Chapter 3

To study the influence of various parameters on decolorization and detoxification of colored effluents by selected marine fungi producing these enzymes
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Natural pigments used for coloring textiles have been replaced by "fast colors" which do not fade on exposure to light, heat and water.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 $7 \times 10^5$ 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
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In 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. These persistent 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 CO₂, H₂O 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
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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. Interestingly, 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 MnCl₂. 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
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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_{2}O_{2}-generating enzymes, in various combinations, which play an important role in the
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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)
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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).

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\begin{align*}
\text{H}_2\text{O} & \quad \text{Laccase} \\
\text{O}_2 & \quad \text{Laccase}_{\text{ox}} \quad \text{Mediator} \quad \text{Mediator}_{\text{ox}} \quad \text{Substate} \quad \text{Substate}_{\text{ox}}
\end{align*}
\]

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).
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<table>
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<tr>
<th>ABTS</th>
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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 H₂O₂-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 H₂O₂-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 H₂O₂-dependent oxidation of a wide variety of non-phenolic lignin model compounds and aromatic pollutants. It is the strongest known fungal peroxidase 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; Martinez, 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$_2$O$_2$, 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$_2$O$_2$, 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$_2$O$_2$, extra-cellularly (Shah and Nerud, 2002). The generation of oxygen radicals and activity of peroxidases requires the presence of extra-cellular H$_2$O$_2$. 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.

![Diagram](image)

**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 (Munoz 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
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(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 (Kachlishvili 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.
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 cglec1 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).
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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 °C respectively (Yang et al., 2004; Asgher et al., 2007). For MnPs, optimum pH of 4-7 and optimum temperature of 40-60 °C 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 °C 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 °C) 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 O2 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
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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$_2$O$_2$-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)

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

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).
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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 (Oiler 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
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(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) KNO₃, 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 (CuSO₄) 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
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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 bagasse 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 °C 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 °C. Each of the effluents mentioned above having final concentration of 1000 color units was incubated at 30 °C 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 °C) 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
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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 (LC₅₀ 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 quadrupole-time of flight mass spectrometer (Model Qstar XL, Applied Biosystems, Rotkreuz-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
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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 °C 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
Decolorization and detoxification of effluents by marine fungi and their enzymes

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 °C 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 °C 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 °C 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 x 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$_2$O). Effluents after each stage of treatment were freeze-dried. The lyophilized samples were dissolved in D$_2$O 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 °C 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 cm²). 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), chicken 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 °C. 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 spectrophotometrically 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, CuSO₄ 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 ultrafiltration 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 100 ml Erlenmeyer flasks containing 10 ml of RB4 at a concentration of 1000 mg L⁻¹, dissolved with 0.1M Na acetate buffer (pH 5.0). Partially purified laccase solution (10 μl) 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°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 RB4 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 H₂O 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°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)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TEA</th>
<th>TEB</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Color (Pt-Co units)</td>
<td>1,44,180</td>
<td>52,500</td>
</tr>
<tr>
<td>Absorbance maxima (nm)</td>
<td>505</td>
<td>667</td>
</tr>
<tr>
<td>COD (mg L⁻¹)</td>
<td>30,000</td>
<td>20,000</td>
</tr>
<tr>
<td>Total phenolics (g L⁻¹)</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Dye components</td>
<td>Azo dye-20</td>
<td>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</td>
</tr>
<tr>
<td>Total solids (g L⁻¹)</td>
<td>0.254</td>
<td>0.51</td>
</tr>
<tr>
<td>Carbonates (g L⁻¹)</td>
<td>30.0</td>
<td>36.0</td>
</tr>
<tr>
<td>Na⁺ (g L⁻¹)</td>
<td>0.043</td>
<td>0.013</td>
</tr>
<tr>
<td>Ca⁺ (g L⁻¹)</td>
<td>0.03</td>
<td>0.009</td>
</tr>
<tr>
<td>SO₄ (g L⁻¹)</td>
<td>7.23</td>
<td>1.23</td>
</tr>
<tr>
<td>Cl (g L⁻¹)</td>
<td>150.0</td>
<td>191.7</td>
</tr>
<tr>
<td>PO₄ (g L⁻¹)</td>
<td>0.021</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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 KNO₃ 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: Component Dyes of the effluents, their C.I. name, maximum visible absorbance and chemical structure (Where available)

<table>
<thead>
<tr>
<th>C.I. Name</th>
<th>Absorbance Maxima (nm)</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive Blue 4</td>
<td>599</td>
<td><img src="image1" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Reactive Blue 140</td>
<td>654</td>
<td><img src="image2" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Reactive Blue 160</td>
<td>609</td>
<td><img src="image3" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Reactive Red 11</td>
<td>545</td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Reactive Yellow 145</td>
<td>419</td>
<td><img src="image5" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Reactive Green 19</td>
<td>634</td>
<td><img src="image6" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Effluents</th>
<th>Control (without effluent)</th>
<th>TEA#</th>
<th>TEB#</th>
<th>MSW#</th>
<th>BL#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biomass (g L⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>5.3</td>
<td>7.1</td>
<td>4.0</td>
<td>7.5</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>4.1</td>
<td>5.2</td>
<td>5.6</td>
<td>2.7</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.2</td>
<td>7.5</td>
<td>4.8</td>
<td>7.1</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.9</td>
<td>4.5</td>
<td>3.7</td>
<td>9.5</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Corn steep liquor</td>
<td>4.8</td>
<td>4.5</td>
<td>4.7</td>
<td>3.1</td>
<td>7.3</td>
<td></td>
</tr>
</tbody>
</table>

|                         | Laccase Activity (U L⁻¹) |                            |      |       |      |      |
| KNO₃                    | 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) |                            |      |       |      |      |
| KNO₃                    | 0         | 353                        | 0    | 0     | 2421 |
| Glycine                 | 12        | 508                        | 38   | 394   | 1679 |
| Glutamic acid           | 0         | 0                          | 0    | 450   | 2372 |
| Beef extract            | 25        | 493                        | 0    | 604   | 2178 |
| Corn steep liquor       | 46        | 1760                       | 222  | 0     | 671  |

|                         | LiP Activity (U L⁻¹) |                            |      |       |      |      |
| KNO₃                    | 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 (%)  |                            |      |       |      |      |
| KNO₃                    | -         | 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%.
Fig. 3.5: *In vitro* decolorization of effluents with 1000 color units each using 10U of crude laccase obtained from different nitrogen sources namely, KNO₃, Glycine, Glutamic acid, Beef extract and Corn steep liquor. Decolorization was monitored from 0 to 48 h. The 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 continuous 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.
Decolorization and detoxification of effluents by marine fungi and their enzymes

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
Decolorization and detoxification of effluents by marine fungi and their enzymes

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 hyrsina*. 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.

<table>
<thead>
<tr>
<th>Cultures (Phylum)</th>
<th>TEA</th>
<th>TEB</th>
</tr>
</thead>
<tbody>
<tr>
<td># 2a (Basidiomycete)</td>
<td>32.3 ± 5.4</td>
<td>6.8 ± 1.9</td>
</tr>
<tr>
<td># 15V (Basidiomycete)</td>
<td>27.7 ± 2.3</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td># 16V (Ascomycete)</td>
<td>38.9 ± 4.8</td>
<td>41.3 ± 4.6</td>
</tr>
<tr>
<td># C3 (Ascomycete)</td>
<td>43.0 ± 5.7</td>
<td>29.8 ± 2.3</td>
</tr>
</tbody>
</table>

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 $\lambda_{\text{max}}$ of reactive yellow 145 fell into this spectral range (Table 3.3).

![Absorbance spectra](image.png)

**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,
Decolorization and detoxification of effluents by marine fungi and their enzymes

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.

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Concen of the effluent (%)</th>
<th>Basidiomycete</th>
<th>Ascomycete</th>
<th>Controlb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NIOCC #2a Cerrena unicolor</td>
<td>NIOCC #15V Coriolopsis byrsina</td>
<td>NIOCC #16V Endothia sp.</td>
</tr>
<tr>
<td>Biomass (g L⁻¹)</td>
<td>Controla</td>
<td>3.6 ± 0.2</td>
<td>4.1 ± 0.5</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.0 ± 0.3</td>
<td>4.6 ± 0.5</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.9 ± 0.7</td>
<td>5.3 ± 0.9</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>4.2 ± 1.7</td>
<td>4.4 ± 0.8</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td>Laccase (U L⁻¹)</td>
<td>Controla</td>
<td>2,015 ± 615.8</td>
<td>1,951 ± 74.5</td>
<td>52 ± 12.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>141.9 ± 42.6</td>
<td>82.4 ± 16.8</td>
<td>32.8 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>46.3 ± 4.5</td>
<td>39.8 ± 11.6</td>
<td>41.5 ± 12.1</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>38.4 ± 7.4</td>
<td>40.9 ± 5.9</td>
<td>47.8 ± 3.6</td>
</tr>
<tr>
<td>% Decolorization</td>
<td>20</td>
<td>44.4 ± 1.5</td>
<td>31.2 ± 2.1</td>
<td>48.0 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>55.6 ± 1.6</td>
<td>29.9 ± 0.3</td>
<td>27.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>23.9 ± 5.8</td>
<td>26.7 ± 7.2</td>
<td>31.9 ± 4.3</td>
</tr>
<tr>
<td>24 h-LC₅₀</td>
<td>20</td>
<td>14.2 ± 1.4</td>
<td>28.0 ± 14.1</td>
<td>18.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.3 ± 0.1</td>
<td>5.0 ± 1.1</td>
<td>5.7 ± 1.3</td>
</tr>
<tr>
<td>% reduction in total phenolics</td>
<td>20</td>
<td>19.3 ± 0.9</td>
<td>28.1 ± 1.1</td>
<td>21.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>22.6 ± 2.5</td>
<td>24.9 ± 6.6</td>
<td>21.7 ± 5.4</td>
</tr>
<tr>
<td>% reduction in COD</td>
<td>20</td>
<td>48.3 ± 3.0</td>
<td>62.9 ± 11.3</td>
<td>90.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>20.6 ± 11.7</td>
<td>28.5 ± 4.4</td>
<td>24.1 ± 6.8</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Concentration of the effluent (%)</th>
<th>Basidiomycete</th>
<th>Ascomycete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>NIOCC #2a</td>
<td>Cerrena unicolor</td>
<td>NIOCC #15V</td>
</tr>
<tr>
<td>Biomass (g L⁻¹)</td>
<td>Control*</td>
<td>3.6 ±0.2</td>
<td>4.1 ±0.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.9 ±0.2</td>
<td>3.7 ±0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.9 ±1.3</td>
<td>4.3 ±0.5</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>3.1 ±0.1</td>
<td>4.2 ±0.2</td>
</tr>
<tr>
<td>Laccase (U L⁻¹)</td>
<td>Control*</td>
<td>2,015 ±615.8</td>
<td>1,951 ±74.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5,015 ±519.7</td>
<td>4,165 ±95.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>534 ±24.9</td>
<td>145 ±20.3</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>855 ±61.9</td>
<td>284 ±85.7</td>
</tr>
<tr>
<td>% Decolorization</td>
<td>Control*</td>
<td>76.4 ±0.9</td>
<td>61.5 ±8.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>42.8 ±1.6</td>
<td>32.4 ±3.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>38.6 ±3.0</td>
<td>38.8 ±4.7</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>57.9 ±14.4</td>
<td>38.2 ±9.9</td>
</tr>
<tr>
<td>24 h-LC₅₀</td>
<td>Control*</td>
<td>13.5 ±3.4</td>
<td>13.7 ±2.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7.9 ±1.3</td>
<td>9.5 ±2.7</td>
</tr>
<tr>
<td>% reduction in total phenolics</td>
<td>20</td>
<td>68.5 ±5.3</td>
<td>52.9 ±6.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>82 ±4.1</td>
<td>70 ±15.3</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>83 ±2.4</td>
<td>67 ±11.2</td>
</tr>
<tr>
<td>% reduction in COD</td>
<td>20</td>
<td>43.7 ±7.0</td>
<td>52.3 ±7.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>46 ±1.6</td>
<td>37 ±8.8</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>48 ±1.2</td>
<td>55 ±15.2</td>
</tr>
</tbody>
</table>

All the parameters were estimated on day 6 after addition of the effluent
* 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 uninoculated 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 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) 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.

![Decolorization graph](image)

Fig. 3.14: Effect of mediators on decolorization of TEA (1% concentration) after 72h incubation with culture supernatants of four fungi at 30 °C. 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.*

<table>
<thead>
<tr>
<th>Laccase (U ml⁻¹)</th>
<th>Decolorization (%)</th>
<th>TEA (20%)</th>
<th>TEB (20%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NIOCC #2a (C. unicolor)</td>
<td>T. versicolor</td>
</tr>
<tr>
<td></td>
<td>Hours</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>14 ± 2.3</td>
<td>18 ± 3.8</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>26 ± 1.9</td>
<td>21 ± 4.9</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>33 ± 3.7</td>
<td>29 ± 5.6</td>
</tr>
<tr>
<td>20.0</td>
<td></td>
<td>39 ± 7.3</td>
<td>39 ± 4.7</td>
</tr>
</tbody>
</table>

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.
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>
<thead>
<tr>
<th>Source of the effluents</th>
<th>Effluents Analysed</th>
<th>Sugar Mill Effluent</th>
<th>Biotech Pharmaceutical Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters analysed</td>
<td>ROF (Reverse Osmosis Feed)</td>
<td>ROR (Reverse Osmosis Reject)</td>
<td>CAT I (Conventional aeration tank Inlet)</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>7.4</td>
<td>7.7</td>
</tr>
<tr>
<td>COD (g L⁻¹)</td>
<td>30.8</td>
<td>52.8</td>
<td>23.2</td>
</tr>
<tr>
<td>Total Phenolics (g L⁻¹)</td>
<td>1.9</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Color Units (Pt-Co Units)</td>
<td>72,500</td>
<td>49,000</td>
<td>52,000</td>
</tr>
<tr>
<td>Total Reducing Sugars (g L⁻¹)</td>
<td>3.8</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Total Solids (g L⁻¹)</td>
<td>0.04</td>
<td>0.061</td>
<td>0.025</td>
</tr>
<tr>
<td>Sulphates (SO₄²⁻) (g L⁻¹)</td>
<td>0.12</td>
<td>0.21</td>
<td>0.82</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>96.0</td>
<td>98.9</td>
<td>338</td>
</tr>
</tbody>
</table>

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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.
Decolorization and detoxification of effluents by marine fungi and their enzymes

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

<table>
<thead>
<tr>
<th>Effluent</th>
<th>Treatment</th>
<th>% Decolorization</th>
<th>% Reduction COD</th>
<th>% Reduction Phenolics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After whole-culture treatment (Step 2)</td>
<td>Followed by biosorption (Step 3)</td>
<td>After whole-culture treatment (Step 2)</td>
<td>Followed by biosorption (Step 3)</td>
</tr>
<tr>
<td>ROF</td>
<td>Without sonication</td>
<td>20± 3.9</td>
<td>49± 2.3</td>
<td>38± 6.5</td>
</tr>
<tr>
<td></td>
<td>With sonication</td>
<td>41± 1.7</td>
<td>63± 3.3</td>
<td>56± 4.4</td>
</tr>
<tr>
<td>ROR</td>
<td>Without sonication</td>
<td>27± 2.4</td>
<td>50± 3.8</td>
<td>38± 4.6</td>
</tr>
<tr>
<td></td>
<td>With sonication</td>
<td>40± 3.3</td>
<td>61± 2.4</td>
<td>50± 2.8</td>
</tr>
<tr>
<td>CAT 1</td>
<td>Without sonication</td>
<td>27± 2.7</td>
<td>50± 3.3</td>
<td>53± 2.5</td>
</tr>
<tr>
<td></td>
<td>With sonication</td>
<td>56± 2.7</td>
<td>75± 2.5</td>
<td>71± 1.3</td>
</tr>
<tr>
<td>CAT 0</td>
<td>Without sonication</td>
<td>32± 4.1</td>
<td>53± 4.6</td>
<td>29± 6.3</td>
</tr>
<tr>
<td></td>
<td>With sonication</td>
<td>60± 8.4</td>
<td>78± 2.6</td>
<td>49± 0.9</td>
</tr>
</tbody>
</table>

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.
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.
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).

<table>
<thead>
<tr>
<th>Effluents</th>
<th>Treatments</th>
<th>Effluent after sonication (Step 1)</th>
<th>whole-fungus treatment of sonicated effluent followed by biosorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without any treatment (control)</td>
<td>whole-fungus treatment of unsonicated effluent</td>
<td>whole-fungus treatment of unsonicated effluent followed by biosorption</td>
</tr>
<tr>
<td>ROF</td>
<td>86 (48-130)$^a$</td>
<td>234 (178-331)$^b$</td>
<td>290 (202-366)$^{bc}$</td>
</tr>
<tr>
<td>ROR</td>
<td>114 (66-172)$^a$</td>
<td>237 (160-325)$^b$</td>
<td>244 (170-327)$^b$</td>
</tr>
<tr>
<td>CAT 1</td>
<td>165 (110-229)$^a$</td>
<td>269 (194-354)$^b$</td>
<td>285 (204-378)$^b$</td>
</tr>
<tr>
<td>CAT 0</td>
<td>96 (59-139)$^a$</td>
<td>214 (138-332)$^b$</td>
<td>335 (207-452)$^c$</td>
</tr>
</tbody>
</table>

LC$_{50}$ values are in µl ml$^{-1}$. Higher LC$_{50}$ 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 0.05%).
Fig. 3.17: Decolorization, laccase and MnP production by NIOCC #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 by 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.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Unloaded biomass</th>
<th>Band Positions (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 0 h</td>
<td>After 48 h</td>
</tr>
<tr>
<td>-OH and/or –NH stretching (3500-3000 cm⁻¹)</td>
<td>3292.3</td>
<td>3292.3</td>
</tr>
<tr>
<td>-CH stretching (3000-2800 cm⁻¹)</td>
<td>2922.0</td>
<td>2927.7</td>
</tr>
<tr>
<td>Amide-I / Amide-II band (1800-1500 cm⁻¹)</td>
<td>1681.8</td>
<td>1651.0</td>
</tr>
<tr>
<td></td>
<td>1637.5</td>
<td>1635.5</td>
</tr>
<tr>
<td></td>
<td>1533.3</td>
<td>1537.2</td>
</tr>
<tr>
<td>Mixed region (1500-1200 cm⁻¹)</td>
<td>1463.9</td>
<td>1469.7</td>
</tr>
<tr>
<td></td>
<td>1240.1</td>
<td>1247.9</td>
</tr>
<tr>
<td>Polysaccharide region (1200-900 cm⁻¹)</td>
<td>1076.2</td>
<td>1062.7</td>
</tr>
<tr>
<td>Finger print region (900-700 cm⁻¹)</td>
<td>-</td>
<td>777.3</td>
</tr>
</tbody>
</table>

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.
Decolorization and detoxification of effluents by marine fungi and their enzymes (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 $\delta$ 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, $\delta$ 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 $\delta$ 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.
Decolorization and detoxification of effluents by marine fungi and their enzymes

An 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_{8}H_{22}N_{6}S_{2}C_{12}) is given in the Table 3.3. It is an anthraquinonic dye of molar mass of 637.4 and $\lambda_{\text{max}}$ 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)
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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).

![Graph showing absorbance vs wavelength for different treatments of RB4.]

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: 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.
Decolorization and detoxification of effluents by marine fungi and their enzymes (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. 3.25: 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* nauplii 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**

<table>
<thead>
<tr>
<th>Toxicity tests</th>
<th>Control (D/W)</th>
<th>Untreated</th>
<th>Direct Sorption</th>
<th>Enzyme treated</th>
<th>Enzyme treated followed by Sorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemia test (24h-LC$_{50}$)*</td>
<td>--</td>
<td>48(43-95)*</td>
<td>57(48-68)*</td>
<td>51(45-59)*</td>
<td>99(79-135)*</td>
</tr>
<tr>
<td>Phytotoxicity (48h)$^5$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germination index (%)</td>
<td>--</td>
<td>57.6(0.4)*</td>
<td>63.9(1.7)$^{ab}$</td>
<td>66.9(1.7)$^b$</td>
<td>91.4(4.5)$^c$</td>
</tr>
<tr>
<td>Dry weight (root)</td>
<td>2.6(0.1)*</td>
<td>0.6(0.03)$^b$</td>
<td>1.0(0.02)$^c$</td>
<td>1.0(0.0)$^c$</td>
<td>1.8(0.04)$^d$</td>
</tr>
</tbody>
</table>

*LC$_{50}$ values are in ul ml$^{-1}$. Upper and lower confidence limits are within brackets.
$^5$ 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 decolorization 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
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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,
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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 (Zümrüye 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 ≥ 0.8) between the laccase titer and decolorization in the present studies. On the other hand, studies involving
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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 \( \text{H}_2\text{O}_2 \) 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 LC50 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 (Rodriguez-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%
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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. 1-62. 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
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oxygen-dependent laccase activity as it helps in degassing and removal of H₂S 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
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(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 *Tremetes 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-Tielo 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.,
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
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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 hypochlorite 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 anthraquinone-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 assess 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.