (Prigione et al., 2008a, b). Heat-inactivation of biomass increases hydrophilicity of the surface (Bayramoğlu and Arica, 2007). Autoclaved fungal biomass (2 g wet weight) of *Aspergillus oryzae* strain Y-2-32, removed 65% of melanoidin pigments within 4 days by adsorption

(Ohmomo et al., 1988). Biosorption potential of microbial exopolymeric substances (EPS) is well known (Gadd, 2009). Basidiomycetous fungi are reported to produce large amounts of EPS (Smith et al., 2002). These polymeric substances form a sheath around fungal hyphae. They are highly hydrophilic and become gel-like by absorbing water (Bes et al., 1987). Additionally, microbial biomass acts as an ion exchanger by virtue of reactive groups available on the cell surfaces (Gadd, 2009). Mucoraceous fungi rich in chitosan are a good source of biosorption of dyes (Prigione et al., 2008a. b), similarly the white-rot fungi with their high EPS content might offer a novel source of biosorption of industrial effluents.

The basidiomycetous fungus NIOCC #2a produced 2.3 g of EPS L^{-1} of the LN medium. It showed CNS (carbon:nitrogen:sulphur) ratio of 4.5:0.76:10, and therefore appeared to be sulfated polysaccharide (D'Souza et al., 2006). The EPS forms aggregates around fungal mycelium and stains with alcian blue (Raghukumar et al., 2006). The heat-inactivated mycelia of NIOCC #2a also stained with alcian blue indicating that EPS was not affected by autoclaving. Thus, it might play a role in biosorption of melanoidin pigments in the present study. Bayramoğlu and Arica, (2007) reported biosorption of textile dyes by the white-rot fungus *Trametes versicolor*, although the role of EPS was not mentioned.

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 (Philipps & Vincenzini, 1998), marine fungi should be screened for EPS production.

In order to support the above data of decolorization and detoxification of raw hybrid technology, a spectrometric analysis (ESI MS) was carried out. For this purpose CAT O was selected as a representative effluent (Fig 3.19). The

disappearance of higher molecular mass peaks after sonication and appearance of new peaks in the lower region after step-2 confirm degradation (Fig. 3.19 A-C). This leads to the hypothesis that sonication hydro-mechanically shears the melanoidin pigment particles and makes them available for enzymatic degradation. This was evident by the increase in the number of peaks in the lower region. Also, formation of several new peaks in higher molecular mass range indicates simultaneous dimerization/polymerization of the components in the effluent (Fig. 3.19 C). The disappearance of the peaks after step-3 indicates biosorption of the degraded components (Fig. 3.19 D).

Aromatic compounds are some of the major contributors of toxicity (Raghukumar et al., 2004). According to NMR data, a substantial decrease in aromatic character of the treated effluent was observed. Sonication reduced the absorbance of the effluents in the UV region which may be attributed to a reduction in aromatic compounds as suggested by Beltran et al. (2000). This was further confirmed by disappearance of chemical shifts in the H^1 NMR spectra (Fig. 3.20).

Molasses-based effluents containing high COD and color are some of the most difficult effluents to treat. Each method has its own advantages and disadvantages and therefore a combination of different techniques would help in resolving this problem. Low frequency sonication for a short duration is an eco-friendly and cost-effective approach. Sonication combined with cellulase treatment (Sangave and Pandit, 2006), sonication/ozone treatment followed by mixed microbial consortium to treat distillery waste water has been tried with some success (Sangave et al., 2007). Decolorization and detoxification of molasses spent wash with lignin- degrading fungi has been reported (Miyata et al., 2000; D'Souza- Ticlo et al., 2006; Raghukumar et al., 2006; Thakker et al., 2006). Although removal of metals and decolorization of dye-containing effluents through biosorption has been extremely successful (Gadd, 2009; Prigione et al.,

2008), its use in treatment of molasses-based effluents has not been reported widely.

Sonication of the molasses-based effluents in the first step removed the foul odor and turbidity. It increased their accessibility to enzymatic degradation by the ligninolytic fungus in the next step. This was evident from enhanced reduction in color, COD, total phenolics and toxicity. Biosorption using heat-killed wet biomass of the same fungus decolorized the effluent further. Such a hybrid technology combining sonication followed by whole-culture treatment for decolorization and detoxification and subsequent biosorption of the residual color eliminates use of chemicals as is generally practiced in advanced oxidation processes. The successful application of the same three step-processes for treatment of four different effluents strengthens present findings. Besides, it will offer a huge saving in precious fresh water used for diluting the effluent before its release for meeting the zero discharge regulation of pollution control boards. Thus, a three step combinatorial technology for decolorization and detoxification of recalcitrant molasses-based pollutants is recommended.

3.5.4 *Fractionation of molasses-based effluents and effect of laccase*

The molecular weight, structure and elemental composition of melanoidins is strongly influenced by the ratio of initial components and type of reactions as well as reaction conditions such as temperature, reaction time, pH etc. (Chandra et al., 2009). This is one of the main reason because of which molasses-based effluents behave differently for the enzymatic decolorization. In the present study it was observed that although molecular weight distribution of the fractions of four effluents was almost same, CAT I and CAT O showed better decolorization with partially purified laccase. Laccase mediators were able to enhance this decolorization in certain fractions of the four effluents (Fig. 3.21). Again, the positive effect of HBT was variable for the different effluents. Higher molecular weight fractions of ROF and ROR were decolorized well whereas; color removal

in medium range fractions was more in CAT I and CAT O (Fig. 3.21). In a study, smaller molecular weight fractions of molasses waste water (MSW) from the stillage of an alcohol factory were decolorized rapidly while the larger molecular weight fractions were hardly decolorized by acetogenic bacteria. But in case of anaerobically treated MSW, the same strain was able to remove color of all the molecular weight fractions. The decolorization activity of this strain was suggested to be sugar oxidase dependent (Sirianuntapiboon et al., 2004a). In another study only the larger molecular weight fractions of melanoidin pigment solution were decolorized by a *Citeromyces* sp (Sirianuntapiboon et al., 2004b). Dahiya et al. (2001) also reported that the larger molecular weight fractions of melanoidin were decolorized effectively than smaller molecular weight fractions using white-rot fungus *P. Chrysosporium*.

Also, during the present study certain fractions of the effluents polymerized in the presence of HBT (Fig. 3.21). This indicates that because of the polymerization of certain melanoidin pigments during enzymatic treatment, effective color removal is negated. Strategy to remove these components before enzymatic treatment may be helpful for getting better results.

3.5.5 *Enzymatic degradation and sequential remediation of RB4*

Anthraquinone dyes belong to the most frequently used group of synthetic colorants in dying and textile industry. Synthetic dyes used are recalcitrant to remove by conventional wastewater treatments such as adsorption, photo-oxidation, coagulation, flocculation, photo-degradation and chemical degradation. Moreover, the main disadvantage related with chemical methods (as Fenton reagents oxidation, ozonations, photochemical degradations and sodium hypoclorite addition) is the formation of toxic compounds resulting from the cleavage of the chromophoric groups (Robinson et al., 2001b). Reactive textile dyes are highly water-soluble anionic dyes.

The present study was focused on the degradation of a highly water-soluble anionic dye reactive blue 4 (RB4) as an investigation model. This is an anthroquinone-based dye with dichlorotriazine group as reactive site. This dye has been well characterized by Epolito et al., (2005). The color removal by electro-chemical oxidation, photo-Fenton process and wet peroxide oxidation of this dye has already been investigated (Carneiro et al., 2005; Carneiro et al., 2007; Gözmen et al., 2009). In the present study, attention was paid to metabolite identification, capacity of the enzyme for mineralization and demonstrating a sequential remediation process.

In the first step of treatment, the dye was subjected to the enzymatic degradation using partially purified laccase from NIOCC #2a. The dye was decolorized to the maximum in 12 h (Fig. 3.23) after which no change in the spectral pattern was observed although more than 60% activity of the enzyme was still there, indicating degradation products were not substrates of enzyme. The increase of absorbance in the UV spectrum during this period (Fig. 3.23) suggests the gain of smaller phenolic compounds resulting from the degradation of parent dye molecule. The ultra performance liquid chromatography elution profile of the dye considerably changed during incubation period supporting change in the aromatic character of the parent dye (Fig 3.24). The number of peaks at lower retention times increased gradually as the incubation period progressed (Fig. 3.24), indicating the formation of more polar oxidation products. The enzymatic degradation resulted into 61% of color removal. An anthraquinonic dye RBBR was decolorized by 80% using purified laccase from *Trametes* sp. (Yang et al., 2009). However, these results cannot be compared with present study as the initial dye concentration and method of color measurement was different.

Identification of probable degradation products was carried out by the aid of high resolution Electrospray Ionization mass spectrometric (ESI-MS). This analysis is being used as the most efficient tool for the analysis of reaction mixture resulting from such chemical/enzymatic transformation. Comparison of

the spectra of the initial dye solutions and the degraded solutions after enzymatic treatment can help us to understand the biodegradation process. The fingerprint of the reaction products (Fig. 3.25) resulted in identification of probable transformed molecules (Fig. 3.26). Transformation/degradation pathway for the reactive dye degradation has been proposed by several authors (Liu et al., 2010; Osma et al., 2010). Since laccase acts by free radical mechanism, in the present study pathway for the RB4 degradation has not been suggested. Instead, the probable molecules present during the different intervals of enzyme treatment have been listed (Fig. 3.26).

The degradation products of the dye solution were subjected to sorption over the powdered mycelium of the NIOCC #2a. The lyophilized biomass makes sorption process quick and facilitates the treatment of large volumes of effluents (Tigini, et al., 2010). Dried and lyophilised granular biomasses may help overcome conservation, robustness and separation issues (Aksu and Çağatay, 2006). Bayramoğlu et al. (2006) suggested that physical and chemical modification methods can be used to maximize the dye removal efficiency by the fungal biomass. Thus, these results are very important from an applicative point of view because it allows overcoming some difficulties in the industrial exploitation of biosorption. The adsorption equilibrium reached soon and this resulted in further decolorization (Fig. 3.23). The decrease of the absorbance in the whole of the UV spectrum confirmed the sorption of low molecular weight phenolic compounds over the fungal biomass. As against, the dye solution directly subjected to sorption also decolorized but the UV spectrum showed no considerable change. Also it took longer time to reach equilibrium. This confirms that the smaller polar compounds resulting due to enzymatic degradation were better candidates for the sorption (Fig. 3.23). In our earlier studies using submerged culture of the same fungus at the same concentration of dye, about 80% of color removal occurred during six days. Here in the present study more

than 93% decolorization was achieved in ~12.0h, which proves the above strategy will be highly suitable for remediation of such dyes.

The toxicity of many reactive dyes is known to be enhanced by irradiation or photo-oxidation. Reaction intermediates of anthraquinonic dyes, in particular have been found to increase following photo modification (Lizama et al., 2002). The dye solution was not completely mineralized after enzymatic treatment (Fig. 3.21). The toxicity against *Artemia* larvae was not reduced significantly after this step due to the formation of polar phenolic compounds. In the same manner dye solution directly subjected to sorption also showed no reduction in toxicity (Table 3.13). The two step treatment resulted in the maximum reduction in toxicity against *Artemia* larvae.

Untreated dyeing effluents are being discharged into water bodies and this water is used for agriculture. Thus, it is of concern to asses the phytotoxicity of the dye before and after remediation. Osma et al. 2010 reported that the degradation products of a reactive dye after laccase treatment showed less phytotoxicity. But in our case it was not significantly reduced after the enzymatic treatment. The two fold decrease in the phytotoxicity after the sequential treatment (Table 3.13) favors worthiness of the method applied.

In conclusion, above finding indicates that the enzymatic oxidation of RB4 can be adopted as a green chemistry approach for industrial applications and in the field of waste water treatment. Also, these results, confirmed that the two step treatment combining enzymatic degradation followed by biosorption yielded a substantial and rapid decolorization and detoxification of RB4.

Table 3.10: A comparison of the decolorization, reduction in COD and phenolics

Effluent	Treatment	% Decolorization		% Reduction COD		% Reduction Phenolics	
		After whole-culture treatment (Step 2)	Followed by biosorption (Step 3)	After whole-culture treatment (Step 2)	Followed by biosorption (Step 3)	After whole-culture treatment (Step 2)	Followed by biosorption(Step 3)
ROF	Without sonication	20± 3.9	49± 2.3	38± 6.5	41± 7.1	30± 3.2	48± 7.4
	With sonication	41± 1.7	63± 3.3	56± 4.4	62± 4.1	65± 8.9	74± 5.2
ROR	Without sonication	27± 2.4	50± 3.8	38± 4.6	48± 5.9	26± 10	36± 6.3
	With sonication	40± 3.3	61± 2.4	50± 2.8	63± 3.5	61± 14.2	67± 6.2
CAT I	Without sonication	27± 2.7	50± 3.3	53± 2.5	59± 4.8	37± 10.7	44± 8.1
	With sonication	56± 2.7	75± 2.5	71± 1.3	74± 1.6	49± 8.2	58± 5.1
CAT O	Without sonication	32± 4.1	53± 4.6	29± 6.3	46± 6.7	46± 1.4	53± 2.8
	With sonication	60± 8.4	78± 2.6	49± 0.9	53± 1.4	52± 4.0	61± 2.4

P values obtained by one way-analysis of variance (ANOVA): (I) between unsonicated and sonicated effluents in step 2 for color, COD and phenolics were 0.008, 0.002 and 0.06, respectively, (II) for color, COD and phenolics between unsonicated and sonicated effluents in step 3 were 0.01, 0.01 and 0.03, respectively and (III) between step 2 and step 3 for reduction in color, COD and phenolics were 3.17E-07, 0.004 and 0.0002, respectively.

Table 3.11: A comparison of toxicity test (LC_{50} values) of the unsonicated and sonicated effluents, after the whole fungal treatment (Step 2) followed by biosorption (Step 3).

Effluents	Treatments					
	Without any treatment (control)	whole-fungus treatment of unsonicated effluent	whole-fungus treatment of unsonicated effluent followed by biosorption	Effluent after sonication (Step 1)	whole-fungus treatment of sonicated effluent (Step 2)	whole-fungus treatment of sonicated effluent followed by biosorption (Step 3)
ROF	86 (48-130)^{a}	234 (178-331)^{b}	290 (202-366)^{bc}	112 (72-184)^{a}	338 (270-447)^{cd}	387 (279-547)^{d}
ROR	114 (66-172)^{a}	237 (160-325)^{b}	244 (170-327)^{b}	115 (68-171)^{a}	294 (201-433)^{bd}	328 (223-479)^{d}
CAT I	165 (110-229)^{a}	269 (194-354)^{b}	285 (204-378)^{b}	164 (94-267)^{a}	345 (240-496)^{c}	398 (287-572)^{c}
CAT O	96 (59-139)^{a}	214 (138-332)^{b}	335 (207-452)^{c}	88 (53-129)^{a}	251 (176-303)^{b}	362 (250-504)^{c}

LC50 values are in µl ml^{-1}. Higher LC50 values indicate greater reduction in toxicity. Upper and lower 95% confidence limits are within brackets. Different letters indicate significant differences between the same effluent (Tukey's test at the level 60.05%).

4.1 *Introduction*

Fungal laccases often occur as multiple isoenzymes expressed under different cultivation conditions (e.g. inducible or constitutive isoforms) (Susla et. al., 2007). Most are monomeric proteins, although dimeric and tetrameric laccases have also been described (Marques and Peralta et. al., 2003). Study at the molecular level reveals that several species produce a variety of isoenzymes. More than one isoenzyme is produced by most of the white-rot fungi. Eight different isoenzymes are produced by *P. ostreatus*, six of which have been isolated and characterized (Palmeri et. al., 2003). Many fungal species, e.g., *Coriolopsis rigida*, *Dichomitus squalens*, *Physisporinus rivulosus*, and *Trametes gallica*, produce isoenzymes that are closely related, both structurally and in their catalytic properties. In *P. chrysosporium*, production of different laccase isoenzymes was detected in cell extract and in the culture medium (Dittmer and Dhawale, 1997). Such diversity in laccase isoenzymes was first attributed to post-translation modifications of the same gene product, but the characterization of several laccase gene families suggested that at least a part of this biochemical diversity could be the result of the multiplicity of gene in fungal genomes (Ong et. al., 1997). Chen et al. (2003) reported that the molecular basis for the production of different isoenzymes is the presence of multiple laccase genes in fungi.

For studying the gene families and the copy number of laccase gene, genomic DNA library construction is useful. Due to the high sequence conservation of the laccase signature, sequencing has been exploited to design DNA probes, hybridization to clone, and finding one or more laccase gene from a variety of organisms. Genes that encode laccase isoenzymes in *P. ostreatus* have been cloned and sequenced (Giardina et al., 1995; Giardina et al., 1999).

The ecological role for the large variety of MnP, LiP or laccase isoenzymes in ligninolytic fungi is not yet clear. The diversity and function of ligninolytic genes in soil-inhabiting ascomycetes has not yet been elucidated, despite their possible role in plant litter decay processes. Studies of the diversity of functional genes are an important tool in microbial ecology (Ohkuma et al.,

1999). One of the most important criteria in phylogenetic systematics is the issue of homology (for details see Wiley et al. 1991; Henning et al. 1999). In terms of molecular data, three types of homology are defined: (i) orthology, when the sequences have a single and the same ancestor; (ii) paralogy, when they originate from a gene duplication event, and (iii) xenology, when they originate by horizontal gene transfer (Nei and Kumar, 2000). The two or more laccase genes in different organisms could be the result of duplication events, after which the enzymes evolved to perform similar biochemical processes (Castilho et al., 2009). A laccase gene family in which the gene encoding two of five laccase was located on the same chromosome was found in *Tremetes villosa* (Yaver and Golightly, 1996). Valderrama et al. (2003) reported that a single monophyletic branch exists for fungal laccases and that laccase isozyme genes may have evolved independently, possibly through duplication-divergence events. Multiple ligninolytic peroxidases are products of multiple genes and/or results of differing posttranslational modifications (Conesa et al., 2002). Genes encoding isoenzymes are differentially regulated and may be inducible or constitutively expressed during the life of the cell. Kilaru et al. (2006) identified seventeen different laccase genes, nine of which were active, while analyzing the complete sequenced genome of *Coprinopsis cinerea*. Genetic analysis has revealed that isoenzymes often originate from different laccase genes in the genome.

In addition to analyzing changes that have occurred in the evolution of different organisms, the phylogenetic analysis of a group of related protein sequences is a determination of how they might have evolved. When a gene family is found in an organism or group of organisms, the evolutionary relationships among the gene products can help to predict which ones might have an equivalent function. These functional predictions can be tested by genetic experiments (Valderrama et al., 2003). As an example, laccases are members of an ancestral group of copper-dependent oxidoreductases along with ascorbic acid oxidases and ceruloplasmin (Messerschmidt and Huber, 1990). Although significantly divergent at the protein sequence level, the similarity among them is

evident at the tertiary structure level of the redox sites (Solomon et al., 1996). The striking conservation at the active site level of very different copper-containing oxidases suggest that the enzyme activity linked to three different copper sites must have been a very early biological event (Solomon et al., 1996), therefore laccases are probably ancient enzymes from an evolutionary point of view (Messerschmidt and Huber, 1990).

Based on multiple sequence alignments of more than 100 laccases, four ungapped sequences regions (L1-L4) were evidenced in laccases (Kumar et al., 2003). The copper ligands include 12 amino acids that are housed within these conserved regions. Moreover, four loop regions (I, II, III, and IV) were identified and suggested to be involved in substrate binding on the basis of 3D structure superimposition (Larrondo et al., 2003). These conserved domains are highly helpful for the designing of gene specific primers.

Two reconstruction methods, maximum parsimony and distance, are generally used to find the evolutionary tree or trees that best account for the observed variation in a group of protein sequences (Valderrama et al., 2003).

Dedicated databases, namely CAZy [http://www.cazy.org] (Cantarel et al., 2009) and FOLy [http://foly.esil.univ-mrs.fr] (Levasseur et al., 2008) have been designed to annotate the genes involved in the (hemi)cellulolytic and ligninolytic processes, respectively.

Fungal laccases have been extensively exploited for industrial purposes and there is a wealth of information available regarding their reaction mechanism, biological role and several molecular aspects, including cloning, heterologous expression and transcriptional analyses. Gene amplification and expression in appropriate hosts could be promising for abundant production and affordable price of LDE, as is already the case with laccases used commercially in the pulp and paper industry (Wesenberg et al., 2003). Further potential benefits of genetically improved LDE could be extended substrate range, catalytic activity and stability for industrial application of LDE.

Industrial enzyme market is valued at $2 billion per annum with a potential growth rate of 3 to 5%. Laccase stake in this market is about 40% thus making it a potential $800 million market (Vincogen Corporation, annual report, 2003-2006). Reducing the costs of laccase production by optimizing the fermentation medium is the basic research for the industrial applications. Another approach is the overproduction of laccase in a suitable host. Yeasts are suitable as hosts for heterologous protein production because they combine a high capacity of growth, the easy manipulation of unicellular organisms and eukaryotic post-translational modifications. Moreover heterologous expression of a gene in hosts like unicellular eukaryotes makes the purification process simpler and cost effective. Laccase genes have been heterologously expressed in the yeasts (Kiiskinen et al, 2004; Necochea et al., 2005; Piscitelli et al., 2005; Colao et al., 2006; Bleve et al., 2008). The latter led to the very promising results opening the way to the use of direct mutagenesis or *in vitro* evolution for improvement of laccase for industrial applications. However, until now, the expression of filamentous fungal metalloenzymes including laccases, in production systems such as *Saccharomyces cerevisiae*, *Aspergillus niger* or *Trichoderma reesei* has met only limited success in terms of recombinant protein yields (Conesa et al., 2001). Thus the major challenge is to increase laccase production in heterologous expression system for efficient and economical application for industries. Also, recently Hong et al. (2007) expressed a new laccase gene isolated from a novel laccase-producing fungus *Trametes* sp 420 in *P. pastoris* obtaining a high laccase yield (8.3×10^4 UL^{-1}).

4.2 *Objective*

The primary objective of this study was to analyze the Lignin degrading enzyme gene sequence polymorphism in the various fungi belonging to ascomycete and basidiomycete. An attempt was made for extraction of complete coding sequence for a laccase gene from NIOCC #2a and its expression in a suitable heterologous host.

4.3 *Materials and Methods*

4.3.1 Screening of marine-derived fungi for lignin-degrading gene-specific sequences

Organisms and culture conditions

The four basidiomycetes (NIOCC #2a, NIOCC #15V, NIOCC #312 and NIOCC #DV2) and two ascomycetes (NIOCC #13V and NIOCC #C3) were used for this study. Cultures were maintained on B&K agar plates at room temperature. The relationships of these isolates have been discussed in chapter 2.

Genomic DNA extraction

The fungi were grown in B&K (broth) medium for 4-5 days. Mycelia were harvested, lyophilized and crushed in a mortar and pestle to a fine powder. Isolation of DNA was carried out following the modified form of standard procedure (Stoeck and Epstein, 2003). The above samples were incubated at 65ºC for two hours in a high salt extraction buffer containing 100 mM Tris-HCl buffer with 8 pH containing 100 mM Na_2EDTA, 100 mM $NaPO_4$, 1.5 M NaCl, 1% cetyltrimethylammonium bromide, 20% Sodium Dodecyl Sulphate and Proteinase K, 100 µg mL^{-1} for obtaining fungal DNA.

Amplification of the LDE gene

Genomic DNA from each of the fungal isolates was used as the template in PCR amplification reactions. The conditions for PCR included an initial hot start incubation (5 min at 94ºC) followed by 34 cycles (denaturation at 94º C for 30 sec, annealing at 55º C for 30 sec and extension at 72º C for 1 min) and a final extension at 72°C for 5 min.

The primers encoding catalytic and conserved domains for each gene (Laccase, LiP and MnP) have been listed in Table 4.1.

Table 4.1: Oligonucleotide sequences used for LDE homology

Gene	Primer designation	5'Sequence3'	Expected amplicon from genomic DNA	Reference
Laccase	Cu1F Cu2R	CAYTGGCAYGGNTTYTTYCA GRCTGTGGTACCAGAANGTNCC	140-300	Luis et al. 2004
	LAC2F LAC3R	GGIACIWIITGGTAYCAYWSICA CCRTGIWKRTGIAWIGGRTGIGG	900	Lyons et al. 2003
	Cu1F LccR	CAYTGGCAYGGNTTYTTYCA RTGRCTRTGRTACCARAANGT	150-250	Pointing et al. 2005
MnP	MnPF MnPR	GMRATGGCCTTCRRTTCYT TTAKGCAGGRCCRTYGAACT	900-1000	Bogan et al. 1996
LiP	LiPF LiPR	SCBAACATYGGYCTYGACGA TCSABGAAGAACTGSGWGTC	450-550	Reddy and D'Souza, 1998

'F' and 'R' represents forward and reverse primers respectively

Construction of LDE clone libraries

Bands corresponding to PCR amplicons were excised from 1% agarose gels (electrophorsed with 100 bp DNA ladder) and purified using the GenElute gel extraction kit (Sigma-aldrich, USA).

Since fungi can contain several alleles and multiple distinct LDEs, amplified products from the isolates were cloned using a pGEMT Cloning Kit (Promega, USA) before sequencing. Amplified products were transformed into *E. coli* cells (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Transformants were grown overnight at 37°C in Luria-Bertani broth containing 100 µg ml^{-1} of ampicillin. The presence of insert was confirmed by PCR with M13 forward and reverse primers. One µl of the broth containing the clone was added to 25 µl of PCR reaction mixture. PCR protocol included an initial hot start incubation (5 min at 94°C) followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. Clones containing positive insert were further processed for plasmid isolation and purification using Millipore plasmid preparation kit (Millipore, USA). Clone libraries were created for all samples that

yielded a PCR product of appropriate size. About 30 clones per cloning reaction were sequenced and analyzed.

Sequencing of the amplified gene fragments and phylogenetic analyses

Cloned products were sequenced in both directions with M13 forward (5' GTAAAACGACGGCCAG 3') and M13 reverse (5' CAGGAAACAGCTATGAC 3') primers

Sequencing of the plasmids was done at the National Centre for Cell Sciences, Pune, India, using the Big Dye Terminator cycle sequencing kit (V3.1, Applied Biosystems, USA) according to the manufacturer's protocol and analyzed in a DNA Analyzer (3,730 DNA Analyzer, Applied Biosystems, USA).

Forward and reverse sequences were assembled using Chromas Pro version 1.34 (Technelysium Pty Ltd, Tewantia, Queensland, Australia). Pairwise alignment of the sequences was carried out using Clustal W2 software (Thompson et al. 1994).

The consensus DNA sequences were used for the GenBank BLASTn and BLASTx searches (Altschul et al., 1997) to look for homologous nucleotide and polypeptide sequences respectively (http://www.ncbi.nlm.nih.gov).

Positions of putative introns were determined by comparing each deduced amino acid sequence to that of its closest identified relative.

4.3.2 *Expression of laccase*

Laccase hyper producing isolate NIOCC #2a was selected for the isolation and expression of a laccase in the suitable host.

Designing of laccase specific PCR primers

For obtaining complete coding sequence of a laccase gene, additional internal primers were designed as is necessary to obtain a specific gene transcript. Several published amino acid sequences of laccases from the organisms which were closely related to the NIOCC #2a were aligned. Primers were designed to be specific for fungal laccases and targeted conserved sequences around copper

binding domains. A total of 26 primers were designed as listed in Table 4.2. The quality of the primers was assessed using softwares such as Primer 3 and Gene Runner.

Table 4.2: Primers designed for the amplification of the coding sequence of laccase

Forward Primer	5'Sequence3'	Reverse Primer	5'Sequence3'
LF122	CTCATTACAGGAAACAAGGGC	**LR524**	TCGTCATCAACATCGTAAAG
LFD122	CTCATYACDGGHAAVAAGGGY	**LFD524**	CTYTACGAYGTYGAYRAYGA
LF311	AGGGTACTAACTGGGCTGATG	**LRD524**	TCRTYRTCRACRTCGTARAG
LFD311	AVGRTACBAACTGGGCYGATG	**LF1171**	CGGTTCCTGTTCTCCTTCAAAT
LF334	TACTAACTGGGCTGATGGTCCCGC	**LR1171**	ATTTGAAGGAGAACAGGAACCG
LFD334	TACBAACTGGGCYGATGGYCCYGC	**LFD1171**	CGGTYCCYGTDBCTYCTBCARAT
LR1425	AAGCGGATGGTGACGTTATC	**LRD1171**	ATYTGVAGRAGVACRGGRACCG
LRD1425	AAVCGGATVGTRACRTTRTC	**LF1311**	CCCTTCCACTTGCACGGTCAC
LR1467	ATGTGGCAGTGGAGGAACCA	**LR1311**	GTGACCGTGCAAGTGGAAGGG
LR1772	ACACAAGATTGTAGACCTCAAA	**LFD1311**	CCYTTCCAYTTGCAYGGKCAC
LRD1772	ACACAAKAWTGTAGACYTCAAA	**LRD1311**	GTGMCCRTGCAARTGGAARGG
LF436	CTGGTATCATAGTCACTTGTC	**LRD1467**	ATGTGGCAGTGRAGGAACCA
LFD436	CTGGTAYCAYAGTCACTTGTC		
LF524	CTTTACGATGTTGATGACGA		

mRNA isolation and generation of cDNA

Total RNA was isolated from the 8-10 day old NIOCC #2a mycelium grown in modified LN medium using PurLink RNA Mini Kit (Invitrogen). Polyadenylated RNA was purified using mRNA purification kit (Macherey-Nagel Gmbh & Co. KG, Germany). First strand cDNA (5' and 3' separately) synthesis was carried out using the SMARTer RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturers protocol.

Selection of suitable gene specific PCR Primers and RACE

Prior to the performance of RACE (rapid amplification of cDNA ends) the efficiency of the laccase specific primers was tested. Several different combinations of primer pairs were used as listed for the amplification of 5' and 3' RACE ready cDNA by temperature-gradient PCR. The amplified gene products

were cloned into pGEM-T easy cloning vector (Promega) and sequenced using vector specific primers. Post sequencing analyses of the products was conducted.

Selected primer pair was used to conduct the RACE (SMARTer RACE cDNA Amplification Kit, Clontech, USA) to get the full coding sequence following the manufacturers protocol.

4.4 *Results*

4.4.1 *Diversity of the lignin degrading enzymes in marine-derived fungi*

Two species of Ascomycota (NIOCC #C3 and NIOCC #16V) and four species of Basidiomycota (NIOCC #2a, NIOCC #15V, NIOCC #312 and NIOCC #DV2) were analyzed to evaluate the diversity of lignin degrading enzymes.

The three sets of laccase primers (Table 4.1) yielded varying results. Although primer set Lac2F/Lac3R was able to amplify the gene sequences in basidiomycetous fungi none of them showed affiliation to the laccase. Whereas; Cu1F/LccR gave positive results only with #2a and #DV2. The primer set Cu1F/Cu2R generated more than a single band (150-250 bp) in all fungi except #16V. Also, largest number of laccase positive inserts was obtained wit Cu1F/Cu2R (Table 4.1).

The LiP gene-specific primer-set amplified sequences in all the fungi with higher number of positive inserts in #312 and #DV2 (Table 4.3). Satisfactory amplification using MnP primer-set could not be obtained for any of the fungi used in this study.

Table 4.3: Frequency of occurrence of positive inserts

Primer Set	Isolates	Frequency (%) of positive insert
Cu1F/Cu2R	#2a	83.3
	#15V	36.6
	#312	30
	#DV2	66.7
	#13V	16.7
Cu1F/LccR	#2a	33.3
	#DV2	50.0
LiPF/LiPR	#2a	3.3
	#15V	10
	#312	40
	#DV2	46.7
	#13V	13.3
	#16V	3.3

Nucleotide sequence analysis with the Blastn tool (nucleotide/nucleotide comparison) did not retrieve significant similarity for many of the sequences analyzed. In spite of this, Blastx (nucleotide/translated sequence comparison) retrieved more than 100 high-scored amino acid sequences for most of the sequences. Overall frequency of positive inserts for laccase was higher in basidiomycetes than in ascomycetes whereas, #312 and #DV2 belonging to Basidiomycota yielded significantly higher homology for LiP gene sequences (Table 4.4). Few of the sequences amplified with LiP gene-specific primer also showed affiliation to manganese peroxidases.

About 49% sequences showing affiliation to laccases were less than 95% similar to their closest relative whereas this figure was 100% for LiP gene sequences.

Many of the sequences, mostly belonging to the two ascomycetes used in this study also showed homology to hypothetical peptides and proteins belonging to enzymes other than LDEs (Table 4.4).

Table 4.4: Results of BLASTx studies

Gene	Primer set	Isolate	Clone No.	Closest relative	Maximum identity	Accession no.
Laccase	Cu1F/ Cu2R	#2a	89F89R	laccase [*Cerrena* sp. WR1]	65%	ACZ58367.1
			2f2r	LAC1 [*Polyporus brumalis*]	92%	ABN13591.1
			10f10r	Lac1 [*Cerrena unicolor*]	83%	ACL93462.1
			18f18r	Laccase[*Panus* sp. HKUCC4062]	100%	AAP78656.1
			58f58r	laccase2 [*Trametes versicolor*]	95%	BAD98306.1
			66f66r	Laccase [*Panus* sp. HKUCC 4062]	100%	AAP78656.1
			82f82r	Laccase [*Panus* sp. HKUCC 4062]	100%	AAP78656.1
			11f11r	Laccase2[*Trametes versicolor*]	89%	BAD98306.1
			19f19r	Laccase2 [*Trametes versicolor*]	90%	BAD98306.1
			27f27r	hypothetical protein MPER_09368 [*Moniliophthora perniciosa* FA553]	81%	XP_002391234.1
			51f51r	hypothetical protein MPER_09368 [*Moniliophthora perniciosa* FA553]	77%	XP_002391234.1
			75f75r	laccase [*Panus* sp. HKUCC 4062]	100%	AAP78656.1
			83f83r	Lac1 [*cerrena unicolor*]	82%	ACL93462.1
			4f4r	hypothetical protein MPER_09368 [*Moniliophthora perniciosa* FA553]	82%	XP_002391234.1
			12f12r	Laccase [*Panus* sp. HKUCC 4062]	100%	AAP78656.1
			20f20r	LAC1 [*Polyporus* brumalis]	100%	ABN13591.1
			28f28r	LAC2 [*Polyporus* brumalis]	100%	AB13592.1
			36f36r	hypothetical protein MPER_09368 [*Moniliophthora perniciosa* FA553]	81%	XP_002391234.1
			52f52r	laccase2 [*Trametes versicolor*]	91%	BAD98306.1
			60f60r	laccase-like multicopper oxidase [uncultured fungus]	87%	ABN79423.1
			68f68r	laccase-like multicopper oxidase [uncultured fungus]	87%	ABN79423.1
			76f76r	laccase [*Panus* sp. HKUCC 4062]	100%	AAP78654.1
			92f92r	laccase [*Panus* sp. HKUCC 4062]	97%	AAP78656.1
			5f5r	laccase [*Panus* sp. HKUCC 4062]	100%	AAP78656.1
			13f13r	Laccase2 [*Lentinus* Sajor-caju]	89%	CAD45378.1
		#15V	F53r53	bilirubin oxidase [*Ganoderma tsunodae*]	92%	BAA28668.1

Table 4.4 contd.

Gene	Primer set	Isolate	Clone No.	Closest relative	Maximum identity	Accession no.
Laccase	**Cu1F/ Cu2R**	**#15V**	F55r55	hypothetical protein PVOR_31659	98%	ZP_07902888.1
			F56r56	laccase [uncultured *Basidiomycota*]	37%	CAF24947.1
			F59r59	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			F61r61	laccase 2 [*Coriolopsis gallica*]	92%	ACR50978.1
			F64r64	glycoside hydrolase family 38	100%	ZP_07900526.1
			F67r67	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			F68r68	laccase [*Pycnoporus cinnabarinus*]	69%	ACH87824.1
			5B07	laccase [*Pycnoporus cinnabarinus*]	69%	ACH87824.1
			5B08	laccase D [*Trametes* sp. 420]	91%	AAW28939.1
			5C07	laccase [*Coriolopsis rigida*]	93%	ADJ95376.1
			5C08	laccase [*Pycnoporus cinnabarinus*]	94%	ACH87824.1
			5D08	bilirubin oxidase [*Ganoderma tsunodae*]	89%	BAA28668.1
			5G07	laccase [*Coriolopsis rigida*]	93%	ADJ95376.1
			5G08	laccase D [*Trametes* sp. 420]	90%	AAW28939.1
		#312	F70r70	aldo/keto reductase [*Paenibacillus vortex* V453]	96%	ZP_07900758.1
			F72r72	hypothetical protein NCU06927 [*Neurospora crassa* OR74A]	57%	XP_959274.1
			F75r75	laccase [*Xylaria* sp. HKUCC 2797]	69%	AAP78659.1
			F78r78	hypothetical protein GLRG_01318 [*Glomerella graminicola* M1.001]	49%	EQ26174.1
			F79r79	alpha-L-rhamnosidase [*Paenibacillus vortex* V453]	95%	ZP_07899802.1
			F83r83	laccase [*Xylaria* sp. HKUCC 2797]	97%	AAP78659.1
			F95r95	laccase [uncultured Basidiomycota]	48%	CAF24981.1
			F86r86	hypothetical protein PVOR_11760 [*Paenibacillus vortex* V453]	98%	ZP_07899221.1
			F91r91	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			5A09	laccase-like multicopper oxidase [uncultured fungus]	46%	ACK99190.1

Table 4.4 contd.

Gene	Primer set	Isolate	Clone No.	Closest relative	Maximum identity	Accession no.
Laccase	**Cu1F/ Cu2R**	**#312**	5C09	laccase [*Schizophyllum commune*]	40%	ADO14328.1
		#DV2	f3r3	Laccase [*Ganoderma lucidum*]	69%	ACR24357.1
			f4r4	laccase hybrid [*Trametes* sp. C30]	100%	ACO53434.1
			F5r5	bilirubin oxidase [*Ganoderma tsunodae*]	100%	BAA28668.1
			F6r6	inorganic phosphate transporter [*Laccaria bicolor* S238N-H82]	84%	XP_001880970.1
			F7r7	integral membrane sensor signal transduction histidine kinase	98%	ZP_07900783.1
			F8r8	Helix-turn-helix type 11 domain-containing protein	75%	YP_003241311.1
			F11r11	hypothetical protein BIFPSEUDO_03391	30%	ZP_03742817.1
			F12r12	laccase hybrid [*Trametes* sp. C30]	100%	ACO53434.1
			F13r13	laccase [*Coriolopsis rigida*]	93%	ADJ95376.1
			F16r16	iron transport multicopper oxidase FET3 [*Aspergillus oryzae* RIB40]	83%	XP_001822739.2
			F19r19	laccase [*Ganoderma lucidum*]	100%	ACR24357.1
			F20r20	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			F21r21	laccase [*Coriolopsis rigida*]	92%	ADJ95376.1
			F27r27	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			F28r28	laccase [*Xylaria* sp. HKUCC 2797]	97%	AAP78659.1
			F29r29	bilirubin oxidase [*Ganoderma tsunodae*]	100%	BAA28668.1
			F30r30	laccase [uncultured Basidiomycota]	30%	CAD62512.1
			F35r35	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			F36r36	laccase [*Xylaria* sp. HKUCC 2797]	97%	AAP78659.1
			F37r37	bilirubin oxidase [*Ganoderma tsunodae*]	92%	BAA28668.1
		#13V	F39r39	hypothetical protein GLRG_01318 [*Glomerella graminicola* M1.001]	48%	EFQ26174.1
			F43r43	laccase [*Xylaria* sp. HKUCC 2797]	97%	AAP78659.1
			F44r44	laccase [*Xylaria* sp. HKUCC 2797]	97%	AAP78659.1
			F45r45	laccase [*Coriolopsis rigida*]	93%	ADJ95376.1
			F51r51	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1

Table 4.4 contd.

Gene	Primer set	Isolate	Clone No.	Closest relative	Maximum identity	Accession no.
Laccase	Cu1F/ LccR	#2a	61f61r	hypothetical protein MPER_09368 [*Moniliophthora perniciosa*	87%	XP_002391234.1
			93f93r	LAC1 [*Polyporus brumalis*]	91%	ABN13591.1
			22f22r	laccase2 [*Trametes versicolor*]	91%	BAD98306.1
			38f38r	Lac1 [*Cerrena unicolor*]	83%	ACL93462.1
			4A01	LAC2 [*Polyporus brumalis*]	100%	ABN13592.1
			4B01	LAC2 [*Polyporus brumalis*]	100%	ABN13592.1
			4F02	LAC2 [*Polyporus brumalis*]	100%	ABN13592.1
			4G01	LAC2 [*Polyporus brumalis*]	100%	ABN13592.1
			4H01	LAC2 [*Polyporus brumalis*]	100%	ABN13592.1
			4G02	laccase2 [*Trametes versicolor*]	89%	BAD98306.1
			4H02	laccase2 [*Trametes versicolor*]	895	BAD98306.1
		#DV2	4A 03	Laccase [*pycnoporus cinnabarinus*]	97%	AAP78650.1
			4A04	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4B04	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4B04	Laccase [*Pycnoporus cinnabarinus*]	97%	AAP78650.1
			4C03	laccase [*Pycnoporus cinnabarinus*]	97%	AAP78650.1
			4C04	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4D03	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4E03	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4E04	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4F03	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4F04	laccase [*Pycnoporus cinnabarinus*]	95%	AAP78650.1
			4G03	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1

Table 4.4 contd.

Gene	Primer set	Isolate	Clone No.	Closest relative	Maximum identity	Accession no.
Laccase	**Cu1F/ LccR**	**#DV2**	4G04	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4H03	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
			4H04	laccase [*Pycnoporus cinnabarinus*]	100%	ACG75927.1
LiP	**LiPF/ LiPR**	**#2a**	C02	predicted protein [*Laccaria bicolor* S238N-H82]	44%	XP_001875374.1
			4B06	sec7 domain belongs to guanine nucleotide exchange factors	44%	XP_001873875.1
			4C05	adenylate cyclase [*Puccinia graminis* f. sp. tritici CRL 75-36-700-3]	40%	EFP93025.1
			4G05	other/AgaK1 protein kinase	47%	XP_001839463.1
		#15V	A03	peptidase M23 [*Exiguobacterium sibiricum* 255-15]	38%	YP_001814886.1
			E03	hypothetical protein MPER_13114 [*Moniliophthora perniciosa*	51%	XP_002387934.1
			E04	other/FunK1 protein kinase [*Coprinopsis cinerea* okayama7#130]	34%	XP_002911071.1
			4B08	putative oxidoreductase [*Kitasatospora setae* KM-6054]	38%	BAJ30247.1
			4D07	versatile peroxidase-like 1 [*Grifola frondosa*]	65%	ADK60901.1
			4H08	versatile peroxidase-like 1 [*Grifola frondosa*]	655	ADK60901.1
			4E08	other/FunK1 protein kinase [*Coprinopsis cinerea* okayama7#130]	34%	XP_002911071.1
			4F08	hypothetical protein CC1G_10775	28%	XP_001834901.2
		#312	A11	lignin peroxidase isoform J [*Phanerochaete chrysosporium*]	67%	ABT17196.1
			A12	manganese peroxidase precursor [*Ceriporiopsis rivulosa*]	93%	ABB83812.1
			B11	lignin peroxidase isoform J [*Phanerochaete chrysosporium*]	69%	ABT17196.1
			B12	lignin peroxidase precursor [*Phanerochaete sordida*]	54%	BAG85350.1
			C12	lignin peroxidase isoform J [*Phanerochaete chrysosporium*]	695	ABT17196.1
			D11	manganese peroxidase precursor [*Ceriporiopsis rivulosa*]	93%	ABB83812.1

Table 4.4 contd.

Gene	Primer set	Isolate	Clone No.	Closest relative	Maximum identity	Accession no.
LiP	LiPF/ LiPR	#312	D12	lignin peroxidase precursor [*Phanerochaete sordida*]	53%	BAG85350.1
			E11	lignin peroxidase isoform J [*Phanerochaete chrysosporium*]	68%	ABT17196.1
			E12	lignin peroxidase isoform J [*Phanerochaete chrysosporium*]	68%	ABT17196.1
			F12	lignin peroxidase precursor [*Phanerochaete sordida*]	54%	BAG85350.1
			G11	manganese peroxidase precursor [*Ceriporiopsis rivulosa*]	93%	ABB83812.1
			H12	manganese peroxidase precursor [*Ceriporiopsis rivulosa*]	93%	ABB83812.1
		#DV2	A09	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	93%	ADK60909.1
			A10	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	64%	ADK60909.1
			B09	manganese-dependent peroxidase-like protein [*Heterobasidion annosum*]	84%	ACB69799.1
			B10	lignin peroxidase prepropeptide [*Trametes versicolor*]	65%	CAA53333.1
			C09	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	94%	ADK60909.1
			C10	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	94%	ADK60909.1
			D09	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	895	ADK60909.1
			D10	lignin peroxidase prepropeptide [*Trametes versicolor*]	66%	CAA53333.1
			E10	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	92%	ADK60909.1
			F09	manganese peroxidase-like 1 [*Pycnoporus cinnabarinus*]	88%	ADK60905.1
			F10	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	95%	ADK60909.1
			G10	lignin peroxidase prepropeptide [*Trametes versicolor*]	92%	CAA53333.1
			H09	lignin peroxidase prepropeptide [*Trametes versicolor*]	65%	CAA53333.1
			H10	lignin peroxidase-like 1 [*Pycnoporus cinnabarinus*]	92%	ADK60909.1
		#13V	F06	phosphoenolpyruvate-protein phosphotransferase	75%	YP_002306050.1
			G05	hypothetical protein [*Podospora anserina* S mat+]	54%	XP_001912098.1
			A06	predicted protein [*Laccaria bicolor* S238N-H82]	44%	XP_001888003.1
			D06	hypothetical protein [*Podospora anserina* S mat+]	55%	XP_001912098.1
			4B12	transcriptional regulator [Burkholderia cenocepacia MC0-3]	100%	YP_001776897.1

Table 4.4 contd.

Gene	Primer set	Isolate	Clone No.	Closest relative	Maximum identity	Accession no.
LiP	**LiPF/ LiPR**	**#13V**	4D12	ABC transporter related protein [*Paenibacillus vortex* V453]	100%	ZP_07901131.1
			4H12	unnamed protein product [*Sordaria macrospora*]	45%	CBI54786.1
		#16V	A08	hypothetical protein AN7159.2 [*Aspergillus nidulans* FGSC A4]	62%	XP_664763.1
			4B09	hypothetical protein MGG_14098 [*Magnaporthe oryza* 70-15]	56%	XP_001405060.1
			4B10	predicted protein [*Nectria haematococca* mpVI 77-13-4]	75%	XP_003048053.1

4.4.2 *Heterologous expression of a laccase gene*

Whole cell cDNA of the isolate #2a was constructed. Although partial coding sequences of laccase gene after amplification using cDNA as template were retrieved using several primer pairs (Table 4.2) as shown in Fig. 4.1 actual, complete transcript could not be obtained during the RACE using any of these primer pairs.

```
dbj|BAE79811.1|  laccase 1 precursor [Spongipellis sp. FERM P-18171]
Length=516

 Score =  214 bits (515),  Expect = 3e-54
 Identities = 90/100 (90%), Positives = 94/100 (94%), Gaps = 0/100 (0%)
 Frame = -2

Query  379  FHLHGHNFHVVRSAGQTTPNYVDPIVRDVVNTGGTGDNVTIRFTTDNPGPWFLHCHIDWH
200
            FHLHGHNFHVVRSAGQTT NYV+PIVRDVVNTG + DNVTIRFTTDNPGPWFLHCHIDWH
Sbjct  416  FHLHGHNFHVVRSAGQTTANYVNPIVRDVVNTGASPDNVTIRFTTDNPGPWFLHCHIDWH
475

Query  199  LEAGFAVVFAEGVNQTNAANPTPADWNNLCNIYNALADGD  80
            LEAGFAVVFAEG+NQTNAANPTPA WNNLCN+YNAL  GD
Sbjct  476  LEAGFAVVFAEGINQTNAANPTPAAWNNLCNLYNALDSGD  515


gb|ACL93462.1|  Lac1 [Cerrena unicolor]
Length=510

 Score =  209 bits (504),  Expect = 6e-53
 Identities = 88/101 (88%), Positives = 92/101 (92%), Gaps = 0/101 (0%)
 Frame = -2

Query  379  FHLHGHNFHVVRSAGQTTPNYVDPIVRDVVNTGGTGDNVTIRFTTDNPGPWFLHCHIDWH
200
            FHLHGHNFHVVRSAGQ TPNY DPIVRDVVNTG  GDNVTIRFTTDNPGPWFLHCHIDWH
Sbjct  410  FHLHGHNFHVVRSAGQDTPNYDDPIVRDVVNTGAMGDNVTIRFTTDNPGPWFLHCHIDWH
469

Query  199  LEAGFAVVFAEGVNQTNAANPTPADWNNLCNIYNALADGDK  77
            LEAGFAVVFAE VN+T A NPTPA W+NLC +Y+ALADGDK
Sbjct  470  LEAGFAVVFAEAVNETKAGNPTPAAWDNLCTLYDALADGDK  510
```

Fig. 4.1: Sequences (obtained after the amplification of whole cell cDNA) showing affiliations during BLASTx studies

4.4 *Discussion*

4.4.1 *Screening of lignin degrading enzymes from marine-derived fungi*

Lignin degrading enzymes are known to occur as multiple alleles within an organism. Intraorganismal heterogeneity among laccase genes in fungi has been documented by several authors (Mansur et al., 1997; Muñoz et al., 1997; Yaver et al., 1999; Litvintseva and Henson, 2002; Lyons et al., 2003). Multiple laccase genes can be found in many organisms; e.g., *Trametes villosa* has 5 (Yaver et al., 1996), *Pleurotus ostreatus* 4 (Palmieri et al., 1997), *Coprinus cinereus* up to 17 (Kilaru et al., 2006) and *Trametes gallica* has been shown to secrete up to 20 different isonezymes of laccase (Dong et al., 2005). *Podospora anserine* produces three laccase isoenzymes (Fernandez-Larrea and Stahl, 1996) and the basidiomycete *Coprinopsis cinerea* contains eight laccase genes (Hoegger et al., 2004). A report demonstrated the presence of at least two laccase genes in yeast-like ascomycete *Hortaea acidophilla* (Tetsch et al., 2005). The southern hybridization analyses of heterokaryotic *Heterobasidium annosum* indicated the existence of two or more types of laccase genes (Asiegbu et al., 2004). A laccase multi-gene family has been demonstrated in *A. bisporus* and *Pleurotus ostreatus* (Perry et al., 1993; Pezzella et al., 2009). One to six different 'laccase like multi-copper oxidase' genes were found within the *Morchellaceae* sp and *Discinaceae* sp representing 26 different sequence types (Kellner et al., 2007). A high diversity of laccase sequences amongst southeastern US salt marsh fungi have been reported (Lyons et al., 2003). The multiplicity of sequence types yielded by single organisms likely represents distinct laccase genes within the same isolate and possibly allows for broader substrate specificity and may confer an ecological advantage in the competition for space and nutrients in the decay system (Lyons et al., 2003).The present study indicated presence of multiple distinct types of LDEs with abundance of novel sequences among marine-derived fungi (Table 4.4) having less than 95% similarity to the closest relative at the level of amino acid.

Molecular phylogenetics has been increasingly dominant in several biological approaches such as organismal and molecular evolution (Hillis et al., 1996). In one of the analyses it was shown that the laccase from *Flammulina velutipes* was phenotypically and evolutionarily distinct (Castilho et al., 2009). This evidence reinforces the laccase diversity and suggests intra-specific variations occurring in this genetic pool. Comparative phylogenetic analyses using predicted amino acid sequences of a basidiomycete, *Heterobasidion annosum* showed strong similarity to the laccases from other basidiomycetes but least similar to laccases from ascomycete fungi (Asiegbu et al., 2004). A phylogenetic dendrogram analysis of laccase based on partial polypeptide sequences separated ascomycetes from basidiomycetes and no mixed clustering was observed (Asiegbu et al., 2004).

Some reports suggested the importance of ascomycets with laccase for litter degradation in certain habitats such as, the decay of *Spartina alterniflora* leaves in marshland ecosystems (Lyons et al., 2003). Although many basidiomycetous laccases have been analyzed at the molecular level, the study of ascomycetous laccase genes still remains problematic due to a low level of conservation among these genes (Tetsch et al., 2005). In general, laccases in basidiomycetous fungi are highly homologous (42-98% identity on amino acid level) (Cassland and Jönsson, 1999) whereas ascomycetous laccases exhibit much less homology (53-65% identity on amino acid level) (Berka et al., 1997). In the present study also amplification could not be obtained using any of the laccase specific primers from ascomycete #16V while ascomycete #13V yielded a low frequency of positive inserts (Table 4.3). Although these isolates showed presence of laccase during qualitative and quantitative assay (Chapter 2), the lack of a product in laccase-positive isolates might be attributed to the presence of an intron in the regions of the gene where primer binding occurs (Lyons et al., 2003). Also, several sequences showed affiliation to the amino acid sequences other than LDE which might have occurred due to non-specific binding of the primers (Table 4.4).

The successful gene amplification using MnP primer-set could not be obtained during the present studies. Pointing et al. (2005) were also not able to get positive results using this primer pair and suggested that they are too specific to detect MnP genes among diverse taxa, as aresult of their design based upon a single isozyme of MnP in *P chrysosporium*.

The primer pair used for the amplification of LiP genes was able to produce positive results only for #312 and #DV2. Pointing et al. (2005) reported the presence of LiP-like gene sequences among all the LiP producing basidiomycetes using the same primer pair while xylariaceous fungi showed absence of these segments.

The degenerate primers used in the present study only amplify short fragments, which limits such interpretations. More exhaustive information on the gene diversity of LDEs and their ancestors is needed to elucidate the diversification of these genes during evolution. Further statistical and phylogenetic analysis of the present data will be helpful. Designing specific primers that amplify longer fragments in each gene family will be helpful.

Since the existing database of LDE sequences from fungi is limited, the sequences assembled here provide a valuable basis for future studies of these degradative enzymes.

4.4.2 *Heterologous* expression *of laccase from NIOCC #2a*

A thermo- and metal tolerant laccase isozyme, *Lac IID* has been reported from the marine-derived isolate #2a (D'Souza-Ticlo et al., 2009). In the present study an attempt was made to isolate a complete transcriptional unit of laccase from the same fungus and its heterologous expression in the suitable host. In order to optimize the PCR conditions the annealing temperature was varied over a range from 50-70ºC.

Further efforts will be made to characterize the LDEs and to clone and sequence the complete genes. The synthesis of the encoded laccase in an expression system will be carried out.

Summary

Increasing discharge and improper management of toxic and colored industrial effluents have created a great concern over their treatment which is economical and safe. Therefore it is imperative to develop cost effective and efficient methods for their remediation. Microbial decolorization and degradation is a promising green technology for the treatment of such effluents. Mycoremediation is a form of bioremediation where fungi (live or dead) are used to degrade or sequester the waste in the environment. Industrial effluents are mostly extreme in pH and rich in salts such as carbonates, chlorides and sulfates. In light of this, marine fungi or marine-derived fungi with their ligninolytic system hold good advantage for their application in remediation of colored effluents since they are better adapted to perform under such extreme conditions.

The objective of the present study was to isolate fungi from various marine and coastal habitats, screening them for lignin degrading enzyme production, decolorization of industrial effluents and identification of these fungi using molecular tools. The potential isolates were used for the remediation of various colored effluents. Further, homology studies of lignin degrading enzymes in certain marine-derived fungi were done and an attempt was made to extract a laccase from a marine-derived fungi and its expression in a secondary host.

Fungi were isolated from decaying lignocellulosic materials collected from various mangrove swamps of Goa, India and from a coral lagoon of Kavarati Island in the Lakshadweep Archipelago, Arabian Sea. Various techniques namely, particle-plating, moist chamber incubation and single spore isolation method were employed for the isolation of fungi. About 50 of these isolates were screened for the presence of lignin degrading enzymes such as laccase and peroxidases. Laccase producing fungi were observed to be dominating in the mangroves during qualitative plate assay. The growth of these fungi was significantly more in the medium prepared with half strength sea-water (p value=0.000429). Since most of the screened isolates were anamorphs, they were identified by comparison of their

rDNA sequences. Most of the fungi belonged to the phylum Ascomycota and only 3 isolates (NIOCC #15V, NIOCC #50V and NIOCC #DV2) were of the phylum Basidiomycota. Among ascomycetes NIOCC #13V was the best laccase producer whereas NIOCC #2a produced maximum laccase titer among basidiomycetes during quantitative (Spectrophotometric) assay. Basidiomycete #50V, #DV2 and #312 were better MnP producers than rest of the candidates. Several of the screened isolates were used for the removal of color from various azo and anthraquinonic dyes. Although ascomycetes were able to remove color from these dyes, decolorization was significantly higher by basidiomycetes.

The effect of various nitrogen sources incorporated in the growth medium on enzyme production and decolorization of industrial effluents by the marine-derived fungus NIOCC #2a was assesd. Fungal growth was best in the presence of glutamic acid as the nitrogen source when no effluent samples were added whereas; in presence of effluents results were variable depending upon the kind of effluent added. In the same way results were variable for the lignin degrading enzyme production and decolorization depending upon the effluent characteristics. Since color of the effluents was variable and this could be a reason for varied decolorization percentages, this factor was corrected in an *ex situ* experiment but results were still variable. These results indicated that the type of nitrogen source used, not only influence the amount and type of lignin-degrading enzymes produced but also had an effect on the decolorization of effluents.

Four marine-derived fungi belonging to the phylum Ascomycota (NIOCC #C3 and NIOCC #16V) and Basidiomycota (NIOCC #2a and NIOCC #15V) were used for the remediation of two raw textile-mill effluents (TEA and TEB) which were highly variable in their pH and dye composition. Textile effluent A (TEA) contained an azo dye and had a pH of 8.9 and textile effluent B (TEB) with a pH of 2.5 contained a mixture of eight reactive dyes. Each of these fungi decolorized TEA by 30–60% and TEB by 33–80% used at 20– 90% concentrations within 6 days. This was accompanied by two to three-fold reduction in toxicity as

measured by LC_{50} values against *Artemia* larvae and 70–80% reduction in chemical oxygen demand and total phenolics. Mass spectrometric scan of effluents after fungal treatment revealed degradation of most of the components. The ascomycetes appeared to remove color primarily by adsorption, whereas laccase played a major role in decolorization by basidiomycetes. A remediation process involving these two groups of fungi which include instant color removal by adsorption using ascomycetes followed by treatment with crude laccase from basidiomyctes to remove the adsorbed color from the fungal biomass has been demonstrated.

A novel three-step technology for treatment of four molasses-based raw industrial effluents (ROF, ROR, CAT I and CAT O), varying in their COD, color and turbidity is reported. Sequential steps involved in this treatment are; (1) sonication of the effluents, (2) whole-fungal treatment of these by a ligninolytic marine fungus and (3) biosorption of the residual color with heat-inactivated biomass of the same fungus. Sonication reduced the foul odor and turbidity of the effluents. It increased biodegradability of the effluents in the second stage of treatment. Laccase production in the presence of all the four effluents was directly correlated with their decolorization. After the third step, a reduction of 60–80% in color, 50–70% in COD and 60–70% in total phenolics were achieved. Comparative mass and nuclear magnetic resonance spectra indicated increasing degradation of the effluent components after each stage. Toxicity (LC_{50} values) against *Artemia* larvae was reduced by two to five-folds.

The effect of partially purified laccase and its mediator (HBT) on the decolorization (*ex situ*) of various molecular weight fractions of above mentioned molasses-based effluents has been demonstrated. Laccase mediator was able to enhance the decolorization of most of the fractions in all the four effluents. Along with decolorization, polymerization was also evident in certain fractions of CAT I and CAT O.

The ability of partially purified laccase from #2a to decolorize and degrade a model dye, Reactive Blue 4 was determined. An attempt was made to describe the resulting degradation products formed due to enzymatic activity. Also, a quicker and non-conventional process was developed for the decolorization and detoxification of this dye using a combination of enzymatic degradation followed by sorption of the degradation products over the powdered mycelium.

Lignin degrading enzymes (LDEs) produced by different marine-derived fungi were examined for their homology. The four basidiomycetes (#2a, #15V, #312 and #DV2 and two ascomycetes (#13V and #C3) were used for this study. The primer set Cu1F/Cu2R used for the amplification of laccase gene produced best results. The LiP gene specific primer pair amplified sequence in all the fungi with higher number of inserts in #312 and #DV2. Satisfactory amplification using MnP specific primer set could not be obtained for any of the fungi. About 49% of the sequences showing affiliation to laccases were less than 95% similar to their closest relative whereas this figure was 100% for LiP gene sequences.

Extraction of a transcriptional DNA sequence of laccase from #2a was attempted for the expression of this gene in a secondary host. Although partial coding sequences of laccase gene after amplification using cDNA as template were retrieved using several primer pairs, actual complete transcript could not be obtained by using RACE with any of these primer pairs.

Future Prospects

The increasing number of fungal genome sequences clearly demonstrates that their biosynthetic potential is far from being fully exploited. Thus, mining the full-genome sequences of fungi leads to a high speed discovery of genes and gene clusters which are potentially involved in the production of secondary metabolites. Molecular mechanisms will in future largely influence our understanding of taxonomy and its importance in natural product research (Voigt and Kirk, 2011). Species boundaries may need to be re-defined in the light of genome evolution and microbial interactions. The search for new fungal taxa and taxon groups with biotechnological potential and their implementation in naturally occurring organismic alliances will continue to play a significant role for the elucidation of cryptic or novel natural products and their application in various fields such as bioremediation.

Extensive studies on the taxonomy of fungi in mangroves are needed as it will be immensely helpful in keeping the record of metabolites and enzymes produced by these fungi. Both endophytic fungi from marine habitats and obligate marine fungi are one of the least studied groups of fungi. Several techniques are needed to improvise the isolation of yet uncultured fungi from these habitats. A high throughput screening strategy for lignin-degrading marine fungi is required to tap the vast resources in marine ecosystem. Assesment of biological activity by the application of functional genomics will be immensely helpful in targeting such fungi.

Most of the LDEs show optimum activity around 30-35 ºC and at pH of 3-4. Industrial effluents generally have alkaline pH and temperatures above ambient. Moreover, many of the effluents contain inhibitory compounds for enzymatic activity. Therefore, fungi from the marine environment with LDE activity at alkaline pH preferably at higher temperatures should be selectively isolated for the remediation purposes. Gene specific probes for the estimation of enzymatic activity in the natural environments such as marine habitats will be

helpful. A laccase hyper-producing strain such as #2a (D'Souza-Ticlo et al., 2008) or peroxidase producing isolate #312 (Raghukumar et al., 1999), from marine environment using inexpensive growth medium would be a suitable alternative. Understanding the mechanism of degradation and detoxification of industrial effluents by marine lignin degrading fungi are some of the key areas for future research.

White-rot fungi utilize simple radical-generating mechanisms for the degradation of lignin/xenobiotics. However, further understanding of the various enzymes involved in the degradation reactions and their molecular characteristics will be needed. The promising challenge is to integrate the role of various enzymes and come up with a total picture of how lignin is degraded in nature by white-rot fungi. These studies will be helpful in the remediation of homologous xenobiotics.

The major drawbacks in large-scale applications are lack of sufficient enzyme stocks and the cost of auxiliary chemicals such as enzyme mediators. Production of enzymes in fungi for the remediation purpose can be increased by several ways. The activities of the lignin degrading enzymes can be increased by the addition of different low molecular mass mediators, mostly secreted by white-rot fungi themselves. The bidegradation efficiency can be further enhanced by addition of supplementary nutrients and proper process optimization. Rate of biodegradation can also be improved by the use of contaminant adapted/acclimatized and/or genetically improved fungi. Effect of carbon and nitrogen sources and pH of the effluent on the fungus and/or enzyme(s) used for bioremediation should be studied in detail. White-rot-fungi with their enzymes seem to be highly promising for the sustainable treatment of the colored effluents. However, replacing enzymes by simpler compounds that would mimic the behavior of the catalysts would increase the rate of reaction and decrease costs. Moreover, systematic studies using different media suggest that isoenzyme ratios are a key factor for effective pollutant removal (Majeau et al., 2010). The pathway

followed by each enzymatic product (degradation or polymerization) is probably dependent not only on the enzymes and substrates involved but also on reaction conditions, such as pH, humidity, percent oxygen and electrical conductivity as well as on the presence of other compounds (Grinhut et al., 2007). The state of knowledge of parameters controlling LDE production in fungal strains is still contradictory and incomplete.

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 H_2O_2-generating system from the fungal mycelium and thus they require presence of the live fungal biomass for lignin-degradation/remediation. Immobilized peroxidase-producing fungi may be tested for this purpose. Co-immobilization of all the three major LDEs (Laccase, MnP and LiP) or fungal isolates that produce them could be a promising technology for treatment of colored effluents. Biofilms for immobilization of lignin-degrading fungi or their enzymes for continuous use in wastewater treatment is yet another challenge for the future. Isolation, characterization, immobilization and engineering of LDEs for their hyper-activation and thermo-stabilization, and their direct use in industrial processes are the area of potential future research.

The development and preparation of novel enzymes for use in biological remediation remains a key challenge and a safe and economic alternative to commonly and perhaps now redundant, physico-chemical strategies. There are perhaps two approaches: rational and evolutionary (Whiteley and Lee, 2006). With the former, amino acid sequences, functional properties and structural features of different enzymes are compared, and then tested to see if the desired effect is accomplished. In the evolutionary design, a large library of random mutations in proteins is made followed by a selection of enzymes that work well with a particular xenobiotic. In principal, multiple environmental factors would "select" enzymes to meet these challenges. Molecular evolution is a useful tool for evolving enzymes with extended substrate specificities for any recalcitrant

pollutant. Furthermore, this technology is more likely to 'succeed' then rational approaches as the latter requires multiple sets of structural and biochemical information on every enzyme involved. Sequences encoding specific enzymes can be retrieved direct from environmental samples thereby circumventing the process of isolating and screening wild-type organisms. Degenerate primers can be used to amplify conserved segments from these genes by PCR and inserted into the original functional gene. Such an approach allows rapid exploitation of the natural sequence diversity already present in the environment for creation of novel hybrid enzymes (Okuta et al., 1998). With the advent of molecular engineering the principle of developing a synthetic enzyme and the creation of micro assemblers or microchips with the role of the computer as a delivery vehicle cannot be too far into the future. Enzyme properties can be exploited to engineer active-site topology, to enlarge binding pockets and to alter the substrate specificity and stability. Consequently, the ability to modify a protein or structure to make it more stable to such conditions, or make it more resistant to self destruction, or make it target directed and functional in the presence of other toxic elements creates enormous challenges. These will be the tools and scientific technological platforms for the investigation and transformations of any wastewater or biological system. The development of more robust enzyme systems tailored by protein engineering and the search for environment friendly mediators along with future research on heterologous expression are significant hurdles that must be overcome. The search for molecular chaperons and foldases contributing to proper folding and incorporation of the prosthetic groups to ligninolytic enzymes will be helpful (Martínez, 2002). Future research should be targeted towards search for or creating fungal strains with over-producing lignin-degrading enzymes. Recombinant enzymes with these properties or protein engineering of the enzyme should be considered to achieve this goal. Increasing efficiency of these enzymes for electron transfer should be aimed at, producing hybrid enzymes. The structural information currently available should permit in the near

future to redesign ligninolytic enzymes for different biotechnological applications. The enzyme engineering would be to obtain variants with improved stability or modified substrate specificity. Attempts have been made to modify several protein structures such as MnP and Cytochrome c Peroxidase (Wilcox et al., 1998; Yeung wt al., 1997; Wang and Lu, 1999). The stability of a recombinant MnP has been intended to increase after protein engineering (Reading and Aust, 2000). Two functional hybrids of MnP and LiP mimicking versatile peroxidase (VP) have been obtained in an engineered protein (Timofeevski et al., 1999a; Timofeevski et al., 1999b; Mester and Tien, 2001). Such organisms could be used in bioreactors for treatment of waste-waters or scaling up of enzyme productions.

The varying methods to asses decolorization and detoxification are also a perplexing factor towards developing strategies for bioremediation. HPLC analyses for individual dyes are possible in some cases, though this is labour intensive and probably not applicable for monitoring complex transformations. Further studies should be conducted, using advanced analytical techniques, to elucidate the catabolic processes involved in the degradation of distinct dye groups by the lignin degrading enzymes. In the near future, the progress in the field of nanotechnology could provide biochemical engineers powerful tools for studying cell surface and topology to better understand the importance of membrane-bound oxidoreductases and their role in growth-associated degradation of organic contents in the effluents by white-rot fungi.

Biosorption has been proposed as a cheap and effective biotechnology for many years, yet has had extremely limited industrial exploitation to date, even as an addition to conventional pollutant treatment approaches in hybrid technologies. Common suggestions for future research directions include identification of better and more selective biosorbents, more development of biosorption models and identification of biosorption mechanisms, and further assessments of market size, and costs of development. The importance of biosorption in the environment and conventional biotreatment processes perhaps suggests further research should be

directed in these areas. Although ascomycetes are common soil and litter inhabitants, their role in the degradation process has been studied to a lesser extent than that of white-rot basidiomycetous fungi. As the ligninolytic enzyme system of ascomycetes is less prominent the primary mechanism of color removal could be sorption as seen in the present study. The ascomycetes with greater capability of sorption should be screened.

For whole cell bioremediation, microscopic observation should be performed to determine if effective decontamination has really been achieved or if the pollutants have simply been adsorbed onto the biomass or accumulated into the cell compartment. Fluorescence microscopy may be useful to detect pollutant accumulation as demonstrated in the work of Verdin et al. (2006).

It is possible that results obtained at the lab scale may not reflect the situation in natural environments. The efficacy of the fungal isolates in treating effluents from common effluent treatment (CET) plants which contain mixtures of effluents from various industries should also be assessed and attempts should be made to scale up so that can be used at industrial level.

Currently, various physical, chemical and biological processes are mostly applied singly to treat the wastewater. As more insights are gained in understanding the chemical nature of recalcitrance, enhanced transformation and, hopefully, complete mineralization of xenobiotic effluents could be achieved by modular multi-step processes, coupling reduction and oxidation reactions by abiotic or biotic means. This principle seems to be bearing fruit for an increasing range of recalcitrant molecules. More work is required to design a combination of various techniques for remediation purposes.

6.1 Media

6.1.1 Low nitrogen (LN) (Tien & Kirk, 1988) **modified medium:** Glucose and glycine at 3.75% and 0.5% concentration respectively were added to a mineral salts base which contained the following components; 0.0001%, 7% & 10% final concentration of thiamine, trace elements solution and basal salts solution respectively. The pH was adjusted to 4.5 using citrate phophste buffer. The volume was brought up to 100 ml using either D/W or S/W, as per the requirement.

Basal salts solution: KH_2PO_4, $MgSO_4$, and $CaCl_2$ at 2%, 5% & 0.1% respectively, the final volume was made with D/W.

Trace elements solution: 1.5g nitrilic triacetate was dissolved in 800 ml distilled water and the pH was adjusted to 6.5 using 1N KOH to which, the following components were added:

$MgSO_4$	3g
$MnSO_4$	0.5g
NaCl	1g
$FeSO_4.7H_2O$	0.1g
$CaCl2.6H_2O$	0.1g
$ZnSO_4.7H_2O$	0.1g
$CdCl_2$	0.08g
$AlK(SO_4)_2.12H_2O$	0.01g
H_3BO_3	0.01g
$Na_2MoO_4.2H_2O$	0.01g

The solution was made up to 1L with D/W and stored in the dark.

6.1.2 Boyd and Kohlmeyer (B&K) agar medium

Yeast Extract powder	0.1 g
Peptone powder	0.2 g
Dextrose	1.0 g
Agar powder	1.5 g
Seawater/Distilled water	100 ml

6.1.3 Malt Extract Agar (1/5 strength)

Malt Extract Agar Base (MEA) powder	1 g
Agar powder	1.5 g
Seawater	100 ml

6.1.4 Malt Extract Broth

Malt Extract Broth base (MEB) powder	2.0 g
Seawater	100 ml

6.2 Antibiotic Solution

The antibiotic solution contained 400,000 U of procaine penicillin and 1g of streptomycin sulphate in 100 ml of sterile distilled water.

6.3 Dissolved Oxygen Estimation (Wrinklers Method) (APHA, AWWA, WEF, 2005)

A 300-ml glass Biological oxygen Demand (BOD) stoppered bottle was placed below the water surface and filled up to the brim. Immediately without the introduction of air bubbles, 2 ml of $MnSO_4$ followed by 2 ml of KI-azide reagent was added. The bottle was then stoppered with care to ensure no air was introduced. The sample was mixed by inversion several times. A brownish-orange cloud of precipitate indicated the presence of dissolved oxygen. It was allowed to precipitate after mixing several times by inversion. Just above the surface, 2 ml of concentrated H_2SO_4 was added and the bottle was then stoppered and inverted several times to allow the precipitate to get dissolved by the acid. An aliquot of this treated sample (201 ml) was titrated with $Na_2S_2O_3$ using starch as indicator.

The concentration of dissolved oxygen (1 mg L^{-1} dissolved oxygen) in the sample is equivalent to the amount of titerant (Ml) used.

6.4 Enzyme Estimation Methods

6.4.1 Laccase assay: Using ABTS (Niku-Paavola et al., 1988)

Laccase activity in the sample is spectrophotometrically determined by monitoring the rate of product (dark green color) formation due to the enzymatic oxidation of ABTS.

In a 1ml of cuvette, the following components are added;

0.5ml of 2mM ABTS prepared in buffer of desired pH range +0.05 to 0.5 ml of enzyme (to be tested) was added, the same buffer in which the ABTS was prepared in, was used to make up the total volume to 1 ml.

The kinetic reaction is spectrophotometrically measured at 405 nm for 1 min at the desired temperature, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

Calculations:

Laccase (U L^{-1}) = [ΔA_{405}×total vol×dilution factor×10^6] ÷ [€ABTS×Sample vol]

Where,

ΔA_{405} = rate ofreaction i.e. final abs-initial abs ÷ time (min)

€ABTS = molar extinction coefficient of the radical-cation ABTS (35000)

total vol = total volume of reaction mixture (ml)

Sample vol = volume of enzyme used (ml)

Laccase is expressed as enzyme units per liter i.e. U L^{-1} where one enzyme unit is expressed as µmol of the product formed per minute.

6.4.2 Lignin Peroxidase (LiP) assay (Paszezynski et al., 1988)

LiP catalyses the oxidation of veratryl alcohol to veratraldehyde by H_2O_2. Only the aldehyde shows absorbance at 312 nm.

In a 1ml cuvette, the following components are added;

0.5ml of reaction mixture* + 0.5 ml of enzyme solution to be tested

*Reaction mixture (25 ml) = 20ml of 125 mM d-tartaric acid buffer (pH 2.5) + 2.5 ml of 40mM veratryl alcohol + 2.5 ml of 8 mM H_2O_2.

The kinetic reaction is spectrophotometrically measured at 310 nm for 1 min at the desired temperature. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme.

Calculations:

LiP (U L^{-1}) = [ΔA_{310}×total vol×dilution factor×10^6] ÷ [$\epsilon_{Veratrylaldehyde}$×Sample vol]

Where;

ΔA_{310} = rate of reaction i.e. final abs - initial abs ÷ time (min)

$\epsilon_{Veratrylaldehyde}$ = molar extinction coefficient of Veratrylaldehyde (9300)

Total vol = total volume of reaction mixture (ml)

Sample vol = volume of enzyme used (ml)

Lignin peroxidase is expressed as enzyme units per litre i.e. U L^{-1}, where one enzyme unit is expressed as the µmol of the product formed per minute.

6.4.3 Manganese-dependent Peroxidase (MnP) assay (Paszezynski et al., 1988)

MnP catalyses the oxidation of mn^{+2} to Mn^{+3} by H_2O_2 the product Mn^{+3}, forms a transient stable complex with tartaric acid, showing a characteristic absorbance at 238 nm.

In a 1 ml cuvette, the following components are added;

0.8875 ml reaction mixture* + 0.0125 ml of 8 mm H_2O_2 + 0.1 ml enzyme solution to be tested.

*Reaction mixture (17.75 ml) = 4 ml of 500 mM sodium tartarate buffer (pH 5) + 0.2 ml of 10 mM $MnSO_4$ + 13.55 ml D/W.

The kinetic reaction is spectrophotometrically measured at 238 nm for 1 min at the desired temperature, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

Calculations:

MnP (U L^{-1}) = [ΔA_{238}×total vol×dilution factor×10^6] ÷ [$€_{Mn}^{+3}$ ×Sample vol]

Where;

ΔA_{238} = rate of reaction i.e. final abs – initial abs ÷ time (min)

$€_{Mn}^{+3}$ = molar extinction coefficient of Mn^{+3} – tartarate complex (6500)

Total vol = total volume of reaction mixture (ml)

Sample vol = volume of enzyme used (ml)

Manganese dependent peroxidase was expressed as enzyme units per litre i.e. U L^{-1}, where one enzyme unit is expressed as the µmol of the product formed per minute.

6.4.4 Glucose oxidase assay

Glucose Oxidase [GO] catalyses the oxidation of Glucose to Gluconic acid. The generation of H_2O_2 is indirectly measured by oxidation of O-dianisidine in the presence of peroxidase.

Method: The reaction velocity is determined by an increase in absorbance at 460 nm resulting from the oxidation of o-dianisidine per minute at 25 ºC and pH 6.0 under the conditions specified.

Reagents:

0.1 M Potassium phosphate buffer, pH 6.0

1% o-Dianisidine

Peroxidase: Dissolve peroxidase at a concentration of 200 µg ml^{-1} in reagent grade water.

18% Glucose: Allow mutarotation to come to equilibrium by standing overnight at room temperature.

Dianisidine-buffer mixture: Prepare by diluting 0.1 ml of 1% o-dianisidine in 12 ml of o.1 M potassium phosphate buffer pH 6.0. Saturate with oxygen for 10 min within 30 min of use.

Procedure: Set spectrophotometer at 460 nm and 25 ºC. Pipette into cuvette as follows:

Dianisidine-buffer mixture, pH 6.0 (oxygenated) 2.5 ml

18% Glucose 0.3 ml

peroxidase 0.1 ml

Incubate in spectrophotometer for 3-5 minutes to achieve temperature and establish blank rate if any. Add 0.1 ml of appropriately diluted sample and record increase in A_{460} for 4-5 minutes. Calculate ΔA_{460} from the linear portion of the curve.

Calculation: Units $mg^{-1} = \Delta A_{460}\ min^{-1} \div [11.3 \times$ mg enzyme ml^{-1} reaction mixture].

6.5 Chemical Oxygen Demand Estimation

Chemical oxygen demand (COD) was estimated by open reflux method (APHA, AWWA, WEF, 2005).

Most types of organic matter are oxidized by a boiling mixture of Chromic and sulfuric acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced $K_2Cr_2O_7$ is titrated with ferrous ammonium sulfate to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter is calculated in terms of oxygen equivalent.

Reagents:

a) Standard potassium dichromate solution, 0.04167M or 0.25N.

b) Sulfuric acid reagent: Technical grade, crystals of Ag_2SO_4 was added to the conc. H_2SO_4 at the rate of 5.5 g Ag_2SO_4 per kg H_2SO_4. It was left for 1-2 days to get dissolved.

c) Ferroin indicator solution, purchased already prepared.

d) Standard ferrous ammonium sulfate (FAS) titrant, approximately 0.25 M

Procedure:

The sample was appropriately diluted in the 100 ml round bottom flask prior to the estimation. In 10 ml of diluted sample, 0.2 g of $HgSO_4$ was added, and mixed gently with 5 ml of sulfuric acid to dissolve $HgSO_4$. The mixing was done over the ice to avoid possible loss of volatile materials. To this, 5 ml of $K_2Cr_2O_7$ (0.25N) was added and flask was attached to the condenser and turned on cooling water. The remaining sulfuric acid reagent (10 ml) was added through open end of condesor. Swirling and mixing was continued while adding sulfuric acid reagent. Open end of condenser was covered with a stopper to prevent foreign material from entering refluxing mixture and refluxed for 2 h. Afterwards, it was disconnected and the mixture was diluted to about twice its volume with distilled water. After cooling to room temperature, excess $K_2Cr_2O_7$ was titrated with FAS, using 0.10-0.15 ml ferroin indicator. The first sharp color change from blue-green to reddish brown that persisted for 1 m or longer was taken as an end point.

Calculation:

COD as mg O_2 L^{-1} = [A –B) × M ×8000] ÷ ml sample

Where;

A = ml FAS used for blank,

B = ml FAS used for sample,

M = molarity of FAS, and

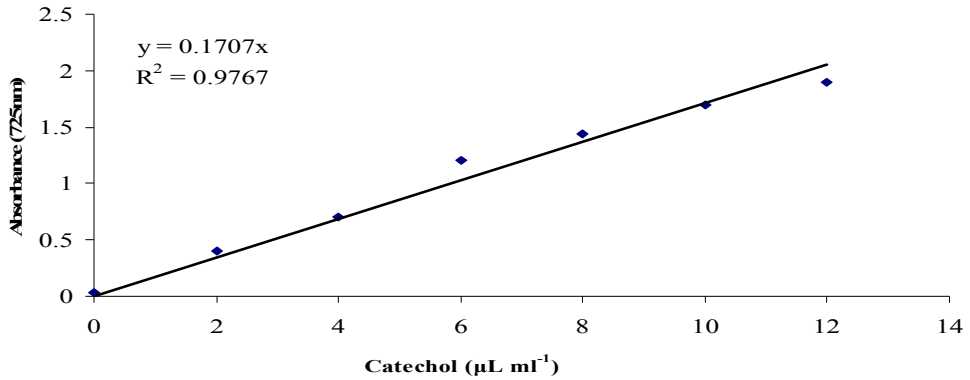
8000 = milliequivalent weight of oxygen × 1000 ml L^{-1}.

6.6 Total Phenolics Estimation

The measurement for Total Phenolics was based on the method reported by Singleton and Rossi, (1965).

Procedure:

The sample (1 ml) was taken in a graduated 25 ml test tube and 1 ml of Folin-Ciocalteau (F-C) reagent was added followed by 2 ml of Na_2CO_3 solution. Tubes were shaken and boiled in a water bath for exactly 1 min. After cooling under running tap water, the blue solution was diluted to 25 ml with D/W and absorbance was measured at 725 nm. The unkown was read from a tandard curve made from different concentrations of Chatechol. A blank containing all the reagents minus F-C reagent was used to adjust the absorbance to zero.



Standard graph for the estimation of Total phenolics. Catechol (Sigma Chemicals, Mo, USA) was used as the reference standard and Total phenolics were expressed as g L^{-1}

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