# SCREENING, PURIFICATION, CHARACTERIZATION AND APPLICATION OF LACCASE FROM *Pleurotus eous.*

**A PROJECT REPORT**

*Submitted by*

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*in partial fulfillment for the award of the degree*

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## BONAFIDE CERTIFICATE

Certified that this project report **"SCREENING, PURIFICATION, CHARACTERIZATION AND APPLICATION OF LACCASE FROM *Pleurotus eous*"** is the bonafide work of **"NANTHAKUMAR.V, SRIVATSAN.S, VINOTHINI.J"** who carried out project work under my supervision.

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# ABSTRACT

## ABSTRACT

The present study was carried out with the objective of purification, characterization, application and stabilization of laccase from the fungi *Pleurotus eous (P.eous).* Laccase was purified 38 folds by ammonium sulfate fractionation and Ion exchange chromatography. The enzyme had a molecular weight of 64 kDa. The purified laccase was most active at pH 6.0 and optimum temperature was observed at 55°C. Laccase displayed normal Michaelis-Menton kinetics for its substrate guaiacol. Laccase activity was suppressed by metal ions like $Al^{3+}$$Fe^{2+}$ $Fe^{3+}$ $Mg^{2+}$ & $Mn^{2+}$ and SDS, were in 100% inhibition was found in 40mM and 50mM of $Fe^{2+}$and $Al^{3+}$ and other metal ions showed partial inhibition. Decolourization studies were carried out using crude filtrate of *Pleurotus eous* alone and also in combination of spore suspension of *Bacillus subtilis.* It was observed that using consortium of microorganism, a complete decolourization of both paper and textile effluents were achieved within a day thus proving to be an efficient method of treating waste water containing dye stuffs at low cost. Drying experiments were carried to test the efficiency of sugars like sucrose, lactose, maltose and mannitol in conferring storage stability and thermostability on laccase. Out of the sugars tested, sucrose was found to be the best enzyme stabilizer; the lyophilized enzyme was stable upto 30 days at 55°C.

# TABLE OF CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2'-azino bis (3-ethylbenzthiazoline-6-sulphonic acid) |
| BSA | Bovine serum albumin |
| EDTA | Ethylene diamine tetra-acetic acid |
| LiPs | Lignin peroxidases |
| LMS | Laccase-mediated systems |
| MnPs | Manganese peroxidases |
| NB | Nutrient broth |
| PAGE | Polyacryl amide gel electrophoresis |
| PAHs | Polycyclic aromatic hydrocarbons |
| PDA | Potato-Dextrose-Agar |
| PDB | Potato dextrose broth |
| SDS | Sodium dodecyl sulphate |
| TLC | Thin layer chromatography |
| TNT | 2, 4, 6-trinitrotoluene |

# CHAPTER I

# INTRODUCTION

# 1. INTRODUCTION

The laccase class of enzymes is widely distributed throughout nature and exhibits a wide range of roles and substrate specificity. They are sometimes also referred as polyphenol oxidases (PPOs) and are a part of the multi-copper oxidase family .The laccase was first discovered in the tree *Rhus vernicifera* more than a hundred years ago (Yoshida, 1883). The plant and fungal laccases are similar in that they have low substrate specificity, reacting with a host of derivatized phenols with $K_m$ of a few millimolar. The lack of substrate specificity often leaves the natural role of laccases in question, but this versatility has allowed new roles to be found for many in several commercial processes, including delignification, decolourization of industrial waste, and wine clarification (Mayer and Staples, 2002).

Laccase, a kind of polyphenol oxidase containing copper atoms, can oxidize an array of organic and inorganic substrates, including mono-, di-, and polyphenols, aminophenols, methoxyphenols, as well as metal complexes like ferrocene, ferrocyanide or iodide, by concomitant four-electron reduction of oxygen to water. Laccase typically contains 15–30% carbohydrate. Laccase usually has an acidic isoelectric point and has a molecular mass of 60–90 kDa. Laccase is encoded by a family of genes and produced in the form of multiple isozymes. It has been proven that genes encoding laccase isozymes were differentially regulated (Soden *et al.*, 2001).

Laccase can be found in plants, insects and bacteria, but its major source is fungi. In fungi, it is associated with many biological functions such as lignin degradation, removal of potentially toxic phenols, morphogenesis, pigment synthesis, sporulation, phytopathogenesis and fungal virulence. Laccase is considered to be a potentially important industrial enzyme. Laccase can be applied extensively in many fields, which include waste

detoxification and textile dye transformation, delignification of lignocellulosics and cross-linking of polysaccharides, upgrading of wine quality, removal of fermentation inhibitors to increase yield of ethanol, improvement of drug analysis, as well as in construction of new-type of energy producing devices and new enzyme sensors.

The currently used oxidation methods in industries such as textile, food, wood processing, pharmaceutical and chemical are not eco-friendly or economical and also produce unwanted side reactions. Hence, enzymatic oxidation is a potential alternative to chemical methods. Laccases from white-rot fungi such as *Pleurotus sp.* are of particular interest with regard to potential industrial applications, because of their capability to oxidize a wide range of industrially relevant substrates. They are being increasingly used for many industrial purposes such as paper processing, prevention of wine discolouration, and detoxification of environmental pollutants (Gianfreda *et al.,* 1999), oxidation of dye (Abadulla *et al.,* 2000), and production of chemicals from lignin. It catalyzes the one-electron oxidation of a wide range of aromatic substrates including polyphenols, methoxy-substituted phenols, and diamines, with the concomitant reduction of molecular oxygen to water (Bourbonnais and Paice, 1990). Remarkably, laccase based treatment of toxic compounds is highly advantageous to bioremediation technologies since the approach is ecofriendly and the enzyme is effective and produced in large amounts and is often produced constitutively.

The physical and chemical methods currently used to decolourize textile industry effluent face a number of limitations, including high cost and inability to decolourize the broad range of dyes. Attempts to develop biodecolouration processes have largely focused on bacterial cultures and have, in general, been unsuccessful. Hence white rot fungi have been shown

to be capable of degrading several recalcitrant organo-pollutants are applied in industrial processes.

Against this background, our study was designed and carried out with the following major objectives:

- To isolate White-rot fungi (*Pleurotus sp.*) from the foothills of Western Ghats and to screen for laccase enzyme.
- To evaluate the impact of temperature, pH, different substrates, inhibitors on laccase activity.
- To purify laccase using ion-exchange chromatography and its characterization based on sodium-deodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).
- To apply laccase enzyme for the decolourization of dyes, textile and paper effluents.
- To stabilize the enzyme laccase using various sugar additives.

# CHAPTER II

# LITERATURE REVIEW

# 2. LITERATURE REVIEW

## 2.1 Lignocellulose and lignin

The lignocellulosic biomass from plants is a renewable source of food, energy and chemicals. It accounts for more than 60% of the total biomass production(Kuhad *et al.,* 1997). Lignocellulosic waste material is produced in huge amounts in agriculture, forestry and in the pulp and paper industry. The use of this waste is an important way to recycle carbon to energy and food. The lignocellulosic material of plants consists of three main components, namely cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer of glucose units linked with ß-1, 4-glucosidic bonds. Hemicelluloses are heteropolysaccharides consisting of short-branched chains of hexoses, e.g. mannose units in mannans and pentoses such as xylose units in xylans . Extracellular enzymes catalyze the hydrolytic breakdown of cellulose in nature: cellobiohydrolases, endoglucanases and ß-glucosidases produced by fungi and bacteria. The major hemicellulose-degrading enzymes are endoxylanases and endomannases. After cellulose, lignin is the second most abundant renewable biopolymer in nature. It is an essential part of the plant cell wall, imparting rigidity and protecting the easily degradable cellulose from attack by pathogens. Lignin is aromatic, 3-dimensional and amorphous. It is synthesized from phenyl propanoid precursors by polymerization in higher plants. The lignin precursors *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol consist of an aromatic ring and a 3-carbon side chain. In the lignin molecule the precursors form 3 types of subunit: hydroxyphenol- (H-type), guaiacyl- (G-type) and syringyl subunits (S-type). A typical finding for the lignin polymer

is that there is no single repeating bond between the subunits but a random distribution of at least 10 different types of bond, the most common being the ß-aryl ether (*ß*-O-4) bond .

Due to its complicated structure and nonhydrolysable bonds, lignin is more difficult to break down than cellulose or hemicellulose. The molecular mass (MW) of lignin is high, about 100 kDa or more, which prevents its uptake inside the microbial cell. Thus, the biological degradation of macromolecular lignin must occur through the activity of extra cellular enzymes.

**2.2 Ligninolytic enzymes**

Lignin breakdown is thought to occur by concomitant action of ligninolytic enzymes. The main extracellular enzymes participating in lignin degradation are haeme-containing lignin peroxidase (ligninase, LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and Cu-containing laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2). A new group of ligninolytic haeme-containing peroxidases, combining structural and functional properties of the LiPs and MnPs, are the versatile peroxidases (VPs). The VPs are capable of oxidising $Mn^{2+}$ and phenolic compounds, as well as nonphenolic aromatic compounds such as veratryl alcohol. These types of peroxidase were isolated from the white-rot fungi *Pleurotus eryngii* (Camarero *et al.,* 1999) and *Pleurotus ostreatus* (Cohen *et al.,* 2001), *Lentinula edodes* Mn-dependent peroxidase also oxidizes veratryl alcohol (D'Annibale *et al.,* 1996), while MnP from *Panus tigrinus* is able to degrade nonphenolic lignin model compounds (Maltseva *et al.,* 1991). In addition, enzymes involved in hydrogen peroxide production such as glyoxal oxidase and aryl alcohol oxidase (EC 1.1.3.7) are considered to belong to the ligninolytic system.

The lignin-degrading enzymes known so far are extracellular and nonspecific, participating in different oxidative reactions where the aromatic structure of lignin and bonds between the basic units are broken. The resulting small-molecular-weight compounds can then be transported inside the cell for further breakdown by fungi and also bacteria.

### 2.2.1 Manganese peroxidases

The MnPs (EC 1.11.1.13) are haeme-containing peroxidases that are produced by lignin degrading fungi. MnPs oxidize phenolic compounds to phenoxy radicals by oxidation of Mn (II) to Mn (III) with $H_2O_2$ as an oxidant. Mn (III) is chelated by organic acids (e.g. oxalate or malate in nature). Chelated Mn (III) oxidizes phenolic lignin compounds to phenoxy radicals that degrade spontaneously (Hofrichter, 2002). MnP oxidizes a wide range of compounds from lignin to polycyclic aromatic hydrocarbons. The MW of extracellular fungal MnPs varies from 40 to 50 kDa and the isoelectric point (pI) is usually acidic (pI 3-4), but neutral MnPs have also been found.

### 2.2.2 Laccases

The laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) are multicopper phenol oxidases, which oxidize phenolic compounds to phenoxyl radicals. Laccases were first isolated from plants but are also present in fungi and some bacteria (Thurston, 1994). Laccase activity has been demonstrated in higher plants, some insects and a few bacteria. However, most known laccases are of fungal origin. In plants, they participate in wound response and lignin synthesis. The fungal laccases are involved in lignin degradation, as well as in several other functions including pigmentation, sporulation, pathogenicity and detoxification. Laccases have been tested in many industrial applications, such as pulp

bleaching, textile dye decolourization, detergents, bioglueing and detoxification. Laccases are usually the first ligninolytic enzymes secreted to the surrounding media by the fungus. The typical MW of fungal laccase is 60-80 kDa and the isoelectric point is between pI 3 and pI 4, but variations on these values occur.

Laccases catalyze the oxidation of a wide variety of substrates, including various phenolic compounds and anilines. The one-electron oxidation of the reducing substrate occurs concomitantly with the four-electron reduction of molecular oxygen to water. When oxidized by a laccase, the reducing substrate loses a single electron and usually forms a free radical. The unstable radical may undergo further laccase-catalysed oxidation or non-enzymatic reactions including hydration, disproportionation, and polymerisation.

Laccases belong to the blue multi-copper oxidase family (Messerschmidt, A. *et al.,* 1990), which includes ascorbate oxidase and mammalian plasma ceruloplasmin. These proteins have a minimum of one mononuclear copper site containing one type-1 Cu (blue Cu) and a trinuclear copper site containing one type-2 Cu (normal Cu) (Solomon, E.I. *et al.*, 1996)and two type-3 Cu (coupled binuclear Cu). Substrates are oxidized near the mononuclear site, and the electrons are transferred to the trinuclear site, where the molecular oxygen is reduced. Neither the electron transfer mechanism nor the oxygen reduction to water is fully understood.

**2.2.3 Lignin peroxidases**

The LiPs (EC 1.11.1.14) are haeme-containing peroxidases. They were first found in the lignin-degrading fungus *Phanerochaete chrysosporium*. LiPs catalyse the oxidation of nonphenolic aromatic

compounds. The resulting cation radicals are further decomposed chemically (Conesa *et al.,* 2002; Martínez, A.T. 2002).

**2.3 White-rot fungi**

The only organisms reported to degrade lignin efficiently are the white-rot fungi that under natural conditions mostly colonize dead or living wood. Wood-degrading fungi are divided into 3 groups based on the type of rot they cause in wood: white-rot, brown rot and soft-rot fungi. White-rot fungi attack the lignin component of wood and leave the cellulose and hemicellulose less affected. Those white-rot fungi that degrade lignin rather than cellulose are called selective degraders. Selective lignin degraders are especially interesting from the standpoint of biotechnological applications, since they remove lignin and leave the valuable cellulose intact. Lignin degradation by these fungi is thought to occur during secondary metabolism and typically under nitrogen starvation. However, a wide variety of lignin degradation efficiency and selectivity abilities, enzyme patterns and substrates enhancing lignin degradation are reported from these fungi.

Combinations of extracellular ligninolytic enzymes (LiP, MnP and laccase) are important for lignin degradation. Based on their ligninolytic enzyme patterns, wood-rotting fungi can be divided in to 3 groups: 1. LiP-, MnP- and laccase-producing, 2. MnP- and laccase-producing and 3. LiP- and laccase-producing fungi. The most common group among the white-rot fungi is the MnP- and laccase-producing group (Hatakka, 2001). A total of 6 LiP and 4 MnP isoenzymes have been characterized, some only on lignocellulose-containing media, from the best studied selective lignin degrader, the whiterot fungus *Phanerochaete chrysosporium* (Farrell *et al.,* 1989; Stewart,P. and Cullen,D, 1999).

## 2.4 Distribution of laccase in prokaryotes

Until recently laccases were found only in eukaryotes, e.g. fungi, plants, insects (Mayer and Staples, 2002). Now there is increasing evidence for the existence of proteins in prokaryotes with typical features of the multi-copper oxidase enzyme family (Alexandre, G. and Zhulin, I.B, 2000; Claus, 2003). Corresponding genes have been found in gram-positive and gram-negative bacteria, including species living in extreme habitats e.g. in *Oceanobacillus iheyensis* or *Aquifex aeolicus* and in the archaebacterium *Pyrobaculum aerophilum.* Early report of laccases in actinomycetes were based on rather non-specific substrate reactions, but have been verified for *Streptomyces griseus* (Freeman *et al.*, 1993).

The first convincing data for prokaryotic laccase activity was presented for *Azospirillum lipoferum* (Givaudan *et al.*, 1993). It is a multimeric enzyme, composed of a catalytic subunit and one or two large chains. After SDS-PAGE, three bands are present with molecular masses of 48.9, 97.8 and 179.3 kDa (Diamantidis *et al.*, 2000). The laccase, which was heterologously expressed in *Escherichia coli* revealed the typical copper binding domains of laccases and two additional potential copper binding sites near the N-terminus. The latter may be responsible for the tyrosinase activity of the enzyme. A laccase like enzyme activity was found in spores of *Bacillus sphaericus* strain (Claus, M. and Filip, Z., 1997). Recently the spore coat protein CotA of *Bacillus subtilis* has been identified as laccase (Hullo *et al.*, 2001).

## 2.5 **Structure of laccase**

Fungal laccases often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular mass of the monomer ranges from 50 to 100 kDa. An important feature is a covalently linked carbohydrate moiety (10-45%), which may contribute to the high stability of the enzymes. The crystal structure of the fungal laccase from *Coprinus cinerius* is now available (Ducros *et al.*, 1998). More recently the spore coat protein CotA of *Bacillus subtilis* has been identified as laccase (Hullo *et al.*, 2001) and the crystal structure has been presented.

For the catalytic activity a minimum of four copper atoms per active protein unit is needed:

Type 1 : paramagnetic 'blue' copper, absorbance at 610nm.

Type 2 : paramagnetic 'non blue' copper.

Type 3: diamagnetic spin-coupled copper-copper pair, absorbance at 330nm.

**Fig 2.1: Crystal structure of a laccase from** *Trametes versicolor.*

The blue spheres show the 4 copper ions involved in the catalytic reaction. The three domains are indicated by the different coloring.

Type 1 copper has a trigonal coordination with two histidines and a cysteine as conserved equatorial ligands and one position usually variable, this axial ligand is methonine in the bacterial (CotA) and leucine or phenylalanine in fungal laccases. It has been widely argued that this axial position ligand strongly influences the oxidation potential of the enzyme, possibly providing the mechanism for regulating its activity. A mutation from phenylalanine to methionine significantly lowered the oxidation potential of the fungal laccase from *Trametes villosa* (Kumar *et al.*, 2003). Type1 copper confers the typical blue color to multicopper proteins, which results from the intense electronic absorption ion caused by the covalent copper-cysteine bond. Type2 copper shows no absorption in the visible spectrum and reveals paramagnetic properties in EPR studies. It is stratergically positioned close to the Type3 copper, a binuclear center

spectroscopically characterized by an electron adsorption at 330nm (oxidized form) and by the absence of an EPR signal as a result of the anti-ferromagnetic coupling of the copper pair. The type 3 copper center is also the common feature of another protein superfamily including the tyrosinases and haemocyanins(Decker and Terwilliger, 2000).

Type 2 and type 3 copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place. Type 2 copper is coordinated by two and type 3 copper atoms by six histidines. A hydroxyl bridge maintains the strong anti-ferromagnetical coupling between the type 3 copper atoms.

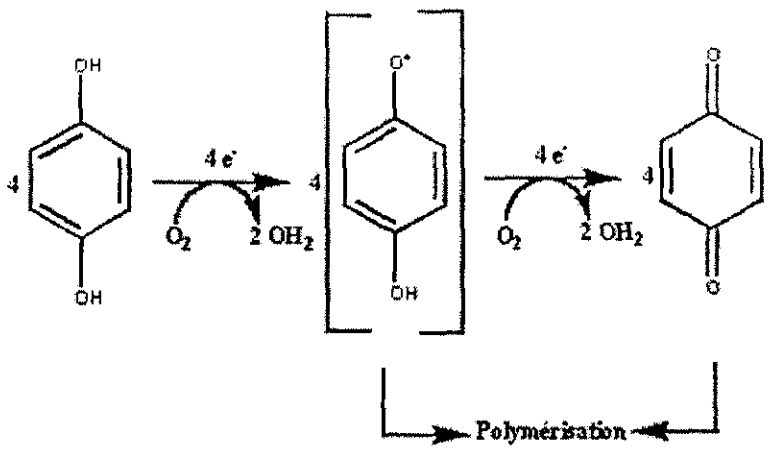
**2.6 Laccase mechanism of action**

Multicopper oxidase belongs to the class of enzyme containing four or more Cu centers in a protein molecule. The minimum functional unit of multicopper oxidase comprises a set of one type 1 Cu, one type 2 Cu, and a pair of type 3 Cus. The type 1 Cu functions as the electron mediator from a substrate to the trinuclear center composed of the type 2 Cu and type 3 Cus, where dioxygen is bound and reduced to two water molecules. All these copper sites are indispensable for the four-electron reduction of dioxygen without the release of activated oxygen species as intermediates.

The action mechanism of laccases, especially the role of the metallic center, remains unknown. It is proposed a two steps mechanism:

1. Copper T1 extracts one electron from the substrate
2. Electron is transferred to the T2/T3 center distant of about 12.5 Ã. After complete reduction of the trinuclear center, the molecular oxygen reduction occurs.

**Figure 2.2: General scheme for phenols oxidation by laccases**




## 2.7 Purification and characterization of laccase

Production of extracellular laccase is a common feature of many higher basidiomycetes fungi, particularly those associated with wood decay or the terminal stages of decomposition of leaf litter. Most of the white rot fungi produce laccase in multiple isoforms (Buswell *et al.*, 1995; Palmieri *et al.*, 2000). Laccases have been purified over the 30 years by many researchers from various fungi in view of characterization. Several purification steps are required to obtain a preparation of free of both pigment and other contaminating proteins. Multiple steps like ultra filtration, precipitation using ammonium sulphate or organic solvents, ion exchange and size exclusion chromatography have been used for the purification of laccases from the culture filtrate.

Electrophoretic separation of crude and purified laccase revealed the presence of multiple isoforms in many fungi. The laccase activity was detected in polyacrylamide gel after the protein separation by incubating the

gel in laccase substrates like guiacol (Coll *et al.*, 1993), ABTS (Niku-Paavola *et al.*, 1990) ,and syringaldazine (Youn *et al.*, 1995).

Wood (1980) reported that laccase protein was composed of several polypeptides when analyzed by SDS-PAGE. Number of laccase isoforms varied between fungal species like *Pycnoporus cinnabarinus* (Schliphake *et al.*, 2000), *Chaetomium thermophyllum* (Cheftz *et al.*, 1998), *Coriolopsis gallica* (Calvo *et al.*, 1998).*Cyathus stercoreus* (Sethuraman *et al.*, 1999), *Marsmius quercophilus* (Dedeyan *et al.*, 2000).

The molecular weight of the fungal laccase varies from 60kDa to 80kDa. However, a very high molecular weight (390 kDa) laccase isoform was detected in *Podospora anserine.* Many excreted proteins from fungi and other organisms have been shown to be glycoproteins. The carbohydrate content of laccase varies from 6.5 to 80%. The highest amount carbohydrate (80%) was detected in the laccase of in *Botrydis cinerea* laccase.

The optimum pH for the purified laccases of fungi varied between 3 and 6.5 and optimal temperatures from 25°C to 80°C. The laccases from *Coriolus hirsutus* and *Coriolus zonatus* are reported as the most thermo-stable laccases, reported so far, showing the thermal transition at 87°C and 92°C (Koroleva *et al.*, 2001). This dramatic loss of enzymatic activity in the temperature ranging from 50 to 65°C caused by a release of Type 2 copper ion. Type 1 copper site as well as the type 3 site is completely disintegrated at temperatures higher than 70°C. Thus, the crucial event for integrity of the copper takes place between 60 and 70°C, whereas over all protein structure is still maintained (Koroleva *et al.*, 2001).

The effect of several potential laccase inhibitors has been studied for many fungal laccases. The most effective inhibition was obtained with sodium azide for many laccases (Coll *et al.*, 1993, Eggert *et al.*, 1996; Sethuraman *et al.*, 1999). In *Cyathus stercoreus* and *Pycnoporus cinnabarinus*, laccase activity was completely inhibited by sodium azide, dithiothreitol and 1-cystein at low concentration. No inhibition of laccases was noticed with kojic acid, EDTA and acetyl acetone upto 8.8 mM (Sethuraman *et al.*, 1999; Eggert *et al.*, 1996). In contrast, the activity laccase in *Coriolus versicolor* was completely inhibited by 0.35 μM kojic acid.

Laccase exhibited phenol oxidase activity towards various lignin related compounds. The laccase of *Pleurots ostreatus* and other fungal laccases had shown high affinity for phenolic compounds containing methylated and hydroxy groups directly attached to the benzene ring but no affinity towards non-phenolic compounds such as p-trans cinnamic acid, and 3, 4-methoxy cinnamic acid (Youn *et al.*, 1995).

## 2.8 **Potential industrial and biotechnological applications of laccase enzyme**

Although oxidation reactions are essential in several industries, most of the conventional oxidation technologies have the following drawbacks: non-specific or undesirable side-reactions and use of environmentally hazardous chemicals. This has impelled the search for new oxidation technologies based on biological systems such as enzymatic oxidation. These systems show the following advantages over chemical oxidation: enzymes are specific and biodegradable catalysts and enzyme reactions are carried out in mild conditions. Enzymatic oxidation techniques have potential within a great variety of industrial fields including the pulp and

paper, textile and food industries. Enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones. Thus, laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a particularly promising enzyme for the above-mentioned purposes. The laccase molecule is a dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites (Gianfreda *et al.,* 1999). This enzyme catalyses the oxidation of ortho and paradiphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions coupled to the reduction of molecular dioxygen to water (Yaropolov *et al.,* 1994). The reported redox potentials of laccases are lower than those of non-phenolic compounds, so these enzymes cannot oxidise such substances. However, it was shown that in the presence of small molecules capable to act as electron transfer mediators laccases were also able to oxidise non-phenolic structures (Bourbonnais and Paice, 1990), expanding, thus, the range of compounds that can be oxidised by these enzymes. Laccase-mediated systems (LMS) have been applied to numerous processes such as pulp delignification (Li *et al.,* 1999), oxidation of organic pollutants (Collins *et al.,* 1996) and the development of biosensors (Kulys *et al.,* 1997) or biofuel cells (Palmore and Kim, 1999). Several organic and inorganic compounds have been reported as effective mediators for the above-mentioned purposes. These include thiol and phenol aromatic derivatives, N-hydroxy compounds and ferrocyanide, respectively. Claus *et al.,* (2002) found that the LMS enhanced dye decolourization and some dyes resistant to laccase degradation were decolourized. Lu and Xia (2004) have recently reviewed the applications of the LMS, which comprise pulp bleaching, textile biofinishing and environmental protection processes.

However, despite that LMS has been studied extensively there are still unsolved problems concerned with mediator recycling, cost and toxicity.

Laccases find applications within the following fields:

### 2.8.1 Food industry

Laccases can be applied to certain processes that enhance or modify the colour appearance of food or beverage. In this way, an interesting application of laccases involves the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity development in clear fruit juice, beer and wine. Laccases are currently of interest in baking due to its ability to cross-link biopolymers. Thus, Selinheimo *et al.,* (2006) showed that a laccase from the white-rot fungus *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough.

Recently, Minussi *et al.,* (2002) have described the potential applications of laccase in different aspects of the food industry such as bioremediation, beverage processing, ascorbic acid determination, sugar beet pectin gelation, baking and as a biosensor. However, they suggested that more studies of laccase production and immobilization techniques at lower costs are needed to improve the industrial application of this enzyme.

### 2.8.2 Pulp and paper industry

The industrial preparation of paper requires separation and degradation of lignin in wood pulp. Environmental concerns urge to replace conventional and polluting chlorine-based delignification/bleaching procedures. Oxygen delignification processes have been industrially introduced, but pre-treatments of wood pulp with ligninolytic enzymes might provide milder and cleaner strategies of delignification that are also respectful of the integrity of cellulose. Although extensive studies have been

performed to develop alternative bio-bleaching systems, few enzymatic treatments exhibit the delignification/brightening capabilities of modern chemical bleaching technologies. One of the few exceptions to this generalization is the development of LMS delignification technologies for kraft pulps. However, all these biobleaching studies were focused on wood pulps and little is known about the efficiency of the LMS on non-wood pulps including those used for manufacturing specialty papers. In this sense, Camarero *et al.,* (2004) explored the potential of LMS to remove lignin-derived products responsible for color from a high-quality flax pulp. They showed the feasibility of LMS to substitute chlorine-containing reagents in manufacturing of this high-price paper pulps. The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibers. For example, laccases can be used in the enzymatic adhesion of fibers in the manufacturing of lignocellulose based composite materials such as fiberboards. Laccases have been proposed to activate the fiber bound lignin during manufacturing of the composites, thus, resulting in boards with good mechanical properties without toxic synthetic adhesives (Felby *et al.*, 1997). Another possibility is to functionalize lignocellulosic fibers by laccases in order to improve the chemical or physical properties of the fiber products. Preliminary results have shown that laccases are able to graft various phenolics acid derivatives onto kraft pulp fibers (Lund and Ragauskas, 2001). This ability could be used in the future to attach chemically versatile compounds to the fiber surfaces, possibly resulting in fiber materials with completely novel properties such as hydrophobicity or charge.

### 2.8.3 Textile industry

The textile industry accounts for two-thirds of the total dyestuff market (Riu *et al.*, 1998) and consumes large volumes of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products (Banat *et al.*, 1996). There are more than 100,000 commercially available dyes with over $7\times10^5$ t of dyestuff produced annually. Due to their chemical structure dyes are resistant to fading on exposure to light, water and different chemicals (McKay, 1979) and most of them are difficult to decolourize due to their synthetic origin. Concern arises, as several dyes are made from known carcinogens such as benzidine and other aromatic compounds (Baughman and Perenich, 1988). Most currently existing processes to treat dye wastewater are ineffective and not economical (Cooper, P., 1995). Therefore, the development of processes based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure (Abadulla *et al.*, 2000), including synthetic dyes currently employed in the industry (Rodríguez Couto *et al.*, 2005). The use of laccase in the textile industry is growing very fast, since besides to decolourize textile effluents as commented above, laccase is used to bleach textiles and even to synthesize dyes (Setti *et al.*, 1999).

### 2.8.4 Other laccase applications

#### 2.8.4.1 Soil bioremediation

Polycyclic aromatic hydrocarbons (PAHs) together with other xenobiotics are a major source of contamination in soil therefore, their degradation is of great importance for the environment. The catalytic properties of laccases can be used to degrade such compounds. Thus, laccases were able to mediate the coupling of reduced 2, 4, 6-trinitrotoluene

(TNT) metabolites to an organic soil matrix, which resulted in detoxification of the munition residue (Durán and Esposito, 2000).

### 2.8.4.2 Synthetic chemistry

In the future laccases may also be of great interest in synthetic chemistry, where they have been proposed to be applicable for oxidative deprotection (Semenov *et al.,* 1993) and production of complex polymers and medical agents (Uyama and Kobayashi, 2002).

### 2.8.4.3 Cosmetics

The cosmetic world has not been indifferent to the application of laccase: for example, laccase-based hair dyes are less irritant and easier to handle than current hair dyes, since laccases replace $H_2O_2$ as an oxidising agent in the dye formulation. More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed.

## 2.9. Decolourization studies

### 2.9.1 Dye classes

A dye is used to impart colour to materials of which it becomes an integral part. An aromatic ring structure coupled with a side chain is usually required for resonance and thus to impart colour. Correlation of chemical structure with colour has been accomplished in the synthesis of dye using a chromogen-chromophore with auxochrome. Chromogen is the aromatic structure containing benzene, naphthalene or anthracene rings. A chromophore group is a colour giver and is represented by the following radicals, which form a basis for chemical classification of dyes when coupled with the chromogen: azo(-N=N-), carbonyl(=C=O), carbon(=C=C=),carbon-nitrogen(>C=NH or –CH=N-), nitroso(-NO or N-

OH);nitro (-$NO_2$ or =NO-OH) and sulfur(>C=S), and other carbon-sulfur groups). The chromogen-chromophore structure is often not sufficient to impart solubility and cause adherence of dye to fiber. The auxochrome or bonding affinity groups are amine, hydroxyl, carboxyl, and sulfonic radicals, or their derivatives. These auxochromes are important in the use of classification of dyes. A listing of dyes by use classification comprises the following:

- *Acetate rayon dyes*: developed for cellulose acetate and some synthetic fibers.
- *Acid dyes:* used for colouring animal fibers via acidified solution (containing sulfuric acid, acetic acid, sodium sulfate and surfactants) in combination with amphoteric protein.
- *Azoic dyes*: contain the azo group (and formic acid, caustic soda,metallic compounds and sodium nitrate); especially for application to cotton.
- *Basic dyes*: amino derivatives ( and acetic acid and softening agents ); used mainly for application on paper.
- *Direct dyes:* azo dyes, and sodium salts, fixing agents, and metallic (chrome and copper) compounds; used generally on cotton-wool, or cotton-silk combinations.
- *Mordant or chrome dyes*: metallic salt or lake formed directly on the fiber by the use of aluminium, chromium, or iron salts that cause precipitation in situ.
- *Lake or pigment dyes*: form insoluble compounds with aluminium, barium,or chromium on molybdenum salts; the

precipitates are ground to form pigments used in paint and inks.

- *Sulfur or sulfide dyes*: contain sulfur or are precipitated from sodium sulfide bath; furnish dull shades with good fastness to light washing and acids but susceptible to chlorine and light.
- *Vat dyes*: impregnated into fiber under reducing conditions and reoxidized to an insoluble colour.

### 2.9.2 Reactive dyes

The reactive dyes are becoming increasingly important in the global market, with their use increasing from 6 to 12% of overall dye consumption between 1980 and 1985. Up to 50% of reactive dyes are hydrolyzed and lost to effluent during the dyeing process. These dyes are mostly azo or phthalocyanine dyes with reactive groups that bind covalently with nucleophilic groups on the textile; this group is usually $-SO_2-CH_2-CH_2-CH_2-O-SO_3-$. Reactive dyes are of particular concern since 20 to 25% in wastewaters is in the form of toxic vinyl sulfone.

### 2.9.3 Biodecolouration vs Biodegradation

Mineralization is the complete decomposition of the molecule into $H_2O$, CO, and inorganics. Biodecolouration of a dye requires only the degradation of the chromophore and may not indicate mineralization of the molecule. This should be distinguished from sorption of the dye to the microbial biomass, as this may result in the removal of the dye from an effluent, but not molecular degradation. The main process of dye removal in many dye biodegradation studies is sorption to microbial biomass.

### 2.9.4 Dye biodegradation

#### 2.9.4.1 Aerobic bacterial degradation

The aerobic degradation of dyes by bacteria is poorly understood, with most studies confined to azo dyes. Little or no degradation has been shown with color removal attributed mainly to sorption. Most instances of aerobic dye decolouration appear to involve facultative anaerobes capable of both aerobic and anaerobic degradation. The mechanism of decolouration for azo dyes is thought to be reductive fission of the azo bonds. Only a few bacteria have been shown to degrade the azo bond aerobically. The enzyme azo reductase, by which most azo dyes are degraded, is inhibited by oxygen. However, the aerobic reduction of simple azo compounds has been demonstrated with species such as *Bacillus subtilis* (Horitsu *et al.*, 1977).

**Table 2.1 : Examples of dye degradation by aerobic bacteria**

| Organisms | Dyes degraded | Concentration | %removed/time |
|---|---|---|---|
| *K. pneumoniac* | Methyl Red | 100 mg/L | 100/2d |
| RS-13 | | | n.a |
| *K. Planticola* | | | n.a |
| *K. terrogena* | | | n.a |
| *S. marcescens* | | | n.a |
| *Salmonella sp.* | | | n.a |
| *A. liquefacians* | Methyl Red | 100 mg/L | 7 d |
| *P. luteola* | Red G | 0.1 %w/v | 76/120 h |
| | Remazol Black B | | 55/120 h |
| | RP2B | | 23/120 h |
| | V2RP | | 51/120 h |
| *P. alcaligenes*<br>*P. mendocina* | 4 di and<br>Triphenylmethane | real effluent | 78/15 h |
| *Pseudomonas sp.* | Orange I<br>Orange II | 50-100 ppm | 100/40 h<br>100/72 h |

### 2.9.4.2 Anaerobic bacterial degradation

There are several reports of azo dye decolouration by anaerobic bacteria. The anaerobic decolouration of azo dyes is usually attributed to azo reductases, which reduce the azo bond to generate colorless aromatic amines. These compounds, which are often toxic, mutagenic, and are carcinogenic, are degraded anaerobically. The generation of these compounds has prevented implementation of anaerobic treatment systems for dye effluents.

### 2.9.5 Effluent Treatment

The coloured effluents resulting from different industries contain considerable amount of different pollutants due to which it is not possible to discharge the untreated water either into the water course or into a municipal sewage without causing damage, since it contains a variety of processed chemicals and dyes. Removal of colour from industrial effluent is a major problem and tighter constraints on discharges are forming waste creators and managers to consider new options for effluent treatment and disposal. Hence decolourization, as well as the methods of decolourization are important. Methods of effluent treatment for dyes may be classified into three main categories: physical, chemical and biological as follows.

**Table2.2: Methods of effluents treatment**

| **Physical** | **Chemical** | **Biological** |
|---|---|---|
| Adsorption | Neutralization | Stabilization |
| Sedimentation | Reduction | Aerated lagoons |
| Floatation | Oxidation | Trickling filter |
| Flocculation | Electrolysis | Activated sludge |

| | | |
|---|---|---|
| Coagulation | Ion exchange | Anaerobic digestion |
| Foam fraction | Wet air oxidation | Bioaugmentation |
| Polymer flocculation | - | - |
| Reverse Osmosis | - | - |
| Ultrafiltration | - | - |
| Ionization radiation | - | - |

Unfortunately, the physical and chemical methods of effluent treatment have high operating costs and of limited applicability. An effective and inexpensive biological treatment system would be of great value.

### 2.9.6 Bioremediation

Bioremediation is a pollution control technology that uses biological systems to catalyze the degradation or transformation of various toxic chemicals to less harmful forms. The general approaches to bioremediation are to enhance natural biodegradation by native organisms (intrinsic bioremediation), to carry out environmental modification by applying nutrients or aeration (biostimulation) or through addition of micro-organisms (bioaugmentation). Unlike conventional technologies, bioremediation can be carried out on-site. Bioremediation is limited in the number of toxic materials it can handle but where applicable, it is cost-effective.

Biodegradation, mineralization, bioremediation, biodeterioration, biotransformation, bioaccumulation and biosorption are some terms with minor subtle differences but often overlappingly used. Biodegradation is the general term used for all biologically mediated breakdowns of chemical

compounds and complete biodegradation leads to mineralization. Biotransformation is a step in the biochemical pathway that leads to the conversion of a molecule (precursor) into a product. A series of such steps are required for a biochemical pathway. In environmental terms, it is importance whether the product is less harmful or not. Biodeterioration refers usually to the breakdown of economically useful compounds but often the term has been used to refer to the degradation of normally resistant substances such as metals, plastics, drugs, cosmetics, painting, sculpture, wood products and equipment. Bioremediation refers to the use of biological systems to degrade toxic compounds in the environment. Bioaccumulation or biosorption is the accumulation of the toxic compounds inside the cell without any degradation of the toxic molecule. This method can be effective in aquatic environments where the organisms can be removed after being loaded with the toxic substance.

**2.9.7 Fungi in bioremediation**

The fungi are unique among microorganisms in that they secrete a variety of extra cellular enzymes. The decomposition of lignocellulose is rated as the most important degradative event in the carbon cycle of earth. Enormous literature exists on the role of fungi in the carbon and nitrogen cycles of nature. The role of fungi in the degradation of complex carbon compounds such as starch, cellulose, pectin, lignin, lignocellulose, inulin, xylan, araban etc. is well known. *Trichoderma reesei* is known to possess the complete set of enzymes required to breakdown cellulose to glucose. Degradation of lignocellulose is the characteristic of several basidiomycetous fungi.

Fungi are good in the accumulation of heavy metals such as cadmium, copper, mercury, lead and zinc. Systems using *Rhizopus arrhizus* have been developed for treating uranium and thorium.

The ability of fungi to transform a wide variety of hazardous chemicals has aroused interest in using them in bioremediation.The white rot fungi are unique among eukaryotes for having evolved non-specific methods for the degradation of lignin; curiously they do not use lignin as a carbon source for their growth (Kirk *et al.*, 1976). Lignin degradation is, therefore, essentially a secondary metabolic process, not required for the main growth process. *P. sordida* was also most useful in the degradation of PAHs from soil. Davis *et al.,* (1993) showed that *P. sordida* was capable of degrading efficiently the three ring PAHs, but less efficiently the four-ring PAHs.

*Phanerochaete chrysosporium* has been shown to degrade a number of toxic xenobiotics such as aromatic hydrocarbons ( Benzo alpha pyrene, Phenanthrene, Pyrene) chlorinated organics (Alkyl halide insecticides, Chloroanilines, DDT, Pentachlorophenols, Trichlorophenol, Polychlorinated biphenyls, Trichlorophenoxyacetic acid), nitrogen aromatics ( 2,4-Dinitrotoluene, 2,4,6-Trinitrotoluene-TNT) and several miscellaneous compounds such as sulfonated azodyes. Several enzymes which are released such as laccases, polyphenol oxidases, lignin peroxidases etc. play a role in the degradative process. In addition, a variety of intracellular enzymes such as reductases, methyl transferases and cytochrome oxygenases are known to play a role in xenobiotic degradation (Barr and Aust, 1994).

Among the fungal systems, *Phanerochaete chrysosporium* is emerging as the model system for bioremediation. The basidiomycetous fungus *Pleurotus ostreatus* has been shown to produce an extracellular hydrogen peroxide dependent lignolytic enzyme which removes the color

due to remozol brilliant blue. Oxidative enzymes play a very major role in biodegradation. Other fungi which can be used in bioremediation are obviously the members of Zygomycetes and the arbuscular mycorrhizal fungi. Aquatic fungi and anaerobic fungi are the other candidates for bioremediation.

Among other fungi used in bioremediation, the yeasts, e.g., *Candida tropicalis*, *Saccharomyces cerevisiae, S. carlbergensis* and *Candida utilis* are important in clearing industrial effluents of unwanted chemicals. *Agaricus bisporus* and *Lentinus edodes* are important in lignocellulose decomposition.Consortia of fungi and bacteria (usually uncharacterised) are used in composting, the most useful waste disposal practice. Phenolic azo dyes have been shown to be oxidized by the enzyme laccase produced by *yricularia oryzae*( Chivukula and Renganathan, 1995).

**Table 2.3 : Examples of dye decolouration by fungi due to adsorption**

| **Cullture** | **Dyes removed** | **Percent removal/time** |
|---|---|---|
| *Neurospora crassa* | Vermelho Reanil | 89-91/24h |
| *Myrothecum verrucaria* | Orange II | 70/5h |
| | Blue 10B | 86/5h |
| | RS (red) | 95/5h |
| *Myrothecum sp.* | RS(Red) | 81-98/24h |
| | Orange II | 25-91/24h |
| | Blue 10B | 58-98/24h |
| *Candida sp.* | Procyon Black | 94/24h |
| | Blue MX2G | 97/24h |
| | Red HE7B | 99/24h |
| | Orange HER | 97/24h |

## 2.10 Stabilization of enzymes

### 2.10.1 Strategies for the stabilization of enzymes

Enzymes are used to perform in an environment quite different from its natural habitat. Most enzymatic reactions are performed in aqueous media, which favors inactivation. Water acts as a reactant in inactivation reactions and also as a lubricant in conformational changes associated with protein unfolding. Therefore, enzyme stabilization under different conditions is an important area of research.

The different methods employed for enzyme stabilization are as follows

1. Immobilization (Ulbrich *et al.,* 1986)
2. Chemical modification (Alexander, 1983)
3. Protein engineering (Stefan, 1993)
4. Inclusion of various additives as enzyme stabilizers (Kristjansson and Kinsella, 1991)

Use of stabilizing additives is a customary practice in enzyme technology and shelf life of enzyme products very much relies upon such additives. However, its use as operational stabilizers has little significance and poor predictability, although in the case of enzymes performing in non-conventional media the use of additives has proved to enhance enzyme activity and stability.

### 2.10.2 Additives as enzyme stabilizers

Hundreds of additives have been proposed as enzyme stabilizers and many of these have been patented with reference to application. The

additives may be substrates (including co enzymes & metal ions), salts, anions, polymers , sugars and glycols (Wiseman, 1993).

**2.10.3 Stabilization using sugars**

Sugars and polyhydric alcohols have been known to protect proteins and in turn enzymes, against loss of activity (Colaco *et al.,* 1992), due to chemicals and heat (thermal denaturation). The basic observations are that these sugars prevent the loss of enzymatic activities by inhibiting the irreversible aggregation and increasing the thermal transition temperature of enzymes. The stabilizing effect of these sugar additives has been recognized for their minimized hydrogen bond-rupturing capacity between the medium and the respective enzyme or to an induced change in topography. An alteration of the kinetic properties of an enzyme, however, should not be interpreted as a reflection of a conformational change without further independent experimental observations. These alterations could also be due to a change in the chemical configuration of the substrate, product, or indeed the enzyme itself in the presence of polyhydric alcohols and sugars. Solvent additives like sugars can affect enzymes by direct interaction with them, or by indirect action through effects on the structure and properties of the solvent, or by a combination of both of these mechanisms.

In order to arrive for an understanding of the stabilizing effects, various sugars like maltose, several workers tested lactose, sucrose and trehalose for their enzyme stability. The idea of using trehalose has its origin in investigation on certain organisms that possess the ability to survive in a complete dehydration state. These organisms said to be in a state of "anhydrobiosis" may persists in the dry state for decades. Many of these organisms including spores of certain fungi, macrocyst of the slime mould *Dictyostelium*, dry active bakers yeast, brine shrimp (*Artemia salina*) cysts.

and the dry larvae and adults of several species of soil dwelling nematodes, contain large amount of sugars, particularly trehalose and their survival in the dry state is correlated with the presence of this molecule (Crowe *et al.*, 1987). When they became wet again, they rapidly swell and resume active metabolism often within minutes.

*Selaginella lepidophylla* (resurrection plant) a desert xerophyte of North America has sucrose and trehalose with approximately 14% of dry weight, with trehalose being the major component (90%) (Adams *et al.*, 1991). In dry cysts of *Artemia* trehalose often constitutes more than 20% of dry weight. Trehalose is particularly effective at stabilizing dry phospholipid bilayers biological membranes and proteins mainly, enzymes (Crowe *et al.*, 1987)

It is well-established fact that many sugars stabilize proteins in solution and afford cryoprotection to isolated enzymes. However, freeze-drying is generally thought to be more disruptive to enzyme function than freeze thawing or thermally induced perturbations. An extreme situation has been observed with L-asparaginase, which is fully active after freeze thawing but inactivated over 80% by freeze-drying. Despite the stresses to which proteins are subjected during dehydration, it has been found that a great degree of protection is provided by sugars. The most dehydration-sensitive enzyme tested to date is phosphofructokinase, purified from rabbit skeletal muscle. This enzyme is completely and irreversibly inactivated during freeze-drying, a characteristic that makes it especially suited for use in testing stabilizing compounds. Of the organic solutes several studied, trehalose has been known to be a superior stabilizer in providing protection to biological macromolecules against dehydration. This explains that it is not

just the type of sugar moiety present that is important for enzyme stabilization but the subunit orientation is also critical.

The preferential interaction of proteins with solvent components was measured in aqueous lactose and glucose systems by using a high precision densimeter. In all cases, the protein was preferentially hydrated; i.e., addition of these sugars to an aqueous solution of the protein resulted in an unfavorable free-energy change. This effect was shown to increase with an increase in protein surface area, explaining the protein stabilizing action of these sugars and their enhancing effect of protein associations. Correlation of the preferential interaction parameter with the effect of the sugars on the surface tension of water, i.e., their positive surface tension increment, has led to the conclusion that the surface free energy perturbation by sugars plays a predominant role in their preferential interaction with proteins. Other contributing factors are the exclusion volume of the sugars and the chemical nature of the protein surface.

Studies of numerous proteins with infrared spectroscopy have documented that unfolding is a general response of unprotected proteins to freeze-drying. Some proteins those are unfolded in the dried solid aggregate during rehydration, whereas others refold. It has been proposed for the latter case that aggregation is avoided because refolding kinetically out-competes intermolecular interactions. In contrast, with proteins that normally aggregate after rehydration, minimizing unfolding during freeze-drying with stabilizer has been shown to be needed to favor the recovery of native protein molecules after rehydration.

# CHAPTER III

# MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

## 3.1 Culture and culture conditions

The white rot fungi were isolated from foothills of Western Ghats, Tamil Nadu and Karnataka, India and was identified to be *Pleurotus eous* (*P.eous*) by Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. The organism were maintained in Potato-Dextrose-Agar slants (PDA) at 4°C. The fungus was transferred to PDA plates and incubated for 7–8 days at 27°C. Inoculum containing $10^6$ spores/ ml was prepared and was then transferred to a sterilized 250-ml Erlenmeyer flask containing 10 g wheat bran (obtained from local market) moistened with distilled water (1:1, w/v). The flasks were incubated at 27°C for 15–20 days. Crude culture filtrate was obtained by adding 30 ml of distilled water to the flasks and filtering through a muslin cloth. The filtrate was again centrifuged at 10,000 X g for 10 min. The supernatant was used as the enzyme source.

*Bacillus subtilis* was obtained from Tamil Nadu agricultural university, Coimbatore, Tamil Nadu, India. *Bacillus subtilis* was subcultured and maintained on nutrient agar medium at 4°C and subsequently grown in 250-ml Erlenmeyer flask containing 100ml nutrient broth (NB).

## 3.2 Qualitative assay for laccase

*Pleurotus eous* were screened for laccase production by growing them on basal medium containing 10mM guaiacol. The basal medium contains (per liter of distilled water): 0.5 g of $KH_2PO_4$, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of $NH_4NO_3$, 0.1 g of KCl, 0.02g of $FeSO_4.7H_2O$, 0.05 g of $Ca(NO_3)_2.4H_2O$, 2.0 g of malt-extract (HiMedia chemicals) and 15 g of agar (Himedia Chemicals). Basal medium was autoclaved in flasks and cooled to 55°C, when the following were added aseptically: 5-ml of 1 M

KOH; 0.4-ml of guaiacol; 30 mg of streptomycin sulphate; 30 mg of Penicillin G (Na salt); 4 mg of benomyl (as Benlate 50 WP) in 2 ml of 1:1 acetone-70% ethanol. The medium was stirred and poured into petridishes. A 10 mm agar plug taken from fungal colony was used to inoculate 250-ml Erlenmeyer flask containing 100-ml potato dextrose medium. The culture was incubated at 27°C and shaken at 100rpm. The production of intense brown colour around the fungal colony in the guiacol-containing medium was considered as a positive reaction for presence of laccase activity

**3.3 Enzyme extraction**

The whole wheat bran growth medium was extracted with three volumes of distilled water each for 6 hours at room temperature .The extract was filtered through filter paper, centrifuged at 10000rpm for 30 minutes and the supernatant was used as the enzyme source

**3.4 Enzyme assay**

Enzyme activity was assayed by using 50µl of crude filtrate; 950µl of 10mM guaiacol in 0.1 M acetate buffer containing 10% (v/v) acetone, pH 5.0 (Palmieri, G. 1993). The enzyme blank consists of 50µl of crude filtrate and 950µl of 0.1M acetate buffer and the substrate blank consists of 50µl 10mM guaiacol in 0.1 M acetate buffer containing 10% (v/v) acetone and 950µl of 0.1M acetate buffer. The contents were mixed well and incubated at 30°C for 5 minutes (Hosoya, T. 1960; Mliki, A. and Zimmermann, W. 1992). The brown colored formed was spectrophotometrically read at 460nm using Beckman DU-530 Spectrophotometer. Enzyme activity was expressed in units/ml. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1µmol guaiacol per minute.

In order to rule out the role of peroxidase oxidation and prove the oxidation only by laccase, the enzyme was pre incubated with catalase (1000

units $ml^{-1}$) for 30 min at 30° C prior to assay to remove any endogenous hydrogen peroxide (Pointing *et al.*, 2000). Similarly, manganese independent peroxidase activity was measured by adding hydrogen peroxide (1mM final concentration) to the laccase assay mixture and to subtract the activity due to laccase alone (Pointing *et al.*, 2000). Also, lignin peroxidase activity was determined by measuring the production of veratraldehyde from veratryl alcohol at 310 nm in glycine-HCl buffer (pH 3.0) at 30° C, upon addition of hydrogen peroxide (1 mM concentration) (Kirk *et al.*, 1998).

Further, aryl alcohol oxidase activity was assayed under the same conditions without the addition of hydrogen peroxide and manganese dependent peroxidase activity was measured by the oxidation of phenol red at 431 nm in the presence of 100 μM $MnSO_4.5\ H_2O$ in glycine-HCl buffer (pH 3.0) at 30° C, upon addition of hydrogen peroxide (0.5 mM final concentration) (Pointing *et al.*, 2000).

**3.5 Protein quantification**

Protein concentration was measured by the method of Lowry *et al.*, (1951) using Bovine serum albumin (BSA) as standard. Protein assay mixture consisted of 0.05ml of sample, 0.15ml of distilled water and 1.0ml of alkaline copper reagent. The contents were incubated for 10 minutes at room temperature. Then 0.1ml of 1.0N Folin's reagent was added. Again contents were incubated for 30 minutes at room temperature. A reaction mixture containing distilled water instead of the sample was used as blank. Blue colour developed was read at 640nm using Beckman DU-530 Spectrophotometer. Absorbance values obtained were compared against standard BSA. Standard protein solutions containing 0-0.1mg of BSA were used for preparing standard graph.

### 3.6 Purification of Laccase

#### 3.6.1 Ammonium sulphate fractionation

After the extraction of enzyme, it was purified by ammonium sulphate precipitation. The quantity of ammonium sulphate required to attain a particular saturation was calculated using the following formula p/z

Salt required = [533(final concentration – initial concentration)] / [100-(0.3* final concentration)]

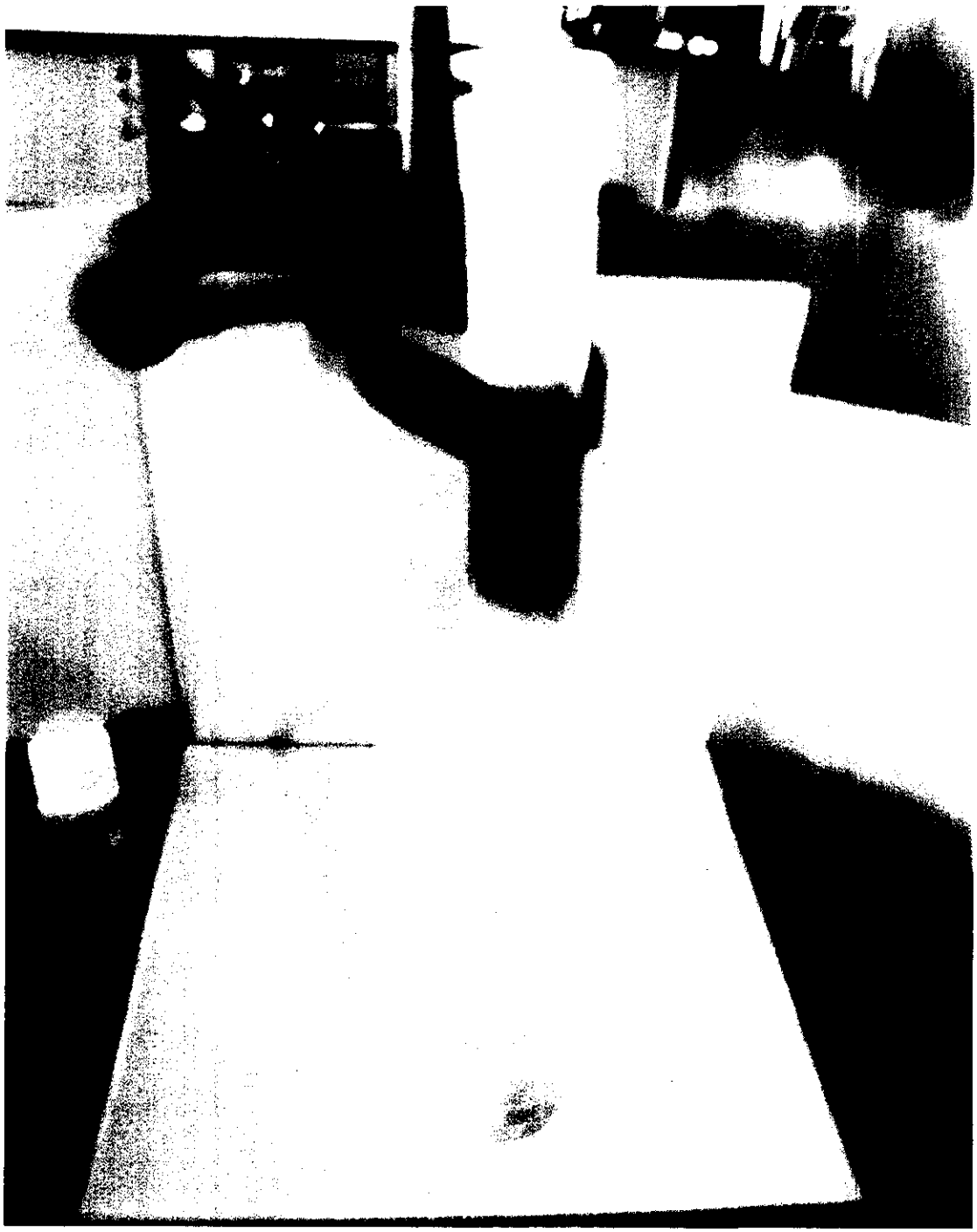
The supernatant was brought to 0-50 percent saturation of ammonium sulphate by the addition of required quantity of the salt (31.35g/100ml) and stirring continuously on ice for three hours. The solution was centrifuged (10000 rpm, 10 minutes, 4 °C), the supernatant was saved and brought from 50 to 70 percent saturation (13.49g ammonium sulphate/ 100ml). The insoluble proteins were collected by centrifugation (10000 rpm, 10 minutes, 4 °C) and dissolved in minimum volume of 20mM sodium acetate buffer pH 5.6.

The precipitated fractions were dialyzed against the same buffer with three changes of buffer. Dialysis was carried out at 4 °C.

#### 3.6.2 Ion exchange chromatography

The final step in enzyme purification was the ion exchange chromatography of laccase obtained after dialysis. For this the column was packed with CM-cellulose previously equilibrated with 20mM sodium acetate pH 6.After washing with 20mM sodium acetate buffer, the enzyme was eluted with potassium chloride. Fraction volume of 1ml was collected. Laccase activity of the fractions was performed with 10mM guiacol in sodium acetate buffer.

**Fig 3.1: Purification of laccase on CM-Cellulose-Ion exchange chhromatography**



### 3.6.3 Thin layer chromatography

Slurry of the absorbent was prepared. The slurry was coated over the glass plate at the thickness of 0.25mm. The plates were allowed to dry in room temperature for 15-30mins. The plates were heated in an oven to activate the absorbent. The sample was applied over the adsorbent. The sample was allowed to dry so that spotting can be done repeatedly for a more concentrated sample spot. The developing solvent was poured into the tank to a depth of 1cm. The TLC plate was placed vertically into the tank with the spotted end dipped in the solvent. Separation of the compound occurs as the solvent moves upwards. Once the solvent reaches the top of the plate, it was removed from the tank and was proceed for further identification.

## 3.7 Characterization

### 3.7.1 Molecular Weight Determination

The molecular mass of laccase was determined by SDS-PAGE. SDS-PAGE (10 percent) was run with Tris-Glycine-SDS buffer system. The sample was loaded onto the well at a constant current of 20mA till the dye front crossed the separating gel. After completion of the run, comassie brilliant blue staining of the gel was done to visualize the proteins. Lysozyme and Bovine serum Albumin (BSA) were used as the protein marker.

### 3.7.2 Effect of temperature on laccase activity

For temperature studies using guaiacol as substrate, the reaction mixture contained 50 µl crude supernatant, 50 µl of 10mM guaiacol and 900 µl of 100 mM sodium phosphate buffer at pH 6.0 were incubated at different temperature 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C for 15

minutes. The brown colour formed was spectrophotometrically read at 460nm using Beckman Spectrophotometer.

### 3.7.3 Effect of pH on laccase activity

For pH studies using guaiacol as substrate is carried out as follows, the reaction mixture contained 50 µl crude supernatant, 50 µl of 10mM guaiacol and 900 µl of 100 mM sodium acetate buffer at different pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 900 µl of 100 mM sodium phosphate buffer adjusted to different pH values of 6.0, 6.5, 7.0, 7.5, 8.0. The substrate blank consists of 50µl 10mM guaiacol and 950µl of appropriate buffer. The contents were mixed well and incubated at 55°C for 15 minutes. The brown colour formed was spectrophotometrically read at 460nm using Beckman Spectrophotometer.

### 3.7.4 Inhibitor Studies

For inhibitor studies the reaction mixture contained 50 µl crude supernatant, 50 µl of 10mM guaiacol in deionised water and 900 µl of 100mM sodium phosphate buffer at pH 6.0. 400µl of appropriate inhibitor ($Al^{3+}$ $Fe^{2+}$ $Fe^{3+}$ $Mg^{2+}$ & $Mn^{2+}$), in the concentration range of 10mM to 50mM and with 5 % 3%and 1% SDS. The substrate blank consists of 50µl 10mM guaiacol in deionised water and 950µl of appropriate buffer. The contents were mixed well and incubated at 55°C for 15 minutes. The brown colour formed was spectrophotometrically read at 460nm using Beckman Spectrophotometer.

### 3.7.5 Effect of substrate concentration

For studying the effect of substrate concentration using guaiacol as substrate, the reaction mixture contained 50-µl crude supernatant, 50 µl of 10mM guaiacol (2mM-12mM) and 900 µl of 100 mM sodium phosphate buffer at pH 6.0 were incubated at 55°C for 15 minutes. The brown colour

formed was spectrophotometrically read at 460nm using Beckman Spectrophotometer. The apparent $K_m$ and $V_{max}$ values were determined.

### 3.8 Decolourization studies

#### 3.8.1 Dyes and monitoring of decolourization

Decolourization in solid medium was assessed by the visual disappearance of colour from the plates. Decolourization in liquid medium could be seen visually and was measured spectrophotometrically at the 0maximum visible wavelength of absorbance for each of the dyes.

#### 3.8.2 Solid plate decolourization studies

Solid medium in petriplates were prepared in Potato dextrose broth (PDB) with 1.5% (w/v) agar, and an aliquot of an individual dye to a final concentration of 100ppm. Plates, each containing one of the dyes were inoculated with the culture. The culture was incubated at 27°C, except those inoculated with *Trametes hirsuta,*which were incubated at 37°C. The plates were incubated for 10 days. Uninoculated plates served as controls for abiotic decolouration. The experiment was performed in duplicate for each culture.

#### 3.8.3 Decolourization of individual dyes

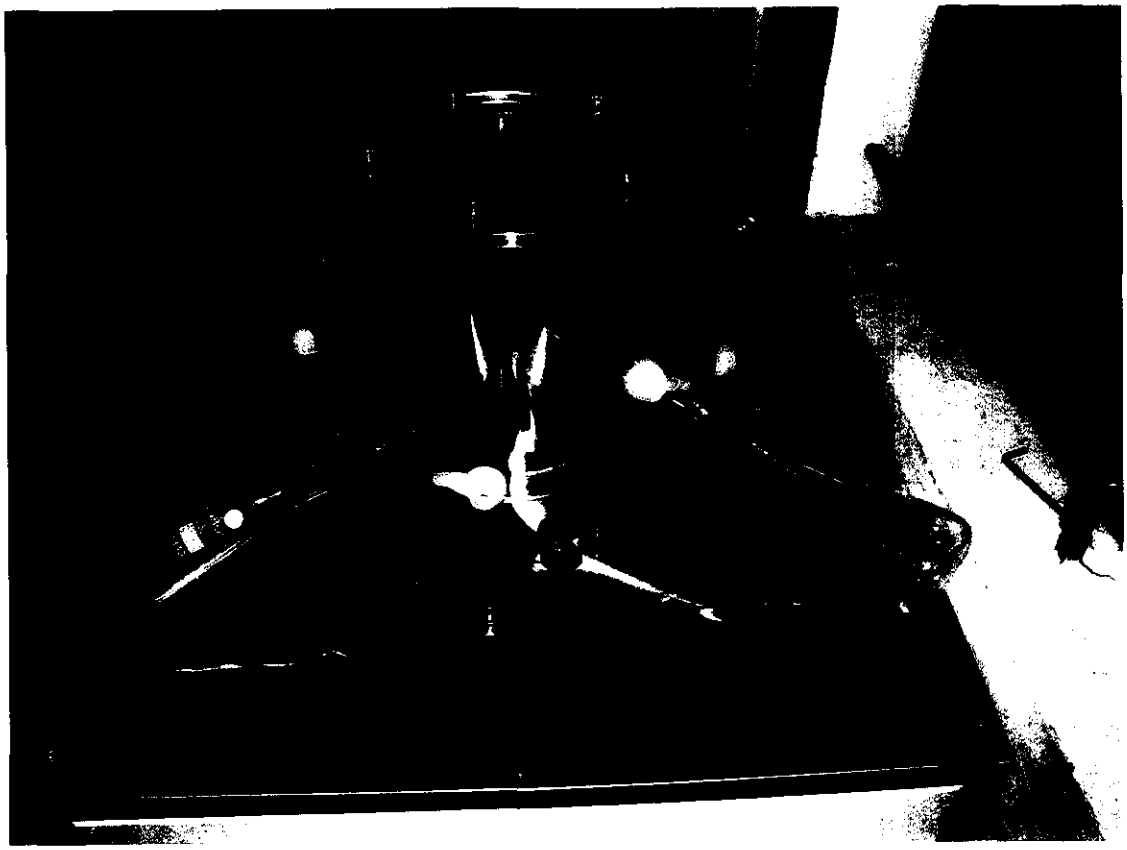
In this work, the Reactive dyes used are Reactive yellow, Reactive blue, Reactive green, Reactive red and Reactive purple. These dyes were obtained from 'Together Textile mills', Kanuvai, Coimbatore, Tamil Nadu. These dyes are widely used by textile industries located in and around Coimbatore. Textile effluent was collected from Together Textile mills, Kanuvai, Coimbatore, Tamil Nadu. Paper mill effluent was collected from Seshasayee paper Board, Erode, Tamil Nadu. The crude filtrates of *Pleurotus eous* and *Bacillus subtilis* CotA spore suspensions were checked for decolourization of dyestuff as well as textile and paper effluents.

Different concentrations of dyestuff solutions were prepared by dissolving the powdered dyestuff in distilled water. The concentrations of dyes used in the present study were 0.1–0.5 %( w/v). The wavelength scanning was used to identify the $\lambda_{max}$ of each of the dyes used. They are, for Reactive Blue ($\lambda_{602\ nm}$), Reactive Yellow ($\lambda_{401\ nm}$) Reactive Green ($\lambda_{620\ nm}$), Reactive purple ($\lambda_{560\ nm}$) Reactive red ($\lambda_{550\ nm}$), paper mill and textile effluents ($\lambda_{220\ nm}$).

**3.9 Stability studies-Stabilization of laccase using sugar additives**

Drying experiments were carried out to test the ability of different sugars (sucrose, lactose, maltose and mannitol) in conferring storage stability and thermostability to laccase. Here enzymes were dried in the presence of different sugars in the ratio (1:10 w/w) of different sugars like sucrose, lactose, maltose and mannitol. To stabilize the laccase activity at high temperature, purified enzyme protein in enzyme solution, respective sugar in of sugar solution and sodium phosphate buffer at pH 5.0 were mixed and lyophilized. A control was an identical preparation of the enzyme, devoid of any sugar, organic acid but subjected to lyophilization. After lyophilization, the dried enzyme in the microfuge tubes was stored in an incubator oven at various temperatures (37°C, 55°C) for various length of time before assay. That is, the enzymes were assayed at $5^{th}$, $10^{th}$ , $20^{th}$ and $30^{th}$ day after freeze-drying.

**Fig 3.2: Stabilization of laccase by Lyophilization**



# 4. RESULTS AND DISSCUSSION

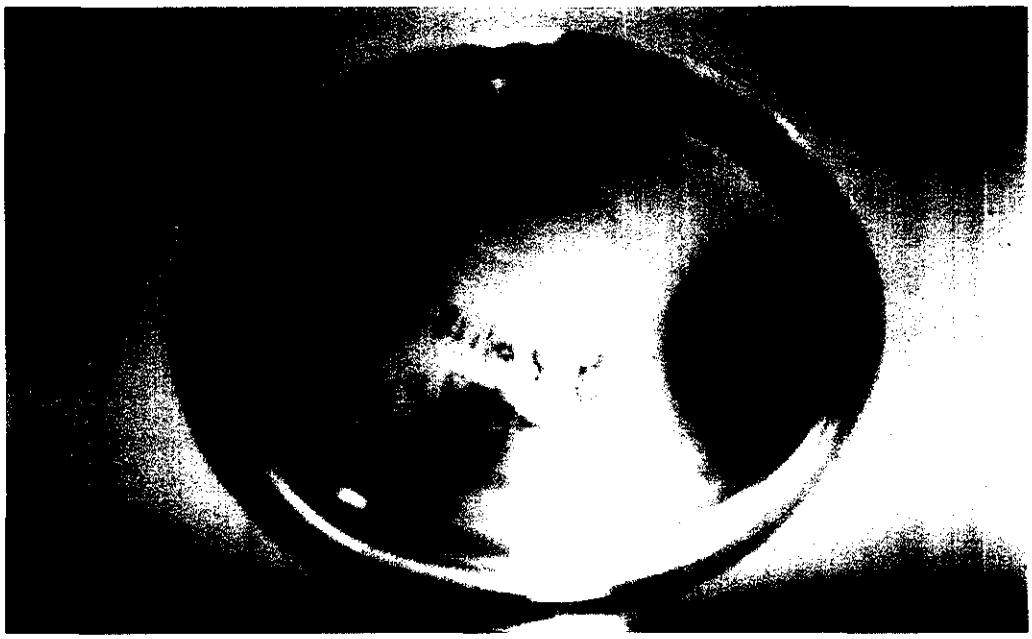
## 4.1 Enzyme source

The enzyme source was culture filtrate of *Pleurotus eous* grown by submerged fermentation technique, at 27°C for 5 days.

## 4.2 Qualitative assay for laccase

Intense brown colour was produced around the fungal colony in the guiacol-containing medium which is considered as a positive reaction for the presence of laccase activity.

**Fig 4.1: Screening of laccase in Basal medium containing guaiacol.**



## 4.3 Enzyme assay

Enzyme assay was performed as mentioned in Chapter 3. ( Materials and Methods). *Pleurotus eous* crude filtrates showed high activity of laccase (4.8 U $ml^{-1}$), Mn-independent peroxidase (0.6 U $ml^{-1}$), and lignin peroxidase (0.10 U $ml^{-1}$). Aryl alcohol oxidase activity was found to be negligible.

# CHAPTER IV

# RESULTS AND DISSCUSSION

### 4.4 Purification of laccase

Laccase was purified from culture filtrate. A purification of upto 38 fold was obtained through ammonium sulphate fractionation and ion exchange chromatography steps.

#### 4.4.1 Crude extract

The crude enzyme extract from the *Pleurotus eous* had a specific activity of 56 units $mg^{-1}$.

#### 4.4.2 Ammonium Sulphate fractionation

The crude extract was subjected to ammonium sulphate fractionation. Differential fractionation of protein in the crude extract was done by a range of ammonium sulphate fractionations (10 to 100%). The supernatant and precipitate of each fraction were tested for laccase activity. Laccase activity in the supernatants started decreasing after removing 50% of saturated ammonium sulphate fraction. In the precipitate laccase activity reached a maximum in the 70% saturation. Ammonium sulphate fractionation yielded a laccase preparation having 1.5 folds higher specific activity over the crude extract. The enzyme yield after ammonium sulphate precipitation was 58%.(table 4.1)

#### 4.4.3 Ion exchange chromatography

Ion exchange chromatography of the enzyme on CM-cellulose showed that all detectable activity was present less than one symmetrical peak from fifth to tenth fraction. Laccase activity was found to be maximum in the eighth fraction. Fractions 7, 8 and 9 were pooled. This purification step yielded a laccase preparation having 38 fold over the crude extract. The specific activity was 2212 units/ mg protein and the enzyme yield was 28.2%. (table 4.1)

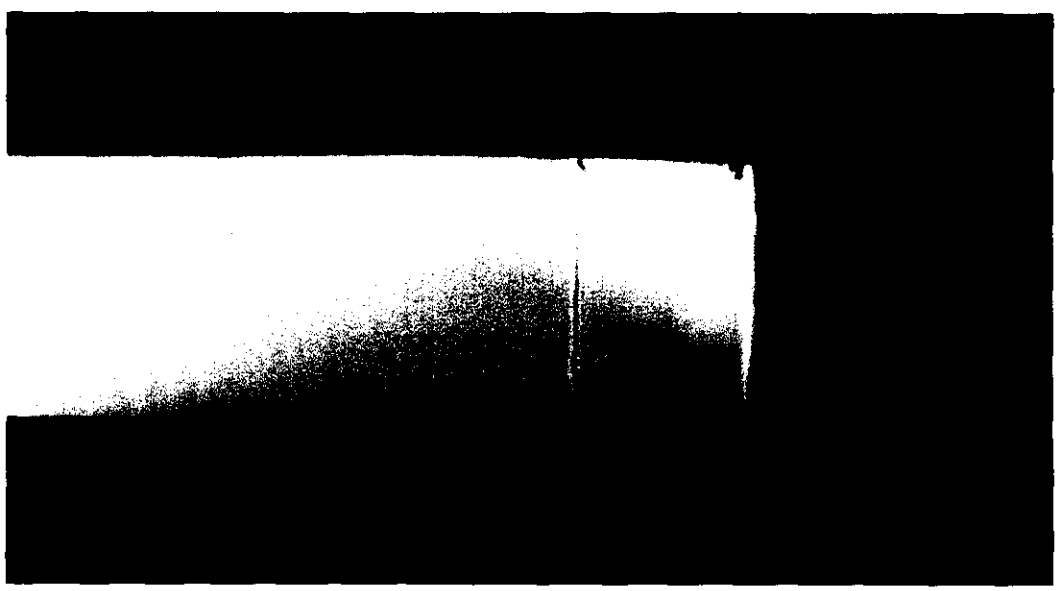
**Table 4.1: Purification of laccase from *Pleurotus eous***

| Treatment | Total volume (ml) | Total enzyme units | Total protein (mg) | Specific activity (units/mg) | Enzyme Yield | Purification fold |
|---|---|---|---|---|---|---|
| Culture filtrate | 85 | 55930 | 1003.00 | 56 | 100.0 | 1.00 |
| Ammonium sulphate precipitaton | 85 | 32439 | 356.00 | 91 | 58.0 | 1.62 |
| CM-cellulose | 18 | 15772 | 7.13 | 2212 | 28.2 | 39.50 |

### 4.4.4 Thin layer chromatography

A blue color developed when ABTS was used as the substrate, which indicates laccase activity.

**Fig 4.2: TLC showing Laccase activity with ABTS as substrate**



## 4.5 Characterization of laccase

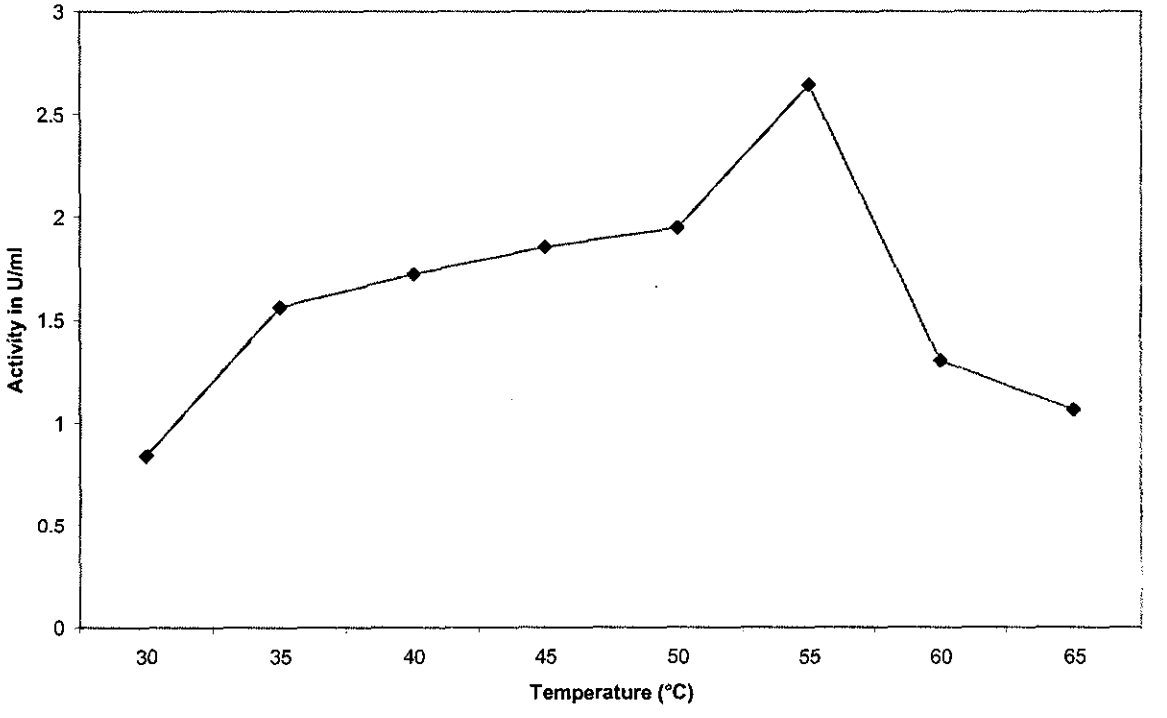
### 4.5.1 Molecular mass (MW) of laccase

The molecular mass of laccase was determined by SDS-PAGE. It was found to be 64 kDa.

### 4.5.2 Effect of temperature on laccase activity

The laccase enzyme activity assays was carried out at a temperature range from 30°C-65°C, using guaiacol as the substrate. The optimum temperature for laccase activity was found to be at 55°C. (fig 4.3).

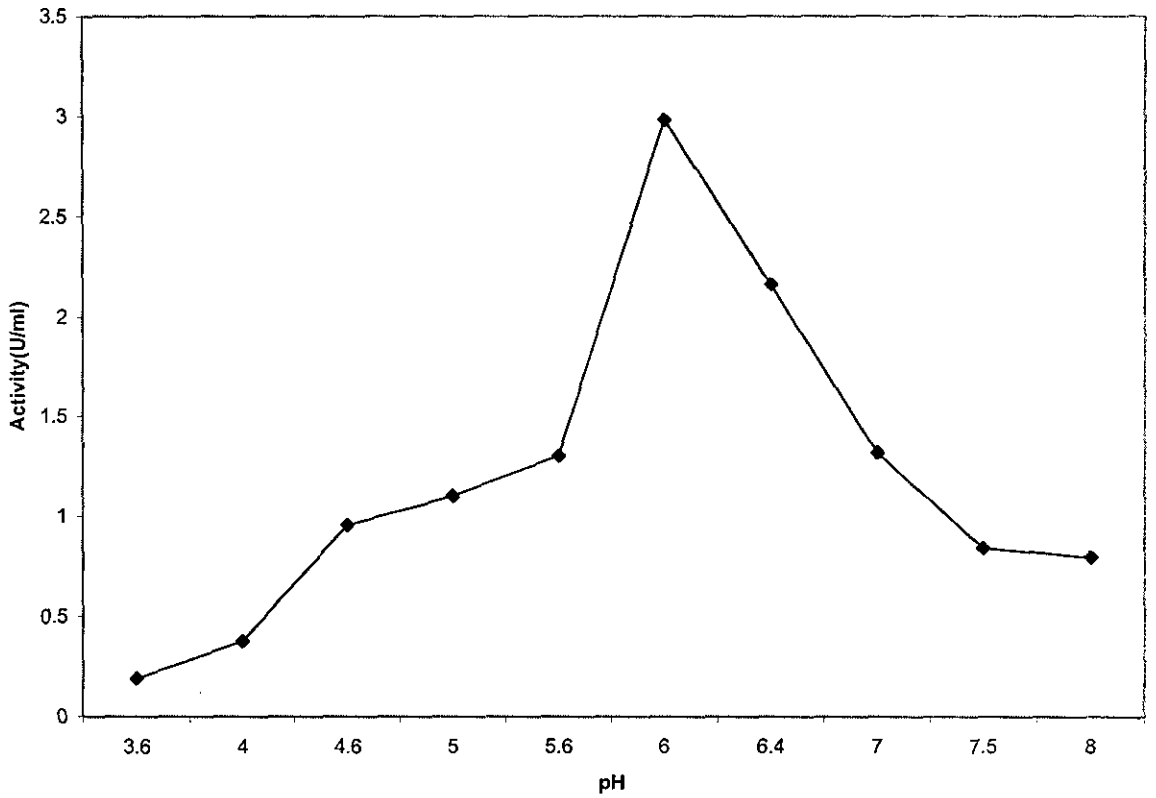
Fig 4.3. Effect of temperature on purified laccase from *P.eous*



### 4.5.3 Effect of pH on laccase activity

The laccase enzyme activity assays was carried out at pH range from pH 3.0 to 9.0, using guaiacol as the substrate. The optimum pH for laccase activity was found to be at pH 6.0. (fig 4.4)

Fig4.4 .Effect of pH on purified laccase from *P.eous*




### 4.5.4 Inhibitor studies

Several inhibitors like $Al^{3+}$ $Fe^{2+}$ $Fe^{3+}$ $Mg^{2+}$ $Mn^{2+}$ and SDS were tested to find out their effect on laccase activity. Out of several inhibitors used 100% inhibition was found in 40mM and 50mM of $Fe^{2+}$and $Al^{3+}$ ( Table 4.2).One interesting finding was that the lower concentration of 1% SDS showed more inhibition than higher concentration (Table 4.3). Other metal ions showed partial inhibition.

Fig 4.5. .Effect of Inhibitors on purified laccase

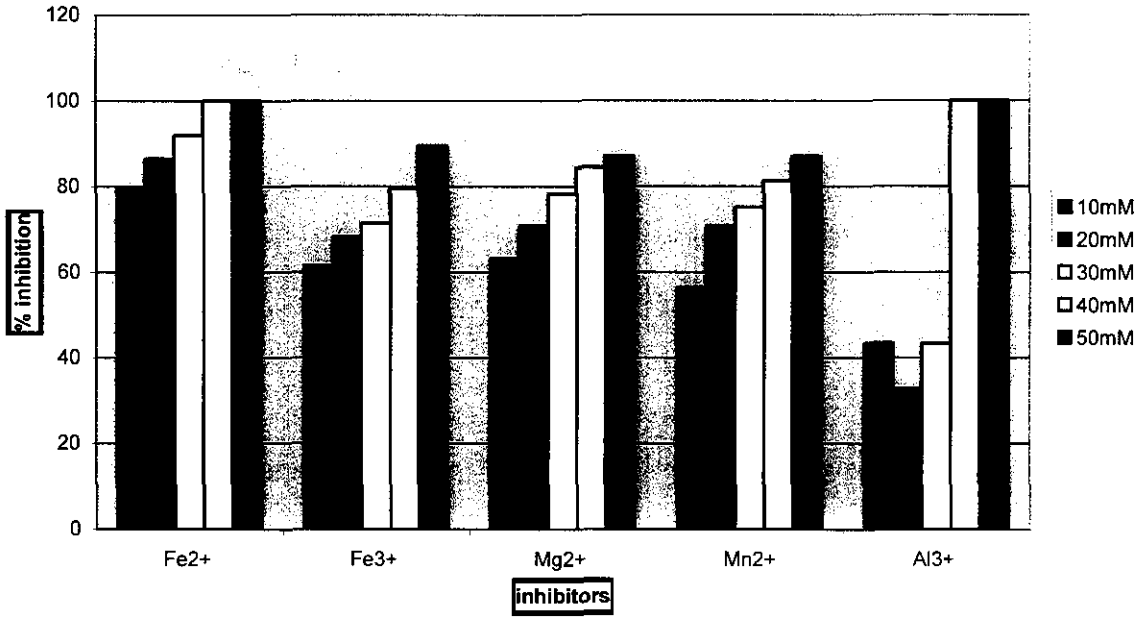



**Table 4.2: Effect of metal ions on laccase activity**

| Metal Ions / Inhibitor concentration | $Al^{3+}$ | $Fe^{2+}$ | $Mg^{2+}$ | $Mn^{2+}$ | $Fe^{3+}$ |
|---|---|---|---|---|---|
| 10mM | 19% | 80% | 63% | 57% | 62% |
| 20mM | 33% | 86% | 71% | 71% | 68% |
| 30mM | 44% | 92% | 78% | 75% | 71% |
| 40mM | 100% | 100% | 85% | 82% | 80% |
| 50mM | 100% | 100% | 87% | 87% | 90% |

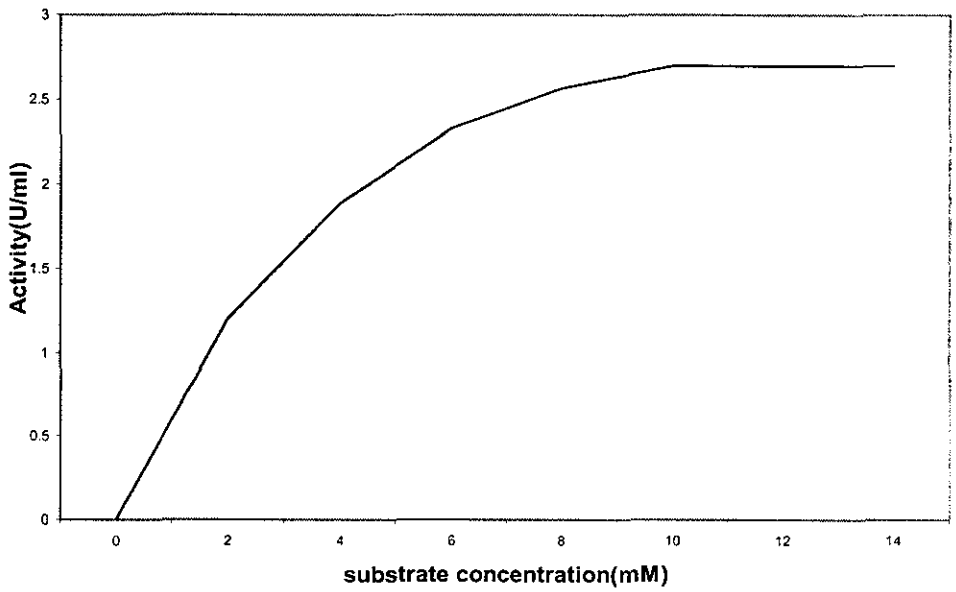
**Table 4.3: Effect of SDS on laccase activity**

| Concentration of SDS | 1% | 3% | 5% |
|---|---|---|---|
| % Inhibition | 76% | 74% | 67% |

### 4.5.5 Effect of Substrate Concentration

Similarly the effect of substrate on laccase activity was carried out using guaiacol at different concentrations (2mM-14mM). Laccase activity was found to be maximum at 10mM substrate concentration. $V_{max}$ ,$K_m$ value was found to be 2.702mM and 1.351mM respectively.(fig 4.6).

**Fig 4.6: Effect of substrate concentration on purified laccase by *P.eous***



### 4.6 Decolourization studies

#### 4.6.1 Agar plate screening

Initial evaluation of dye decolourization was done using solid medium. By day 4, the extent of mycelial growth on the agar plates was comparable for all cultures whether or not any dye was present. Decolourization began with the formation of clear zones around the colonies. Complete decolourization was assessed as the total disappearance of color from the medium, without any visible sorption to the biomass. In a 10-day period, *Pleurotus eous* decolourized the maximum number of dyes to the greatest extent. Reactive yellow was completely decolourized, while there were faint traces of color on the reactive green and reactive blue plates. Reactive red and reactive purple were degraded to lesser extent.

**Fig 4.7 : Decolourization of reactive yellow in PDA by *P.eous***

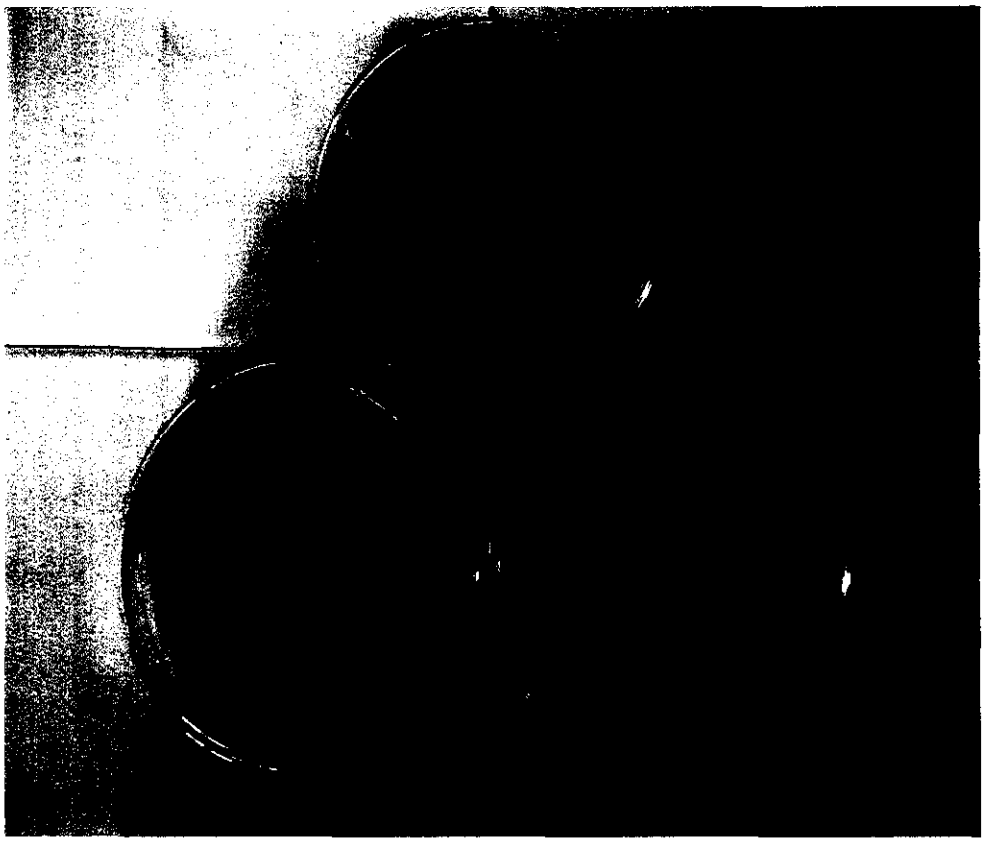


**Table 4.4: Solid plate dye decolourization by *Pleurotus eous* in a 10 day period**

| Reactive dye | Decolourization |
|---|---|
| Reactive yellow | ++++ |
| Reactive green | +++ |
| Reactive red | ++ |
| Reactive blue | +++ |
| Reactive purple | + |

++++ = Complete decolouration

+++ = Faint colour in some regions

++ = Strong colour in some regions

\+ = Small region decolourized

\- = No decolouration



### 4.6.2 Decolourization of individual dyes

On third day of incubation the crude filtrate (0.2-ml) of *Pleurotus eous* was found to decolourize the dye to a greater extent. However, the decolourization was more rapid when *Pleurotus eous* crude extract and *Bacillus subtilis* CotA spore suspensions were used in combination. Similar

work was carried out using *Pleurotus florida* and *Agaricus bisporus* crude filtrates for decolourization of reactive dyes, in which decolourization of Reactive Yellow was 89% followed by Reactive Green, which was 45% (Shanmugam *et al.*, 2005). *Pleurotus eous* filtrate when used individually or in combination with *Bacillus subtilis* CotA spore suspension completely decolourized both paper and textile effluents within 1 day (Table 4.10).

**Fig 4.8 : Decolourization of textile effluent by *P.eous***

**Decolourization of reactive dyes**

**Table 4.5 Treatment of reactive yellow using *P.eous* filtrate and using combinations of crude extracts of *Pleurotus eous* and *Bacillus subtilis* spore suspensions**

| No of Days | *Pleurotus eous* crude extract (% Decolourization) | | | | | *Pleurotus eous* crude extract + *Bacillus subtilis* Spore suspensions (% Decolourization) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% |
| Day1 | 75.7 | 70.5 | 70.4 | 67.6 | 60.38 | 74.3 | 69.8 | 67.8 | 66.6 | 65.3 |
| Day2 | 78.1 | 77.7 | 72.1 | 71.7 | 67.7 | 85.7 | 84.1 | 83.5 | 82.0 | 81.5 |
| Day3 | 86.5 | 84.4 | 81.3 | 78.1 | 74.1 | 90.0 | 88.5 | 87.3 | 86.2 | 84.0 |

The experiment was conducted in duplicate. Decolourization of dyestuffs was carried out using 0.1 to 0.5% (w/v) concentrations. 0.2 ml of crude filtrate of *Pleurotus eous* and *Bacillus subtilis* spore suspensions (1:1, v/v) was used.

**Table 4.6 Treatment of reactive blue using *P.eous* filtrate and using combinations of crude extracts of *Pleurotus eous* and *Bacillus subtilis* spore suspensions**

| No of Days | *Pleurotus eous* crude extract (% Decolourization) | | | | | *Pleurotus eous* crude extract + *Bacillus subtilis* spore suspension (% Decolourization) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% |
| Day1 | 83.8 | 76.8 | 75.4 | 73.6 | 72.4 | 80.4 | 78.2 | 77.6 | 73.1 | 70.1 |
| Day2 | 85.5 | 80.3 | 76.7 | 79.9 | 76.6 | 82.1 | 81.1 | 80.9 | 76.1 | 77.6 |
| Day3 | 85.4 | 83.5 | 81.1 | 80.8 | 77.8 | 86.8 | 84.5 | 83.9 | 82.7 | 82.0 |

The experiment was conducted in duplicate. Decolourization of dyestuffs was carried out using 0.1 to 0.5% (w/v) concentrations. 0.2 ml of crude filtrate of *Pleurotus eous* and *Bacillus subtilis* spore suspensions (1:1, v/v) was used.

**Table 4.7 Treatment of reactive green using *P.eous* filtrate and using combinations of crude extracts of *Pleurotus eous* and *Bacillus subtilis* spore suspensions**

| No of Days | *Pleurotus eous* crude extract (% Decolourization) | | | | | *Pleurotus eous* crude extract + *Bacillus subtilis* spore suspension (% Decolourization) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% |
| Day1 | 83.4 | 76.1 | 74.4 | 72.9 | 71.6 | 85.5 | 84.4 | 78.7 | 72.8 | 71.6 |
| Day2 | 86.1 | 78.6 | 75.4 | 74.4 | 73.3 | 88.3 | 86.6 | 81.4 | 76.9 | 73.4 |
| Day3 | 87.5 | 82.7 | 78.7 | 78.2 | 75.9 | 90.0 | 87.2 | 86.0 | 85.2 | 82.0 |

The experiment was conducted in duplicate. Decolourization of dyestuffs was carried out using 0.1 to 0.5% (w/v) concentrations. 0.2 ml of crude filtrate of *Pleurotus eous* and *Bacillus subtilis* spore suspensions (1:1, v/v) was used.

**Table 4.8 Treatment of reactive red using *P.eous* filtrate and using combinations of crude extracts of *Pleurotus eous* and *Bacillus subtilis* spore suspensions**

| No of Days | *Pleurotus eous* crude extract (% Decolourization) | | | | | *Pleurotus eous* Crude extract + *Bacillus subtilis* Spore suspensions (% Decolourization) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% |
| Day1 | 64.0 | 61.5 | 58.9 | 57.0 | 52.1 | 64.2 | 60.2 | 59.2 | 58.9 | 45.5 |
| Day2 | 69.3 | 65.7 | 62.6 | 61.1 | 60.4 | 70.8 | 65.3 | 63.7 | 63.6 | 51.8 |
| Day3 | 75.2 | 67.8 | 66.4 | 64.0 | 63.9 | 71.3 | 66.7 | 66.1 | 65.3 | 54.3 |

The experiment was conducted in duplicate. Decolourization of dyestuffs was carried out using 0.1 to 0.5% (w/v) concentrations. 0.2 ml of crude filtrate of *Pleurotus eous* and *Bacillus subtilis* spore suspensions (1:1, v/v) was used.

**Table 4.9 Treatment of reactive purple using *P.eous* filtrate and using combinations of crude extracts of *Pleurotus eous* and *Bacillus subtilis* spore suspensions**

| No of Days | *Pleurotus eous* crude extract (% Decolourization) | | | | | *Pleurotus eous* crude extract + *Bacillus subtilis* Spore suspensions (% Decolourization) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% | 0.1% | 0.2% | 0.3% | 0.4% | 0.5% |
| Day1 | 66.5 | 64.9 | 60.5 | 54.7 | 45.1 | 66.6 | 73.0 | 70.5 | 70.1 | 69.1 |
| Day2 | 70.9 | 69.7 | 63.4 | 60.9 | 47.4 | 78.9 | 75.3 | 75.1 | 74.6 | 74.2 |
| Day3 | 75.1 | 71.9 | 65.8 | 61.7 | 55.7 | 81.7 | 77.6 | 77.5 | 77.1 | 75.6 |

The experiment was conducted in duplicate. Decolourization of dyestuffs was carried out using 0.1 to 0.5% (w/v) concentrations. 0.2 ml of crude filtrate of *Pleurotus eous* and *Bacillus subtilis* spore suspensions (1:1, v/v) was used.

**Table 4.10 Treatment of Paper and Textile Effluent using *P.eous* filtrate and using combinations of crude extracts of *Pleurotus eous* and *Bacillus subtilis* spore suspensions**

| No of days | *Pleurotus eous* crude extract (% Decolourization) | *Pleurotus eous* crude extract + *Bacillus subtilis* Spore suspensions (% Decolourization) |
|---|---|---|
| | Paper effluent | Textile effluent |
| Day1 | > 90 % | > 90% |

The experiment was conducted in duplicate. Decolourization of effluents was carried out using 0.2 ml of crude filtrate of *Pleurotus eous* and *Bacillus subtilis* spore suspensions (1:1, v/v).

**4.7 Stability studies**

The result of the drying experiments is given in table (4.11). It shows the recovery of enzyme activity at various temperatures and various lengths of time (i.e. days) before assay against different sugars.

**Table 4.11 -Stability studies using various sugars**

| S.No. | Sugar | Property | Temp (°C) | No. of days | | | |
|---|---|---|---|---|---|---|---|
| | | | | 5 | 10 | 20 | 30 |
| 1 | No sugar | -- | 37 | ++ | + | _ | _ |
| | | | 55 | ++ | _ | _ | _ |
| 2 | Mannitol | NR | 37 | + | + | _ | _ |
| | | | 55 | + | + | _ | _ |
| 3 | Maltose | NR | 37 | ++ | + | + | _ |
| | | | 55 | +++ | +++ | +++ | +++ |
| 4 | Lactose | R | 37 | +++ | ++ | ++ | + |
| | | | 55 | +++ | +++ | +++ | +++ |
| 5 | Sucrose | NR | 37 | +++ | +++ | +++ | +++ |
| | | | 55 | +++ | +++ | +++ | +++ |

\- = 0-10% of initial enzyme activity

\+ = 10-50% of initial enzyme activity

++ = 50-90% of initial enzyme activity

+++ = 90-100% of initial enzyme activity

R - Reducing sugar

NR - non reducing sugar

During storage in moisture free conditions for 5 days at 55°C, the laccase without any sugar was found to retain more than 90% of initial activity. The laccase preparation with maltose retained more than 90% of initial activity upto 30 days. But at 37°C, the enzyme retained only 70% and 36% activity of enzyme activity after 5 and 10 days of incubation,

The laccase stabilized with sucrose and kept at 37°C and 55°C was found to retain more than 90% of initial activity upto 30 days.

The laccase preparation with lactose, at 55°C was found to retain more than 80% of initial activity upto 30 days but at 37°C, the preparation retained only 72%, 42% and 19% of initial activity at 10,20,30 days of incubation respectively.

The results obtained have demonstrated the ability of sucrose in converting normally thermolabile laccase into a thermostabe enzyme while mannitol destabilized the same which otherwise was stable in lyophilized condition. It has been predicted (Arakawa and Timasheff, 1982.) that the preferential exclusion of the protein surface from contact of solvent (normally aqueous medium) by the sugar. This could be the reason for several thermolabile enzymes, which are capable of expressing their activity even at high temperatures in the presence of organic solvents (Zaks and Klibanov, 1984). Further to these general predictions, here we envisage a minimum structural requirement of any sugar additive involved in stabilization of laccase. The striking features include the aldehyde functional group and C-2$\propto$ hydroxyl group of the sugar.

# CHAPTER V

# SUMMARY AND CONCLUSION

# 5. SUMMARY AND CONCLUSION

*Pleurotus eous* was isolated from Western Ghats and screened for laccase production.Laccase was purified 38 folds by ammonium sulphate fractionation and ion exchange chromatography. The enzyme had a molecular weight of 64 kDa. The purified laccase was most active at pH 6.0 and optimum temperature was observed at 55 °C. The enzyme was stable from pH 4.6 to 7.0 and upto 65°C.Laccase displayed normal Michaelis-Menton kinetics and showed a Km value of 1.351mM for its substrate, guaiacol. Laccase activity was suppressed by inhibitors like $Al^{3+}$ $Fe^{2+}$ $Fe^{3+}$ $Mg^{2+}$ $Mn^{2+}$ and SDS. 100% inhibition was found in 40mM and 50mM of $Fe^{2+}$and $Al^{3+}$. Other metal ions showed partial inhibition.

Of several reactive dyes used *Pleurotus eous* was found to decolourize reactive yellow and reactive green to a greater extent. A variety of dyes are used in textile industries that are released in the effluent pose an important environmental problem, which cannot be easily solved. In summary, the low cost of *Pleurotus eous* extracts and *Bacillus subtilis* spore suspensions containing various enzymes as a cocktail may prove to be an alternative source of decolourization and an efficient method of treating waste water containing dye stuffs as well as in treating textile and paper effluents.

Drying experiments were carried to test four sugars for their ability to confer storage stability and thermostability on laccase. The ratio between enzyme protein and sugar was 1:10(w/w). Out of four sugars, sucrose was found to be the best enzyme stabilizer; the lyophilized enzyme was stable upto 30 days at 55°C. It was followed by lactose and maltose. But in contrast mannitol destabilized the enzyme activity.

# APPENDICES

## APPENDICES

APPENDIX I

Acetate Buffer

Stock Solutions

A: 0.2M solution of acetic acid (11.55ml in 1000ml)

B: 0.2 M solution of sodium acetate (16.4g in 1000 ml)

"x" ml of solution A and "y" ml of B is diluted to 100ml with distilled water.

| X | y | pH |
|---|---|---|
| 48.2 | 1.8 | 3.2 |
| 46.3 | 3.7 | 3.6 |
| 44 | 6 | 3.8 |
| 41 | 9 | 4.0 |
| 36.8 | 13.2 | 4.2 |
| 30.5 | 19.5 | 4.4 |
| 25.5 | 24.5 | 4.6 |
| 20 | 30 | 4.8 |
| 14.8 | 35.2 | 5.0 |
| 10.5 | 39.5 | 5.2 |
| 8.8 | 41.2 | 5.4 |
| 4.8 | 45.2 | 5.6 |
| 3.6 | 45.4 | 5.8 |
| 2.4 | 47.6 | 6.0 |

Phosphate Buffer

Stock Solutions

A: 0.2M solution of monobasic sodium phosphate (27.8g in 1000ml)

B: 0.2M solution of dibasic sodium phosphate (53.65g of disodium hydrogen phosphate in 1000ml)

X ml of A, Yml of B diluted to a total of 200ml.

| X | Y | pH |
|---|---|---|
| 5.3 | 94.7 | 8.0 |
| 3.6 | 96.4 | 8.4 |
| 1.9 | 88.1 | 8.8 |
| 0.2 | 99.8 | 9.0 |

APPENDIX II

**Estimation of Protein by Lowry's Method**

Reagents

Stock Standard Bovine serum Albumin

Dissolve 100 mg of BSA and make up to 100ml with distilled water

Working standard solution

Take 10ml of the stock solution and dilute to 100ml with distilled water

2% Sodium carbonate in 0.1N sodium hydroxide (Reagent A)

0.5% Copper sulphate in 1% Potassium sodium tartrate (Reagent B)

Alkaline Copper solution

Mix 50 ml of A and 1ml of B prior to use (ReagentC)

Folin ciocalteau reagent(1:1) Should be freshly prepared

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