PRODUCTION OPTIMIZATION, PURIFICATION AND CHARACTERIZATION OF LACCASE PRODUCED FROM *Ganoderma lucidum*

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Glossary acronyms

(List of abbreviation words)

µg	=	microgram
µl	=	microlitre
ABTS	=	2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
AFEX	=	Ammonia Fiber Explosion
AIL	=	Acid soluble lignin
ASL	=	Acid insoluble lignin
BSA	=	Bovine Serum Albumin
DEAE	=	Diethylaminoethanol
DNA	=	Deoxy-ribose Nucleic Acid
DNS	=	Dinitro salisylic acid
EPR	=	Electronic Paramagnetic Resonance
g	=	gram
GHG	=	Green House Gases
HMF	=	Hydroxy Methyl Furfural
HOBt	=	Hydroxybenzotriazole
Lac	=	laccase
Lig	=	lignin
LiP	=	Lignin Peoxide
LME	=	lignin modifying enzymes
mA	=	milli Ampere
mg	=	milligram
ml	=	milliliter
MnP	=	Manganese Peroxidase
MnP	=	Manganese Peroxide
nm	=	nanometer
PAGE	=	Poly Acrylamide Gel Electrophoresis
PAH	=	Poly cyclic Aromatic Hydrocarbons

PDA = Potato Dextrose Agar

Rpm = Revolution per minute

RT = Room Temperature

SDS = Sodium Dodecyl Sulphate

WRF = White rot fungi

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PRODUCTION OPTIMIZATION, PURIFICATION AND CHARACTERIZATION OF LACCASE PRODUCED FROM *Ganoderma lucidum.*

ABSTRACT

Laccases are copper containing metalloenzyme that catalyze one-electron oxidation of phenolics, non-phenolics, aromatic amines and other electron rich compounds with simultaneous reduction of O_2 to H_2O. Laccases are common enzymes in nature, especially in fungi and higher plants. White rot fungi are of significant interest as they secrete extracellular laccase to degrade lignin to get access to cellulose and hemicelluloses of plants. Laccase are also recognized for their wide application in ethanol production, food industry, dye bleaching, paper and pulp processing, production of chemicals from lignin and many more. In this present study screening of laccase producing fungus was carried out using Guaiacol, 1-naphthol and tannic acid supplemented in PDA. On the basis of zone of oxidation of screening agents *Ganoderma lucidum* was taken for further studies. Laccase production by white rot fungus, *Ganoderma lucidum,* was studied using submerged fermentation in modified Olga medium. Lignin from dried rice straw was extracted. Presence of lignin in the media increased the extracellular laccase production by 110.6 % as compared to the media containing glucose only and 30mM of copper sulphate stimulated laccase production by was 113.7% with enzyme activity reaching as high as 93.1217 U/mL. Optimum temperature was found to be 30 ^{o}C and optimum pH was found to be 5 for higher laccase production. Laccase was purified by ammonium sulphate salt precipitation and by anion exchange chromatography through DEAE sepharose gel column. Native PAGE and ion exchange chromatography indicated the presence of three isoforms of laccase but single band was observed in SDS-PAGE with molecular weight of 43 kDa. Laccase showed highest activity at 30 ^{o}C and at pH 5 respectively. *Ganoderma lucidum* laccase is a thermostable laccase as laccase activity at 70 ^{o}c decreased only by 28.42% after 1 hour incubation. V_{max} and K_m were found to be 0.11 mM and 35.71 µmol/min respectively and k_{cat} was found to be 246.21 min^{-1} for ABTS as substrate. The isolated thermostable laccase can be used for various biotechnological and industrial applications.

Key words : **Laccase, lignin, *Ganoderma lucidum*, ABTS, Guaiacol, 1-naphthol and tannic acid.**

1. INTRODUCTION

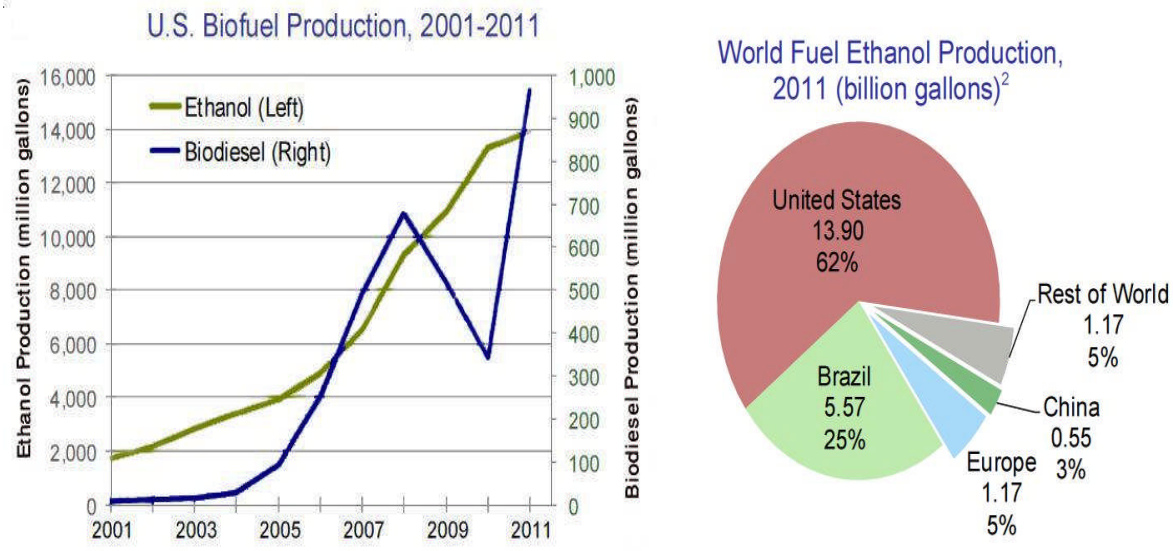
1.1. Background

In recent years attention has been drawn towards bioenergy as a sustainable energy source which may help cope the future energy crisis, increasing demand of energy, rising prices of fuels and increasing concern about global warming from GHG. Biofuels already constitute the major source of energy for over half of the world's population, accounting for more than 90% of the energy consumption in developing countries (Msangi et al., 2007). The carbon balance of biofuels are close to neutral, as lignocellulosic materials absorb as much carbon dioxide in its growth as it releases when it is burnt, when compared with petroleum-derived fuels such as gasoline, diesel, or kerosene and hence biofuels are becoming popular in present scenario of global warming(Graves et al., 2011; Speight, 2011).

Biofuels are a type of fuel whose energy is derived from biological carbon fixation. Global production of biofuels increased 17 percent in 2010 to reach an all-time high of 105 billion liters, up from 90 billion liters in 2009 with the United States and Brazil being the two largest producers of ethanol as in 2010, the United States generated 49 billion liters, or 57 percent of global output, and Brazil produced 28 billion liters, or 33 percent of the total (http://www.worldwatch.org/biofuels-make-comeback-despite-tough-economy).

The production of 1st generation biofuels such as ethanol from sugarcane in Brazil, ethanol from corn in U.S., India and China; biodiesel from oilseed rape in Germany, and palm oil biodiesel in Malaysia are characterized by well maintained commercial markets and technologies. The production processes used to manufacture biodiesel from its feedstock sources differs from that used for bioethanol, as it relies on trans-esterification of oils, whereas bioethanol production relies on the hydrolysis of the constituent grains and sugars of plants into ethanol, under conventional technologies (Msangi et al., 2007). Canada has national mandates for the production of E5 (5 percent ethanol and 95 percent gasoline) and B2 (2 percent ethanol and 98 percent gasoline). But constraints of 1st generation biofuels like – higher food prices due to competition with food crops, expensive technology, limited GHG reduction (except sugarcane ethanol), limited biomass, accelerating deforestation and potentially negative impact on biodiversity necessitated practice of 2nd generation biofuels. The second generation processes use lignocellulosic materials (agriculture, forest residue and non food crops feedstocks) for this purpose and the biosphere has plenty of supplies of lignocellulosic materials to fulfill the future energy needs (Balat et al., 2008). Third-generation biofuels are fuels that would be produced from algal biomass, which has a very distinctive

growth yield as compared with classical lignocellulosic biomass (Lee and Lavoie, 2013). Algae are known to produce biomass faster and on reduced land surface as compared with lignocellulosic biomass. Usually, species such as Chlorella are targeted because of their high lipid content around 60% to 70%; (Liang et al., 2009) and their high productivity (7.4 g/L/d for *Chlorella protothecoides*;(Chen et al., 2011)). Nevertheless, production of algal biomass presents technical challenges such as lipid extraction and dewatering, as well as geographical challenges.



a) b)

Fig. 1. a)-Rate of increase in biofuels production In U.S. from 2001-2011, b)-share of world fuel ethanol production in 2011 A.D. (source: (Lee and Lavoie, 2013)).

1.2. Rationale:

The fact that lignocellulosic biomass is of great importance in biofuels production and paper and pulp industry, has made its effective degradation a subject of research since long time. Lignin is the major limiting factor in degradation of lignocellulosic biomass. Of all the lignin modifying enzymes studied, laccases are considered the major ones responsible for degradation of lignin and it is increasingly being used in the processes like delignification, production of value added chemicals from lignin, paper and plup bleaching, prevention of wine discoloration, waste decolorization and detoxification, bioremediation, biosensors, biofuels cells etc . Laccase has been the subject of research since 19th century and still potent laccase is being searched. In the context of Nepal there are no reports of study about laccase till present date though the fact is well known that Nepal is the habitat of diversified wood rotting fungi. Genetic manipulation of lignin has also been reported to reduce lignin content in plants which would lead to improved ethanol production but such researches have also stated that lignin reduction

below certain threshold can cause large changes in wood chemistry and plant metabolism ultimately leading to low ethanol yield. This also necessitates finding of novel laccase enzyme with higher stability and activity which would prove to be an ideal enzyme for industrial application. However, a serious problem often encountered with industrial exploitation of fungal laccases is its low production levels by the native hosts. Hence, search for potent fungus that can produce significant extracellular laccase enzyme with desirable properties is of utmost importance at present context as development of biofuels in Nepal can provide a great back support to the country's economy by making the country independent in terms of fuels and providing income to the farmers.

1.3. Research hypothesis:

Lingocellulose is one of the major energy sources for obtaining bio-fuels. The main sources of biofuel are cellulose and hemicelluloses in plant biomass but the major obstacle is presented by heteromorphic lignin which encases the cellulose and hemicelluloses. To overcome such obstacle physical and chemical methods are available which are expensive, difficult to operate and polluting. So, biological methods are best ways to obtain biofuels from lignocellulosic biomass. Hence suitable potent enzymes are required for delignification and increased ethanol production. There are numerous fungal species that grow on wood logs and obtain their energy from them. Most of them have laccase as the main enzyme to overcome the lignin barrier and obtain the energy from cellulose and hemicelluloses. The hypothesis of this study is that fungal species of Nepal may have adapted in different ways and some them may be an efficient laccase producer. Study of laccase from such organism to produce potent laccase enzyme and characterization of such enzyme may be applicable industrially for variety of purposes.

1.4. General objective:

The basic objective of this work was the screening of potent laccase producing fungi and optimized production and characterization of laccase enzyme produced by the isolated fungi.

1.5. Specific objectives:

- Screening of potent laccase producing fungi.
- Isolation of lignin from rice straw to use it as substrate for isolated fungi for enhanced laccase production.
- Optimization of production media for over production of laccase.
- Optimization of laccase production from the isolated fungi.
- Purification and characterization of laccase.

2. LITERATURE REVIEW

2.1. Lignin

Plant biomass primarily consists of cellulose, hemicellulose and lignin. After cellulose, lignin is the second most abundant natural polymer found in the woody plants which comprises up to 15-30% of the cell wall (Gold and Alic, 1993). Lignin is a high molecular weight (600–1500 kDa) aromatic amorphous polymer (Buranov et al., 2010; Ross and Mazza, 2010) made up of oxidative coupling of three major monomeric units namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (monolignols) (Kálmán and Réczey, 2007) which are incorporated in the form of phenylpropanoids namely p-hydroxyphenyl, guaiacyl, and syringyl respectively to form lignin (Boerjan et al., 2003); Ralph *et al.*, 2001; Kalman et al., 2007). These monomers are methoxylated to various degress and connected by more than twenty different carbon to carbon (C-C) and ether (C-O-C) linkages such as β-O-4, 4-O-5, β-β, β-1, β-5, 5-5' (Ander and Eriksson, 1976; Kirk, 1985; Li et al., 2008; She et al., 2010) with β-O-4 (β-aryl ether bond) linkage being the most abundant in all lignins (She et al., 2010). Lignins are relatively hydrophobic and are well known for their heterogeneity in lacking a defined primary structure. Gymnosperms (softwood) mainly consists 27-33% of lignin and mainly comprises of guaiacyl type lignin, Dicotyledonous Angiosperms have 18-25% of lignin and mostly guaiacyl-syringyl type lignin is present in them and Monocotyledonous Angiosperms contains 17-24% of lignin and are composed of mixtures of all three (Sarkanen and Ludwig, 1971; Buranov and Mazza, 2008). Lignin fills the spaces between cellulose, hemicellulose, and pectin by covalently linking to the polysaccharides and forming network made up of mainly benzyl-ether, benzyl-ester, acetal and phenyl glycoside bonds (Zhang et al., 2010; Bandounas et al., 2011) between lignin and hemicellulose (Boerjan et al., 2003). The cross linking of lignin with other cell wall components makes it indigestible and it also reduces the accessibility of cellulose and hemicelluloses to microbial digestion. Lignin affects xylem conductance and protects the vasculature from embolism as crosslinking of hydrophilic polysachharide part cell wall and hydrophobic lignin makes water absorption by cell wall difficult (Voelker et al., 2010). Thus, lignin makes the plant cell wall rigid, hydrophobic and affects the xylem conductance (Raven, 1977; Boyce *et al*., 2004) which resists the tissue digestion and decomposition from pathogens and herbivorous (Thurston, 1994; Iyer and Chattoo, 2003; Voelker et al., 2010). Lignin is present in almost all vascular plants but they are absent in bryophytes. This supports the idea that lignin functions for water transportation (Ticlo DTD, 2008). The enzymes implicated in lignin degradation are: (1) lignin peroxidase, which catalyses the oxidation of both phenolic and non-phenolic units, (2) manganese-dependent peroxidase, (3) laccase, which oxidises phenolic compounds to give phenoxy radicals and quinones; (4)

glucose oxidase and glyoxal oxidase for H_2O_2 production, and (5) cellobiose-quinone oxidoreductase for quinone reduction (Thakker et al., 1992). Lignin can be depolymerized by thermochemical methods such as pyrolysis, chemical oxidation, hydrogenolysis, gasification and hydrolysis under supercritical condition but these methods are expensive and environment polluting so, lignin degrading enzymes are of great importance for depolymerization and degradation of lignin.

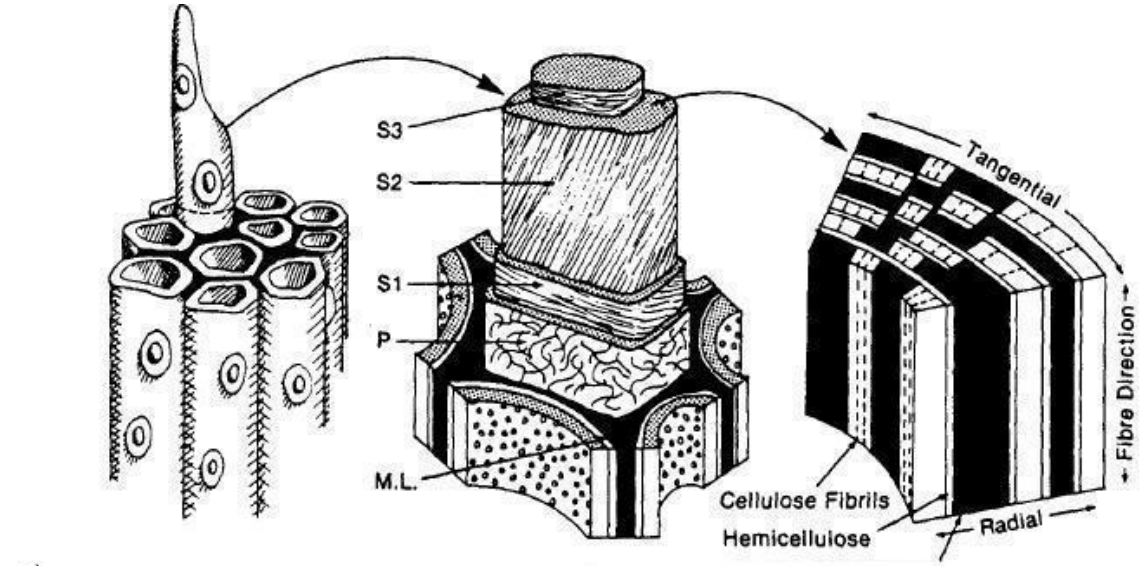


Fig. 2.1 –a) Relationship of hemicelluloses and lignin to the cellulose fibrils, P = Primary cell wall, S1, S2, and S3 = secondary walls, M.L = Middle lamella (Kirk TK, 1985); b-d Monomeric units of lignin b) p-coumaryl alcohol, c) sinapyl alcohol, d) coniferyl alcohol.

2.2. Lignin degrading organism

Wood-rot basidiomycetes fungi are one of the most efficient lignin degraders in nature (Kirk and Farrell, 1987). The enzymology and molecular biology of lignin degradation has been mainly studied in *Phanerochaete chrysosporium* (Gold and Alic, 1993; Kirk and Cullen, 1998). However, many other species of white rot fungi degrade lignin as efficiently as *P. chrysosporium* (Hatakka, 1994). Moreover, several fungi show selectivity for lignin removal (Eriksson et al., 1990). Most fungi are heterotrophs and due to their rigid cell they need to secret extracellular enzymes which can break down complex

polymers into simpler form and then be absorbed as simple nutrients or to reach the targeted nutrient site (Chai, 2008). Fungi can withstand high temperature and wide range of pH (Mille-Lindblom, 2005; Chai, 2008). Extra cellular enzyme system of fungi helps them to tolerate adverse conditions like higher concentration of xenobiotics in comparision to bacteria which have intracellular enzyme system. The degradation systems of most fungi are induced by nutrient depletion, mostly C and N, and not by a particular polymer. This is important because very low concentration of polymer won't repress enzyme synthesis and hence fungi can degrade low concentration of polymer as well.

2.2.1. White Rot Fungi (WRF):

Most of the fungal laccases studied are from the classes of white rot fungi as they are the most efficient lignin degrading microorganism (Kirk and Farrell, 1987; Gianfreda et al., 1999). Fungal attack is an oxidative and non-specific process, which decreases methoxy, phenolic, and aliphatic content of lignin by cleaving aromatic rings, and creating new carbonyl groups resulting in depolymerization and carbon dioxide production (Kirk and Farrell, 1987). White-rot fungi degrade lignin either selectively or non-selectively (i.e. Simultaneous decay) which may be controlled by nutritional factors. Organisms like C*eriporiopsis subvermispora, Dichomitus squalens, Phanerochaete chrysosporium, Phlebia radiata* degrade selectively i.e, lignin and hemicellulose are degraded significantly more than cellulose, while non-selectively WRF like *Trametes versicolor* and *Fomes fomentarius* degrade equal amounts of all components of lignocellulose (Rayner and Boddy, 1988; Tuomela et al., 2000; Hatakka et al., 2001). Some fungi, such as *Ganoderma applanatum*, *Heterobasidion annosum*, and *Phellinus pini*, are capable of carrying out both types of decay (Tuomela et al., 2000). The majority of white-rot fungi grow on hardwoods, except certain species such as *Heterobasidion annosum*, *Phellinus pini*, and *Phlebia radiata* which grow on softwoods (Blanchette, 1995). Almost all white-rot fungi produce manganese peroxidase (MnP) and laccase, but only some of them produce lignin peroxidase (lip) (Hatakka, 1994; Hatakka et al., 2001) . *Pycnoporus cinnabarinus* has been shown to produce laccase as the only ligninolytic enzyme (Eggert et al., 1996) and *Pycnoporus sanguineus* produces laccase as the sole phenol oxidase (Pointing and Vrijmoed, 2000). Lip preferentially cleaves the Cα-Cβ bond in the lignin molecule but is also capable of ring opening and other reactions (Kirk and Farrell, 1987; Hatakka et al., 2001). MnP oxidizes Mn^{2+} which can always be found in wood and soil − to Mn^{3+}. Highly reactive Mn^{3+} in turn oxidizes phenolic rings of lignin to unstable free radicals and decomposition follows spontaneously (Hatakka et al., 2001). Laccase oxidizes phenolic rings to phenoxyl radicals. The main drawback of all heme containing peroxidases is that hydrogen peroxide cause its rapid deactivation and its dependence of Mn^{2+} (for the MnP) or veratryl alcohol (for the LiP). On the other hand, laccase alone is incapable of cleaving the non-phenolic bonds of lignin as well (Kunamneni et al., 2008). Lignin degrading enzymes often combine with other additional

enzyme systems like glyoxal oxidase and superoxide dismutase for intracellular production of H_2O_2; glucose oxidase ; aryl alcohol oxidase and cellobiose dehydrogenase involved in feedback circuits and linking ligninolysis with cellulose and hemicelluloses degradation in nature.

2.2.1.1. *Ganoderma lucidum*

Ganoderma is a genus of polypore mushrooms which grow on wood, and include about 80 species, many from tropical regions and *lucidum* means shiny or brilliant. Because of their extensive use in traditional Asian medicines, and their potential in bioremediation, they are a very important genus economically. In a Chinese medical encyclopedia "Ben Cao Gang Mu" (Materia Medica - Detailed Outline of Medicinal Herb), an ancient medical practitioner Lee Shi Zhen in the Ming Dynasty classified *Ganoderma lucidum* as a "superior herb". *Ganoderma lucidum* (reishi mushroom) can be differentiated from other polypores because they have a double walled basidiospore. They are popularly referred to as shelf mushrooms or bracket fungi. *G. lucidum* contains the largest variety of cellulose-, lignin-, and xylan-digesting enzymes, which are being used in biomass remediation and industrial sludge processing. *Ganoderma* are wood-decaying fungi with a cosmopolitan distribution found especially in temperate and subtropical locations including North and South America, Europe, and Asia. They can grow on both coniferous and hardwood species. It has been found to produce antitumor and hypoglycemic polysaccharides, and immunomodulatory proteins, as well as bioactive oxygenated triterpenoids (Lin and Zhang, 2004).

Ganoderma is one of the mesophilic WRF which secrete extracellular laccase for lignin degradation to get energy from cellulose enclosed by lignin. It has been reported that *G. lucidum* produces two isoforms of 40 and 68 kDa (D'Souza et al., 1999; Zhongyang Ding, 2011); however, the molecular weight of laccase was determined to be 43 kDa by other investigators (Murugesan et al., 2007).

a) b)

Fig. 2.2.*Ganoderma* lucidum fruiting bodies developed in Central Department of Biotechnology a) ventral surface, b)dorsal surface

2.2.2. Brown rot fungi:

Serpula lacrymans, Gloephyllum trabeum, Polyporus ostreiformis and *Meruliporia incrassate* are brown rot fungi causing fungi which attack cellulose and hemicelluloses preferentially. The residual wood left is brown in color. Brown-rot fungi grow more frequently in softwood than in hardwood, particularly favoring top-layer coniferous forest soils (Blanchette, 1995).

2.2.3. Soft rot fungi:

Soft rot is usually caused by fungi belonging to Ascomycetes and Fungi Imerpefecti (Kim and Singh, 2000). They attack the wood causing softening of the surface layer leading to the formation of cavities in wood. Longitudinal cavities formed within the secondary wall of wood cells indicate T1 type of soft rot where as erosion of entire secondary wall occurs in T2 type of soft rot. Soft-rot fungi better tolerate wider ranges of temperature, pH, and oxygen limitation than white- or brown-rot fungi and hence predominate in environments that are too severe for basidiomycetous wood-rotting fungi, such as excessively wet or dry environments, and in substrates rich in heartwood extractives or preservatives (Blanchette, 1991; Blanchette, 1995).

2.2.4. Aerobic bacteria :

Besides the most frequently studied form of laccase i.e., fungal laccase, many bacterial laccases from *Azospirillum lipoferum* (Givaudan et al., 1993), *Marinomonas mediterranea* (Solano et al., 1997). EpoA from *Streptomyces griseus* (Endo et al., 2003), yacK gene of *E. coli* (Kim et al., 2001), CotA of *Bacillus subtilis* and many more have also been purified and characterized. Laccase-like activity has also been found in other bacteria, e.g., CopA protein from *Pseudomonas syringae* (Mellano and Cooksey, 1988) and PcoA protein from *Escherichia coli* (Brown et al., 1995). A bacterial population alone degrades wood extremely slowly, and only in water-logged wood is bacterial attack the major form of decay (Blanchette, 1991; Blanchette, 1995).

2.2.5. Anaerobic bacteria:

According to Colberg and Young, a mixed population isolated from activated sludge was able to cleave the *β*-O-4 linkage of low molecular mass lignin in anaerobic conditions, producing monoaromatic compounds. Mineralization of lignin was 6% (Colberg and Young, 1985). In rumen, up to 50% of lignin is either solubilized or transformed into a soluble lignincarbohydrate-complex and a variable amount is digested, although the biochemical pathways are unknown (Susmel and Stefanon, 1993).

2.3. Laccase

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a one of the major components of the ligninolytic system of white-rot fungi (Munoz et al., 1997), which belongs to multi-copper oxidase family and is widely distributed within plants, insects ,fungi, lichens and bacteria. Laccase has been identified in vegetables like cabbages, turnips, beets, apples, asparagus, potatoes and various other vegetables. Similarly it has been detected in plants like mango, mung bean, peach, pine, prune, etc. (Levine, 1966). Insects like *Bombyx*, *Calliphora*, *Diploptera*, *Drosophila*, *Lucilia*, *Manduca*, *Musca*, *Oryctes*, *Papilio*, *Phormia*, *Rhodnius*, *Sarcophaga* and *Tenebrio* are also reported to have laccase (Madhavi and Lele, 2009). Other enzymes belonging to this family are ascorbate oxidase, mammalian plasma protein ceruloplasmin. It is one of the oldest and most studied enzyme and having molecular mass in range of 40-130 kDa with acidic isoelectric point and contain 15-30% carbohydrate portion (Baldrian, 2006). Laccases are secreted during secondary metabolism as they are secreted under limitation of nutrient levels (mostly N) and is mostly favoured by agitation (Wesenberg et al., 2003). It has been reported to be responsible for a number of diverse and sometimes contradictory functions in organisms like pigmentation in fungi (Clutterbuck, 1972; Clutterbuck, 1990; Aramayo and Timberlake, 1993), plant pathogenicity (Iyer and Chattoo, 2003), secondary cell wall integrity (Ranocha et al., 2002), fungal morphogenesis (Leatham, 1981) and have been proposed to play role in electron transport (Alexandre et al., 1999).

Laccase oxidizes wide range of substrates like phenolic (Bourbonnais and Paice, 1990; Eggert et al., 1996; Yazhong et al., 2003), nonphenolic, PAH (Majcherczyk et al., 1998) and aromatic compounds (Chivukula and Renganathan, 1995) resulting in the formation of reactive radicals, while reducing molecular oxygen to water as a final electron acceptor. Various laccase encoding gene sequences have been reported from a range of ligninolytic fungi; these sequences encode for proteins between 515 and 619 amino acid residues and close phylogenetic proximity between them is indicated by sequence comparisons (Bourbonnais et al., 1995).

First laccase gene was described in the ascomycetous fungus *Neurospora crassa* (Germann and Lerch, 1986). Then two other gene sequences were then reported, one in the *Aspergillus nidulans* (Aramayo and Timberlake, 1990), and the other, in the *Coriolus hirsutus* (Kojima et al., 1990). Similarly two laccase genes were reported in the same chromosome of *Agaricus bisporus* by (Perry et al., 1993), four gene families in *Rhizoctonia solani* (Wahleithner et al., 1996) and *Pleurotus sajor-caju* (Soden and Dobson, 2001); three in *Pleurotus ostreatus* (Giardina et al., 1995), and a family of five genes in different chromosomes of *Trametes villosa* (Yaver and Golightly, 1996).

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2.4. Molecular structure of laccase

The three-dimensional structure of several fungal laccases has been reported which include *Coprinus cinereus* (in a copper type 2-depleted form) (Ducros et al., 1998), *Trametes versicolor* (Piontek et al., 2002), *Pycnoporus cinnabarinus* (Antorini et al., 2002), *Melanocarpus albomyces* (Antorini et al., 2002), *Rigidoporus lignosus* (Garavaglia et al., 2004), *Streptomyces coelicolor (Skálová et al., 2009) a*nd *Lentinus trigrinus* (Ferraroni et al., 2007). The three-dimensional structure of the bacterial laccase has also been reported, the cotA laccase from *Bacillus subtilis* endospore (Enguita et al., 2003; Enguita et al., 2004)

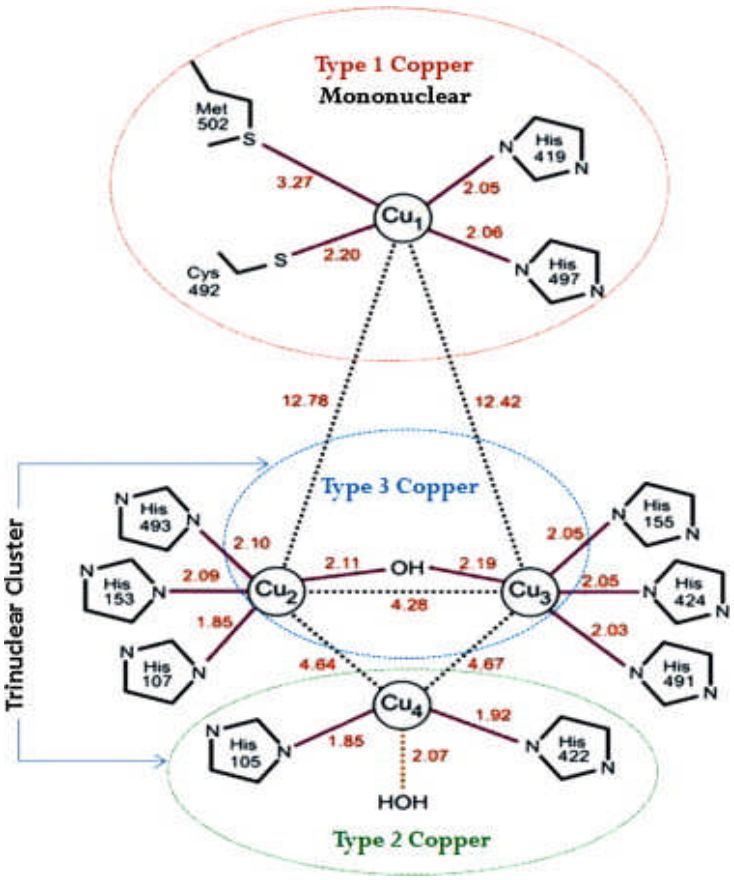

Fig. 2.3. Scheme of t1 (cu 1) and t2/t3 (cu 4 /cu 2 -cu 3) copper sites of laccase cota from *Bacillus subtilis*, with indicated distances between the most important atoms (O. V. Morozova, 2007)

Most fungal laccases are monomeric, dimeric or tetrameric glycoproteins. Laccases are highly N-glycosylated (brown et. al., 2002; Ko et al., 2001; Saparrat et al., 2002, O. Vite-Vallejo et al., 2009) with an important feature of covalently-linked carbohydrate moiety majority of which consists of mannose, N-acetylglucosamine, and galactose. This carbohydrate moiety constitutes about 45% of the protein mass in laccases of plant origin while fungal laccases have lower carbohydrate contents (15-30%) and is supposed to protect the enzyme molecule against proteolysis and inactivation by free radicals (Yoshitake et al., 1993). Laccases have acidic isoelectric point around pH 4.0 (Baldrian, 2006), all of which contribute to its high stability (Durán et al., 2002) and resistance towards protease degradation (O. V. Morozova, 2007). Glycosylation may vary from 20-50% in plants where laccases are involved in polymerization to 5-25% in fungal laccases which are responsible for

depolymerization reaction (Baldrian, 2006). Fungal laccases have molecular mass ranging from 60-90 kDa (Call and Mücke, 1997) and contain four copper atoms which are classified into three types (one type1-Cu, one type2-Cu and a pair of type3-Cu) and are UV/visible and Electronic Paramagnetic Resonance (EPR) spectroscopy detectable. T1 is responsible for the blue color with absorbance approximately at 600-610 nm. The T1 and T3 Cu are linked mainly by His-Cys-His tripeptide, whose Cys ligates the T1 Cu and whose His ligate two T3 Cu, and the T2/T3 Cu are electronically coupled to form a trinuclear cluster. This copper ion can be replaced by mercury or cobalt ions (O. V. Morozova, 2007). Type 2 doesn't give coloration but is EPR detectable while T3 consists of a pair of copper atoms in binuclear conformation with weak absorbance near UV region (shoulder peak at 330nm) but is not EPR detectable because copper ions are paired antiferromagnetically through a hydroxide bridge that makes this site diamagnetic (Thurston, 1994; Messerschmidt, 1997). The redox potential of laccases vary from 0.4 to 0.8 V and the most critical factor determining the E^{o} (laccase) is the coordination sphere of the T1 copper. In most of the blue copper proteins, the copper of T1 is in a distorted tetrahedral (4-fold coordinated) configuration, while in laccase it is trigonal coplanar-coordinated (3-fold coordinated) which is brought about by the presence of ligands supplied by the S-atom of a cysteine and by the Nδ1 nitrogen of two histidines. In other blue proteins like ascorbate oxidase, azurin and plastocyanin there is additional axial ligand in the T1 centre which is contributed by the S-atom of a methionine but in laccase methionine is replaced by either F or L as seen in TvL and C*oprinus cinereus* laccase (CcL) respectively. Since, neither of these aminoacids participate in the coordination, there is absence of that additional ligand so that the copper ion lies almost within the plane formed by the one S and two N ligands leading to slight elevation of the redox potential in laccase (Piontek et al., 2002). Catalytic efficiency of laccase has been shown to be linearly dependent of redox potential of Type I copper (Xu, 1996; Gianfreda et al., 1999). Type II and Type III Cu atoms form a trinuclear cluster with eight imidazoles of histidine as ligands and molecular oxygen gets reduced to two molecules of water in this trinuclear cluster (Messerschmidt and Huber, 1990; Ghindilis, 2000). Fluoride ion has a strong influence on redox potential of the T3 site copper ion and slightly affects the potential of copper ions of the T1 and T2 sites (Reinhammar, 1972; Shleev et al., 2005). In the presence of fluoride ion the potential of the T3 site of laccase from *T. versicolor* is 210 mV lower because of a strong interaction of fluoride ion with the T2/T3 cluster (Reinhammar, 1972). Laccase molecules are usually monomers consisting of three consecutively connected cupredoxin-like domains twisted in a tight globule. On comparing sequences of more than 100 laccases, four conservative regions were revealed which are specific for all laccases (Claus, 2004). One cysteine and

ten histidine residues form a ligand environment of copper ions of the laccase active site and are present in these four conservative amino acid sequences.

But there are some exceptions to the above mentioned properties of laccase. Several non-blue laccases have been reported earlier. Bacillus laccase belongs to the non-blue laccase family. *Pleurotus ostreatus* laccase contains only one copper atom per molecule instead of the usual four, along with two zinc atoms and one iron atom in each protein molecule (Telke et al., 2011). The fungus *P. ribis* produces only one laccase which is a dimer consisting of two 76-kD subunits containing one copper ion, one manganese ion, and two zinc ions (Min et al., 2001). The analysis of the metal ion content of the laccase from *Trametes hirsuta* has shown the presence of copper and manganese in a 3:1 ratio. Enzymes which lack the peak at 600 nm in the absorption spectrum are usually called as laccase-like enzymes because they have the catalytic activity inherent in typical "blue" laccases.

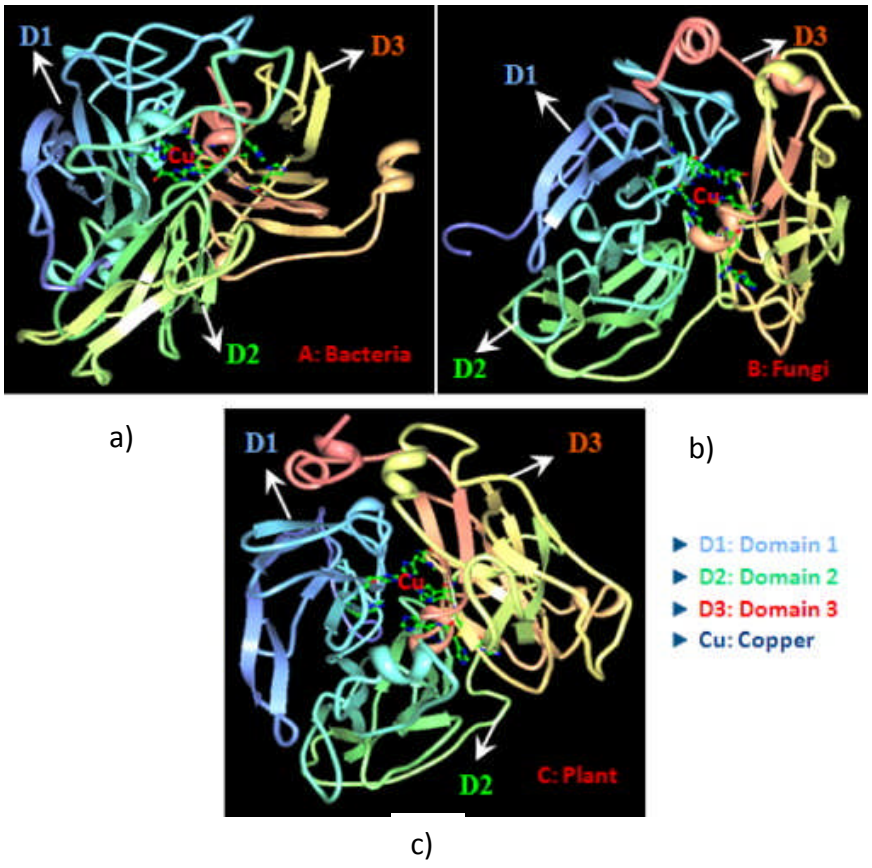

Fig. 2.4. Three dimensional structure of (a) bacterial laccase (*Bacillus subtilis*), (b) fungi laccase (*Tramates versicolor*), and (c) plant laccase (*Populus trichocarpa*). Bacterial laccases have larger binding cavity as compared to those of plant and fungi. ▸ Differences in residues of copper binding site are the basis for their diverse functions (source : (Dwivedi et al., 2011)

2.5. Mechanism of catalysis by laccase:

Laccase catalysis have been proposed to comprise of three major steps

i) Reduction of the mononuclear copper center : here the reducing substrate (usually phenolic compounds) loses an electron to laccase and the electron reduces T1 copper positioned just below the substrate-binding site. Thus oxidized substrate then becomes a radical, which is unstable and may undergo further laccase-catalyzed oxidation (to form quinine from phenol) or non-enzymatic reactions (hydration, disproportionation or polymerization). The electron transfer from substrate to T1 is based on redox potential difference (Madhavi and Lele, 2009). Now the reduced T1 copper oxidizes itself by transferring the electron to the trinuclear copper cluster and thus there are four such mono-electronic reductions of the T1 copper which occur sequentially (Gianfreda et al., 1999).

ii) Internal electron transfer from the type 1 to the type 2 and type 3 copper : based on experimental evidence it has been proposed that oxygen first binds with T2 and any one of the T3 copper atoms which then undergoes asymmetric activation leading to the formation of four O-H bonds during the generation of two molecules of water. The oxygen binding pocket is hypothesized to restrict the access of oxidizing agents other than molecular oxygen (Gianfreda et al., 1999).

iii) Reduction of oxygen to water at the type 2 and type 3 copper site: it takes place at the trinuclear cluster with the concomitant release of water (Claus and Strong, 2009).

The bonds of the natural substrate, lignin, that are cleaved by laccase include, Cα-oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage.

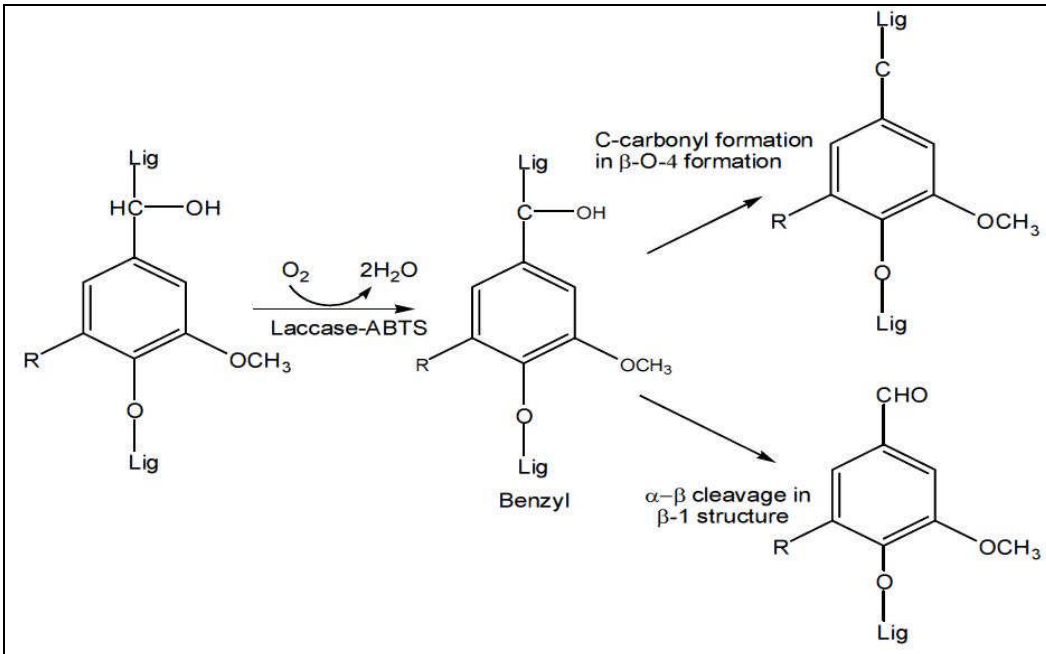

Fig. 2.5. Oxidation of phenolic subunits of lignin by laccase

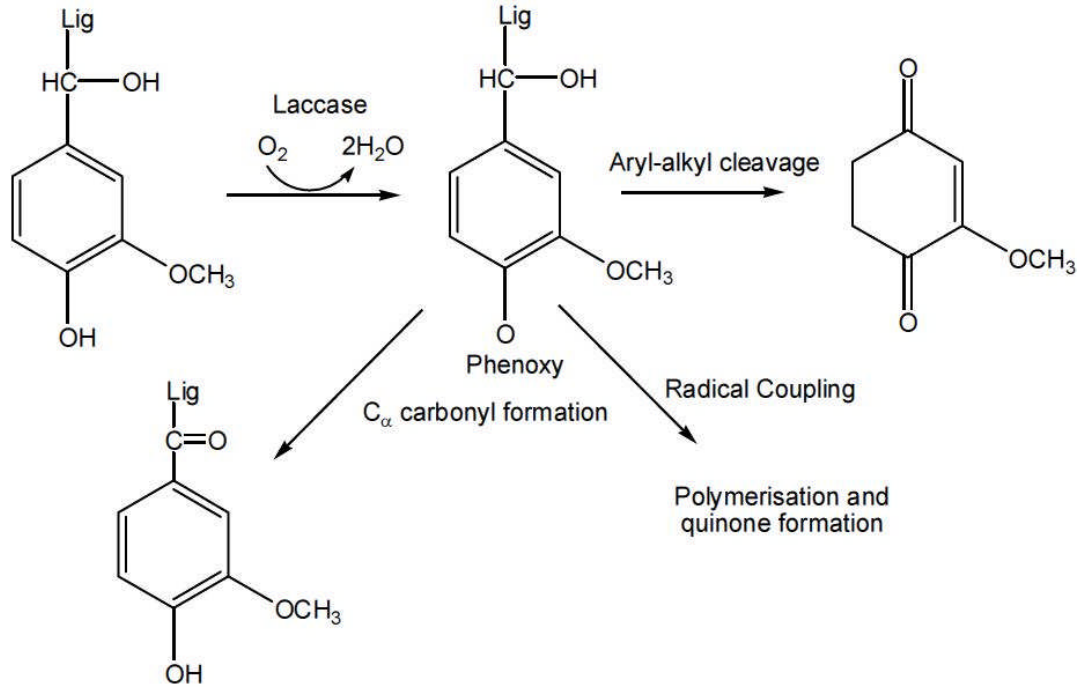

Fig. 2.6. Oxidation of non-phenolic lignin model compounds of lignin by laccase

2.6. Laccase mediator system:

All the substrates of our interest cannot be oxidized by the laccase directly because either they are too large to fit the active site of the enzyme or they have very high redox-potential. Substrates having higher redox potential than type I copper of laccase

cannot be directly oxidized by laccase. This limitation is overcome by the use of so called "mediators" which are intermediate substrates for laccase. These mediators upon oxidization by laccase forms radicals which are able to interact with large or high redox-potential substrates. The oxidized mediator is reduced to the initial state by the compound subjected to oxidation, and thus a closed cycle is created (Bourbonnais et al., 1998). More than 100 mediator compounds have been described but the most extensively investigated laccase mediator is ABTS (2,2'-azinobis-3-ethylbenzthiazoline-6-sulfonate) a synthetic nitrogen-substituted aromatic compound that oxidizes non-phenolic lignin model compounds (Bourbonnais and Paice, 1990).

Bourbonnais and Paice reported that in presence of mediators like ABTS laccase can efficiently oxidize non-phenolic benzylalcohols which led to the findings like some laccases found from Basidiomycetes can catalyze the oxidation of recalcitrant polymers with the help of mediators which have higher redox potentials than laccase itself (Bourbonnais and Paice, 1990; Xu, 1996; Camarero et al., 2005). Although the mediators broadens the substrate range for laccase enzyme, two major factors limit their use i.e., they are expensive and often toxic (Johannes et al., 1998).

Researchers have shown that laccase from different organism react differently with different substrates and mediators (Bourbonnais and Paice, 1990) and thus it is necessary to check out suitable mediator and substrate for particular laccase. It is required that mediators should be good substrate of laccase, stable in both oxidized and reduced form and do not interfere laccase activity. Mostly short listed substrates have been used to demonstrate the efficacy of the enzyme which includes non-phenolic compound ABTS and the phenolic compounds DMP, Guaiacol and Syringaldazine. Laccases in general have shown higher affinity for ABTS and syringaldazine than guaiacol and DMP. However, synthetic mediators are toxic, expensive and generally at concentrations above 1 mM inactivate the laccase. Novel approaches to overcome this hurdles are coming up (from searching for natural mediators such as p-coumaric acid, 4-hidroxybenzoic acid, syringaldehyde etc) to the directed evolution of laccases (Bulter et al., 2003). HBT redox potential has been estimated as 1.1-1.2 V (Xu et al., 2000) the redox potentials of $ABTS^{+\cdot}$ and $ABTS^{2+}$ were evaluated as 0.680 V and 1.09 V respectively (Scott et al., 1993).

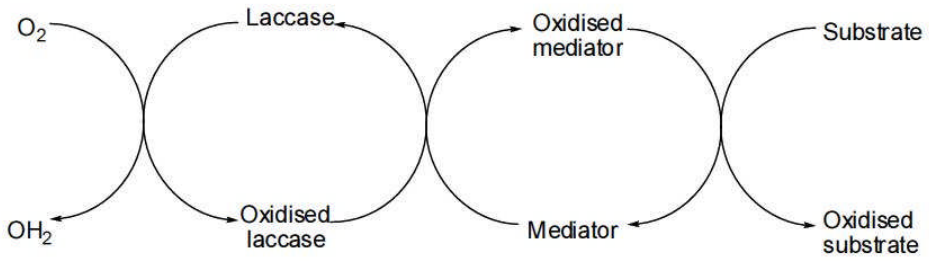

Fig.2.7.a Role of mediator in laccase mediated catalysis (reproduced from (Banci et al., 1999).

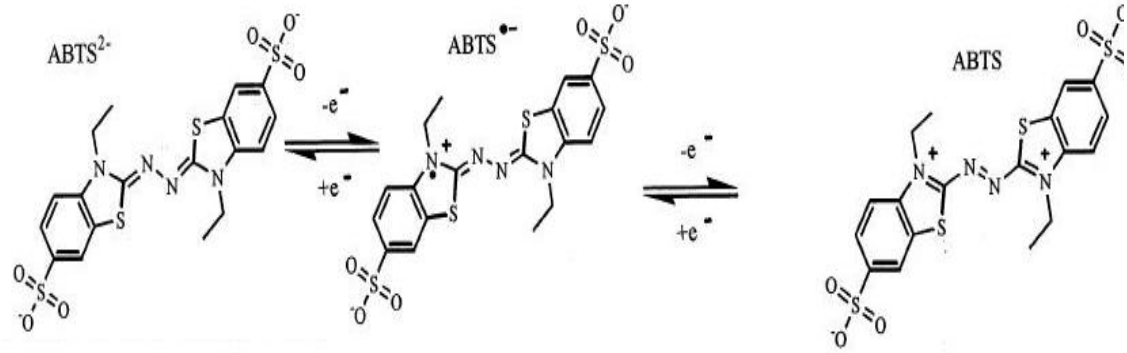

Fig.2.7.b Oxidation of ABTS

2.7. Production of fungal laccases

2.7.1. Induction of laccase production

It has been found that Laccase production is highly dependent on different culture conditions (Heinzkill et al., 1998) and media supporting high biomass does not necessarily support high laccase yields (Xavier et al., 2001). Most often white-rot fungi secrete ligninolytic enzymes during the secondary metabolic phase and are often triggered by nitrogen concentration (Buswell et al., 1995) or when carbon or sulfur become limiting (Heinzkill et al., 1998). Generally laccases are produced in low concentrations by laccase producing fungi (Vasconcelos et al., 2000), but higher concentrations are obtainable with the addition of various supplements to media (Lee et al., 1999). The addition of aromatic compounds such as 2,5-xylidine, lignin, and veratryl alcohol is known to increase and induce laccase activity (Xavier et al., 2001). Many of these inducers resemble lignin molecules or other phenolic chemicals (Farnet et al., 1999). Veratryl alcohol is an aromatic compound famously known to play an important role in the synthesis and degradation of lignin. The addition of veratryl alcohol to cultivation media of many white-rot fungi has resulted in an increase in laccase production (Barbosa et al., 1996). Some of these compounds affect the metabolism or

growth rate while others, such as ethanol, indirectly trigger laccase production (Kunamneni et al., 2007). 2,5-xylidine is also reported to induce pronounced effect on laccase production (Eggert et al., 1996). The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals (Faraco et al., 2002). These inducers can bind to the recognition sites when present in the substrate and induce laccase production. Lee et al. investigated the inducing effect of alcohols on the laccase production by *Trametes versicolor*. The enhanced laccase activity was comparable to those obtained using 2,5-xylidine or veratryl alcohol (Mansur et al., 1997; Lee et al., 1999). It was postulated that the addition of ethanol to the cultivation medium caused a reduction in melanin formation. The monomers, when not polymerised to melanin, then acted as inducers. Cu2+ regulates the transcription of laccase (Baldrian & Gabriel 2002). The addition of low concentrations of copper to the cultivation media of laccase producing fungi generally stimulated enzyme production (Assavanig et al., 1992).

2.7.2. Influence of carbon sources on laccase production

The carbon sources play an important role in enzyme production. There are reports of increased laccase production from different carbon sources like: Mansur et al. showed that fructose induced 100-fold increase in laccase production of Basidiomycete sp. I-62 (Mansur et al., 1997); mandarin peels increased laccase production in *T. versicolor* very effectively (Mikiashvili et al., 2006) and lignocellulosic material (barly bran) increased almost 50-fold laccase activity compared to the control culture with glucose in *T. versicolor* (Moldes et al., 2004*);* Glucose and cellobiose were efficiently and rapidly utilized by *Trametes pubescens* with high laccase activity (Galhaup et al., 2002); replacement of crystalline cellulose or xylan by cellobiose increased laccase activity of *C. unicolor* by 21- and 70-fold, respectively (Elisashvili et al., 2002).

2.7.3. Influence of nitrogen sources on laccase production

Ligninolytic systems are mainly activated during the secondary metabolic phase of the fungus and one of the important triggering factor is nitrogen depletion in the media (Keyser et al., 1978). Monteiro and De Carvalho have reported that high laccase activity is seen with semi-continuous production in shake-flasks using a low carbon to nitrogen ratio (7.8 g/g) (Monteiro and De Carvalho, 1998). Buswell et al. found that laccases were produced at high nitrogen concentrations, although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production (Buswell et al., 1995). Laccase was also produced earlier when the fungus was cultivated in a substrate with a high nitrogen concentration and these changes did not reflect differences in biomass. Elisashvili et al. observed highest laccase activity in *C. unicolor* IBB 62 in a medium with ammonium sulphate as the nitrogen source while D'Souza-Ticlo et al. showed that well

defined organic nitrogen sources such as glutamic acid and glycine were better than beef extract and corn steep liquor for laccase production (Kunamneni et al., 2007). Heinzkill et al. also reported a higher yield of laccase using nitrogen rich media rather than the nitrogen-limited media usually employed for induction of oxidoreductases (Heinzkill et al., 1998).

2.7.4. Influence of pH on laccase production

The pH optima of laccases greatly depend on the type of substrate. When ABTS is used as substrate the pH optima are more acidic and are found in the range of 3.0-5.0 (Heinzkill et al., 1998). Generally, laccases have a bell shaped graph of pH versus its activity with an optimal pH that varies considerably. This variation may be due to changes in the reaction caused by the substrate, oxygen or the enzyme itself. The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to a disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects can play an important role in determining the optimal pH of the bi-phasic laccase enzymes (Xu, 1997). Laccase produced by *Trametes modesta* was fully active at pH 4.0 and very stable at pH 4.5 (Nyanhongo et al., 2002).

2.7.5. Influence of temperature on laccase production

The optimal temperature of laccase can differ greatly from one strain to another. The laccases isolated from a strain of *Marasmius quercophilus* were found to be stable for 1 h at 60 °C. Farnet et al. further found that pre-incubation of enzymes at 40 °C and 50 °C greatly increased laccase activity (Farnet et al., 2000). Another technique that can be used to increase the stability of laccase is to immobilise the enzyme on glass powder by means of air-drying. This technique also has potential for the enzyme to be used on the glass powder matrix in specific biotechnology applications where stability is required (Kunamneni et al., 2007). The laccase from *P. ostreatus* is almost fully active in the temperature range of 40 °C to 60 °C, with maximum activity at 50 °C. The activity remains unaltered after prolonged incubation at 40 °C for more than 4 h (Palmeiri et al., 1993). Nyanhongo et al. showed that laccase produced by *T. modesta* was fully active at 50 °C and was very stable at 40 °C but half-life decreased to 120 min at higher temperature (60 °C) (Nyanhongo et al., 2002).

2.7.6. Inhibition of laccase production

In general, laccases responds similarly to several inhibitors of enzyme activity. Many ions such as azide, halides, cyanide, thiocyanide, fluoride and hydroxide bind to the type 2 and type 3 Cu, resulting in the interruption of internal electron transfer and accordingly therefore inhibition of activity. Other inhibitors include metal ions (e.g. Hg

+2), fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, desferal and cationic quaternary ammonium detergents, the reactions with which may involve amino acid residue modifications, confirmational changes or Cu chelation (Bollag and Leonowicz, 1984).

2.7.7. Heterologous expression

One of the serious problem often encountered in industrial use of fungal laccases is its low production in native hosts. This problem may be overcome by heterologous production in suitable fungal hosts which are capable of producing high amounts of extracellular enzymes.(More et al., 2011). Heterologous production can help in the characterization of individual laccase isoenzymes as well as in avoiding additional production of toxic compounds besides the laccase (Yaver et al., 1999). In order to improve laccase production, fungal laccases have been expressed heterologously in *Saccharomyces cerevisiae* (Larsson et al., 2001), *Trichoderma reesei* (Saloheimo and Niku-Paavola, 1991; Kiiskinen et al., 2004), *Aspergillus oryz*ae (Yaver and Golightly, 1996), *Pichia pastoris*(Jönsson et al., 1997; Soden et al., 2002; Liu et al., 2003; Joo et al., 2008), *Yarrowia lipolitica* (Madzak et al., 2005), *Aspergillus sojae* (Hatamoto et al., 1999), *Aspergillus niger* (Record et al., 2002; Larrondo et al., 2003), *Aspergillus nidulans* (Larrondo et al., 2003), tobacco (LaFayette et al., 1999) and maize (Bailey et al., 2004) and more which are enlisted in table 1. “Selectivity” in expression like, LCC1 laccase from *T. versicolor* was heterologously expressed in *Pichia pa*storis (Jönsson et al., 1997) and not in *S. cerevisiae* (Cassland and Jönsson, 1999) may reflect the inability of yeast to process different laccases post-translationally with the same efficiency (Klonowska et al., 2005).

Table. 1 Laccase production in heterologous hosts (Kunamneni et al., 2008).

Laccase gene	***Production host***	***Laccase Production (mg l^{-1})****	***Reference***
Ceriporiopsis subvermispora lcs-1	*Aspergillus nidulans*	1.5	(Larrondo et al., 2003)
	Aspergillus niger	1.5	
Coprinus cinereus lcc1	Aspergillus oryzae	135	(Yaver and Golightly, 1996)

Melanocarpus albomyces lac1	Trichoderma reesei	920	(Kiiskinen et al., 2004)
Myceliophthora thermophila lcc1	*Aspergillus oryzae*	19	(Berka et al., 1997)
	Saccharomyces cerevisiae	18	(Bulter et al., 2003)
Phlebia radiata lac1	Trichoderma reesei	20	(Saloheimo and Niku-Paavola, 1991)
Pleurotus sajor-caju lac4	Pichia pastoris	4.9	(Soden et al., 2002)
Pycnoporus cinnabarinus lac1	*Pichia pastoris*	8	(Otterbein et al., 2000)
	Aspergillus niger	70	(Record et al., 2002)
	Aspergillus oryzae	80	(Sigoillot et al., 2004)
	Schizophyllum commune	1200	(Alves et al., 2010)
** The reported production levels have been obtained in shake flask cultivation, except in the case of P. radiata and M. albomyces laccases which were produced in a laboratory fermentor*			

2.8. Isoforms of laccase

Laccase producing organisms produce different isoforms of enzyme depending upon the species and culture conditions in which are cultured. Each isozyme may differ from each other depending upon optimum temperature, optimum pH, molecular weight, and affinity to different substrates (Kunamneni et al., 2007). Different number of isoenzymes secreted by different lignin degrading fungi is listed in table 2.

Table. 2 Number of isoforms of laccase produced in different fungus (Madhavi and Lele, 2009)

Organism	**Maximum number of isoenzymes**	**Molecular mass (kDa)**	**References**
Trametes multicolor	5	63	(Leitner et al., 2002)
Trametes trogii	2	70	(Garzillo et al., 1998)
Ganoderma lucidum	3	65-68	(Ko et al., 2001)
Pycnoporus cinnabarinus	1	81	(Eggert et al., 1996)
Coriolus hirsutus	1	73	(Shin and Lee, 2000)
Ceriporiopsis subvermispora	2	71 68	(Fukushima and Kirk, 1995)
Trichoderma	1	71	(Assavanig et al., 1992)
Trametes sanguine M85-2	1	62	(Nishizawa et al., 1995)
Ganoderma lucidum	2	40 68	(D'Souza et al., 1999)
Chaetomium thermophiliu	1	77	(Chefetz et al., 1998)
Chalara paradoxa CH 32	1	67	(Robles et al., 2002)
Pleurotus ostreatus	1	59	(Sannia et al., 1986)
Coriolus hirsutus	1	55	(Koroljova-Skorobogat'ko et al., 1998)
Polyporus versicolor	2	60-65	(Mosbach, 1963)
Botrytis cinerea 61-34	1	74	(Slomczynski et al., 1995)
Monocillium indicum	1	72	(Thakker et al., 1992)
Neurospora crassa	1	65	(Froehner and Eriksson, 1974)
Podospora anserine	3	70 80 390	(Thurston, 1994)

2.9. Application of laccase:

Laccases are one of the oldest enzyme with widespread applications which is solely due to its versatility. Its application ranges from detoxification to pulp bleaching, removal of phenolics from wines, dye decolourization and, organic synthesis (Pilz et al., 2003) bioremediation (Mayer and Staples, 2002), as agents to clean up herbicides, pesticides and certain explosives in soil, in biofuel cells, as a medical diagnostics tool and in the design biosensors (Vianello et al.,2006).

In the recent years, laccases are also being used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and in drug analysis (Srebotnik and Hammel, 2000) and even as ingredients in cosmetics.

The potential of laccase in various biotechnological processes is mainly due to its high non-specific oxidation capacity, lack of a requirement for cofactors and use of readily available oxygen as an electron acceptor (Sharma et al., 2007). Laccase-mediated degradation releases molecular nitrogen and the formation of aromatic amines is thus prevented (Telke et al., 2010).

Very recently laccases have been efficiently applied to nanobiotechnology due to their ability to catalyze electron transfer reactions without additional cofactor. The technique for the immobilization of biomolecule such as layer by layer, micro patterning, and self-assembled monolayer technique can be used for preserving the enzymatic activity of laccases.

2.9.1. DELIGNIFICATION AND PULP BLEACHING:

In the delignification of lignocelluloses and their modification into new compounds like vanillin and guaiacolI, laccases have shown to be useful (Call and Mücke, 1997; Balakshin et al., 2001). In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using ClO_2 and O_3. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods (Carter et al., 1997). In spite of this new method, the pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose (Kuhad et al., 1997).

2.9.2. BIOREMEDIATION

Bioremediation includes all procedures directed for biotransformation of polluting chemical agents into non polluting agents. Laccase can be and has been used to remove or detoxify phenolic and non-phenolic compounds present in different kinds of waste materials that are released from several industrial process like petroleum refining, coal conversion, olive oil production (Aggelis et al., 2003). Polycyclic aromatic hydrocarbons

(PAHs) and xenobiotics are the major source of soil pollution whose degradation are of great imprortance. Laccase have been successfully shown to reduce TNT metabolites to an organic soil matrix (Rodríguez Couto and Toca Herrera, 2006). Similaryly laccases from *P. chrysosporium* and *T. versicolor* were able to convert dikeonitrile derivative, the active form of herbicide isoxafluote, into the inactive benzoic acid analogue with the help of redox mediator (Mougin et al., 2002). Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil In presence of mediators like ABTS and HBT laccases from WRF have shown to oxidize alkenes, carbazole, N-ethylcarbazole, fluorine and dibenzothiophene (Leontievsky et al., 2000). Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants, for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone, to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene. (Kunamneni et al., 2007).

2.9.3. FOOD INDUSTRY

A number of applications of laccase have been demonstrated in food industry also. Laccase can be used in bioremediation, beverage (wine, fruit juice and beer) processing, ascorbic acid determination, sugar beet pectin gelation, baking, and as biosensor and to improve food sensory parameters. Laccase could increase productivity, efficiency and quality of food products without a costly investment and has the advantage of being a mild technology (Minussi et al., 2002).

Epicatechin, ferulic and o-coumaric acids are some of the phenolic compounds which have been removed by the use of laccase during clarification process of wine made from White grape must (Servili et al., 2000). The ability of laccae to cross-link the biopolymers are of current interest and laccase from *Trametes hirsute* has been used to increase the maximum resistance of dough and decrease the dough extensibility in both flour and gluten dough (Selinheimo et al., 2006). Laccases may also be used as oxygen scavenger for better packaging of food (Farneth et al., 2004), for improving quality of vegetable oils, odor removal with laccase has been patented (Tsuchiya and Petersen, 2000; Minussi et al., 2002). To improve the flavor and taste of cacao it was soaked in solutions containing laccase, dried and roasted products. Polyphenol removal from complex mixtures like Musts and wines must be selective to avoid undesirable alterations in their characteristics and use of laccase has aided in removal of polyphenol elimination of wines like stability in acid medium and reversible inhibition of sulphite (Miele, 2009). Haze formation in beers is a persistent problem in the brewing industry so laccase could be added to the wort as an alternative to traditional treatment to remove the excess of polyphenols. A laccase has also been recently commercialized as Suberzyme® for

preparing cork stoppers(Kunamneni et al., 2007; Miele, 2009). Hence the ability of laccase to scavange oxygen can be of great importance in food and beverage industries.

2.9.4. ORGANIC SYNTHESIS

Laccase has provided an environmentally friendly process of polymer production using air without the use of H_2O_2. Various Laccase-catalyzed cross-linking reaction has been reported like - new urushiol analogues for the preparation of "artificial urushi" polymeric films (Japanese traditional coating); laccase induced radical polymerization of acrylamide; preparation of crosslinked enzyme aggregrates with aldehydes and amines leading to improved stability and used in starch oxidation; enzymatic preparation of polymeric polyphenols; and in synthesis of various functional organic compounds including polymers with specific mechanical/electrical/optical properties, textile dyes, cosmetic pigments, flavor agents, and pesticides. laccase can be used for oxidation of hydroxyl groups of sugar derivatives (Marzorati et al., 2005; Baratto et al., 2006), vinblastin synthesis from vindolin and actinocin from 4-methyl-3-hydroxyanthranilic acid which are effective to fight against cancer (Yaropolov et al., 1994) , free radical polymerization on polysulfostyrene matrix resulting in electroconducting water-soluble polyaniline (Karamyshev et al., 2003). Laccases have been employed for the oxidation of functional groups, the coupling of phenols and steroids (D'Acunzo et al., 2006; Ponzoni et al., 2007), the construction of carbon-nitrogen bonds (Mikolasch et al., 2002) and in the synthesis of complex natural products (Barilli et al., 2004) and other various applications of laccase have been descried elsewhere (Kunamneni et al., 2008).

2.9.5. PHARMACEUTICAL SECTOR

Pharmaceuticals are regarded as emerging environmental pollutants since many of them are ubiquitous, recalcitrant and biologically active. Commercial laccase from *Myceliophthora thermophila* showed efficient degradation of several pharmaceuticals such as anti-inflammatory drugs (diclofenac and naproxen) and estrogen hormones (estrone, 17β-estradiol, 17α-ethinylestradiol) where Estrogens were completely degraded after only 15 min while the other types of pharmaceuticals presented higher persistence requiring 1 hour for total removal of diclofenac and 8 h to attain up to 60% of naproxen degradation (Lloret et al., 2010). A novel biosensor based on laccase immobilized on microspheres of chitosan crosslinked with tripolyphosphate has been developed for rutin determination in pharmaceutical formulations (Fernandes et al., 2008). Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc. including triazolo(benzo) cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline (Stahl et al., 2002; Molino et al., 2004). HIV-1 reverse transcriptase inhibitory activity capability of fighting aceruloplasminemia (a medical condition of

lacking ceruloplasmin, a multi-Cu serum oxidase whose ferroxidase activity regulates iron homeostasis) has also been reported for laccase (Kunamneni et al., 2007)

2.9.6. NANOBIOTECHNOLOGY

In the last decade more attention is focused on the applications of nanotechnologies. Since laccases are able to catalyse electron transfer reactions without additional cofactors, their use has also been studied in biosensors or bioreporters to detect various phenolic compounds, oxygen or azides. A biosensor is an integrated biological-component probe with an electronic transducer, thereby converting a biochemical signal into a quantifiable electrical response that detects, transmits and records information regarding a physiological or biochemical change (D'souza, 2001). Laccases can also be applied as biosensors or bioreporters and already a number of biosensors containing laccase have been developed for immunoassays, and for determination of glucose, aromatic amines and phenolic compounds (Simkus et al., 1996; Bauer et al., 1999). Laccase catalysis has been reported to assay other enzymes. Laccase covalently conjugated to a bio-binding molecule has been used as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical, or nucleic acid-detection assays (Karos et al., 2006; Kunamneni et al., 2008; Abari et al., 2012). The bioreporter applications are specially of great interest for the high-sensitivity diagnostic field. In addition to biosensors, laccases could also be immobilized on the cathode of biofuel cells for power generation as reported for small transmitter systems (Palmore, 2004; Patel et al., 2011). Fuel cells are very attractive energy sources, particularly at micro-, mini-, portable-, or mobile-scale, that potentially have higher energy conversion/usage efficiency and lower pollution effect than any of the existing/emerging energy sources. For example, a bio-implantable electrochemical cell system for active implantable medical devices has been described(Choi, 2003) to activate a pacemaker(Southcott et al., 2013). The anode enzyme comprises immobilized glucose oxidase/ glucose dehydrogenase and the cathode enzyme comprises immobilized laccase. Glucose is oxidized at the surface of the anode and oxygen is reduced at the surface of the cathode. The coupled glucose oxidation/oxygen reduction reactions provide a self-generating current source. Laccase may be applied as a biocatalyst for the electrode reactions (Barrière et al., 2004) and they have been patented also (Kunamneni et al., 2008). Laccase-based miniature biological fuel cell is of particular interest for many medical applications calling for a power source implanted in a human body (Barton et al., 2003).

2.9.7. COSMETIC INDUSTRY :

Laccase based hair dyes are less iriitating and easier to handle than current dyes as laccases replace H_2O_2 as an oxidizing agent in dye formulation. Cosmetic and

dermatological preparations containing laccase for skin lightening have also been developed. (Rodríguez Couto and Toca Herrera, 2006). Cosmetic and dermatological preparations containing laccase for skin lightening have also been developed (Rodríguez Couto and Toca Herrera, 2006).

2.9.8. ALTERNATIVE APPLICATIONS

Over 90% of herbicide dymron was shown to be degraded by laccase in the presence of a reaction mediator ABTS within 24 h, while the half-life of dymron is 50 days in soil (Maruyama et al., 2006). Laccase have also been shown useful to prevent β cells from cytokine-mediated apoptosis. It was demonstrated that laccase effectively scavenged peroxinitrite, which can be formed by nitric oxide, and up regulated the expression of antioxidant enzymes, such as manganese superoxide dismutase (MnSOD) and catalase. Interestingly laccase balanced pro- (Bax) and anti-apoptotic (Bcl-2) proteins in terms of both the mRNA and protein levels with a down regulation of cytochrome c protein in RINm5f cells and also amazingly maintained blood glucose concentrations at a normal level with a simultaneous increase in plasma insulin levels during the spontaneous induction of diabetes in NOD mice (Joo et al., 2010). Direct interaction between hepatitis C virus HCV and the laccase at the concentrations of 2.0 and 2.5 mg/ml led to a complete inhibition of virus entry after seven days of incubation and was also capable of inhibiting HCV replication at the concentrations of 1.25 and 1.5 mg/ml after first dose of treatment for four days and at the concentrations of 0.75, 1.0, 1.25 and 1.5 mg/ml after the second dose of treatment for another four days (M EL-Fakharany et al., 2010). Laccase from *Pleurotus cornucopiae* inhibited proliferation of murine leukemia cell line L1210 and human hepatoma cell line HepG2, and reduced the activity of HIV-1 reverse transcriptase with an IC50 of 22 μM (Ho Wong et al., 2010). Similarly laccase from lentinus edodes also showed inhibitory activity towards HIV-1 reverse transcriptase with IC50 equal to 7.5μM but it did not show any antifungal or anti-proliferative activity (Sun et al., 2011). *P. ostreatus* have shown most efficient in degradation of PCB and lindane.

Table . 3. Commercial preparations based on laccases for industrial processes. (source : (Osma et al., 2010)

	Main application	Brand name	Manufacturer
Food industry	Brewing	Flavourstar	Advanced enzyme Technologies Ltd. (India)
	Colour enhancement in tea, etc	LACCASE Y120	Amino Enzyme USA Co. Ltd.

	Cork modification	Suberase	Novozymes (Denmark)
Paper industry	Pulp bleaching	Lignozym-process	Lignozym GmbH (Germany)
	Paper pulp delignification	Novozym 51003	Novozymes (Denmark)
Textile Industry	Denim bleaching	Bleach Cut 3-S	Season Chemicals (China)
	Denim finishing	Cololacc BB	Colotex Biotechnology Co. Ltd. (Hong Kong)
	Denim bleaching	DeniLite	Novozymes (Denmark)
	Denim finishing	Ecostone LC10	AB Enzymes GmbH (Germany)
	Denim finishing	IndiStar	Genencor Inc. (Rochester, USA)
	Denim finishing	Novoprime Base 268	Novozymes (Denmark)
	Denim bleaching and shading	Primagreen Ecofade LT100	Genencor Inc. (Rochester, USA)
	Denim bleaching	ZyLite	ZyLite Zytex Pvt. Ltd. (India)

3. MATERIALS AND METHODOLOGY

3.1. Materials

This study was conducted at lab of Central Department of Biotechnology, T.U. from June 2012 to August 2013. ABTS and Guaiacol were purchased from Hi-media, India and other all chemicals were of reagent grade. All the experiments were done in duplicates.

Table 4. List of selected fungal strains for my research work.

S.N.	Name of plant	Local Name	Plant collection site
1	*Ganoderma lucidium*	Reishi	*Central Department of Biotechnology* Kirtipur, Nepal.
2	*Ganoderma japonicum*		*Central Department of Biotechnology* Kirtipur, Nepal.
3	*Shittake*	Oak mushroom straw	Central Agricultural Technology, Imadole, Gwarko, Nepal

3.2. Screening of laccase producing fungi

Guaiacol (0.02% in PDA), 1-naphthol (5mM/L in PDA) and tannic acid (0.5% in PDA) (Kiiskinen et al., 2004; More et al., 2011) were used for screening of laccase production by the selected fungal strains and color change due to oxidation of screening agents was observed for several days. Guaiacol was added to the media before autoclaving, Tannic acid was autoclaved separately before addition to the media and 1-naphthol was autoclaved along with the media. Laccase produces reddish brown color when it reacts with guaiacol, deep purple color when it reacts with 1-naphthol, and brown color with tannic acid. On the basis of higher and faster color development *Ganoderma lucidum* was selected for laccase production.

3.3. Lignin isolation

Lignin was isolated from rice straw as described by (Minu et al., 2012). Dried rice straw was powdered and oven dried over night at 105 ^{O}C. It was then hydrolysed at 120 ^{O}C for 60 min using 1%(wt/wt) sulphuric acid, and the resulting residue was then subjected to delignification process at 120 ^{o}C for 60 min using alkaline peroxide (1.5% wt/wt NaOH and 0.5% wt/wt H_2O_2). In all steps the total solids used was 10% wt/wt and remaining 90% included 89% wt/wt water and 1% wt/wt sulphuric acid. And in the delignification step 10% wt/wt was acid treated rice straw and remaining 10% was made up of 88.5 % wt/wt water and 1.5 % wt/wt NaOH and 0.5 % wt/wt H_2O_2. Thus obtained liquid part called as black liquor contains silica and lignin while the solid part contains cellulose. Then lignin was isolated from the black liquor by two step treatments. When the pH was dropped to 7 then silica was obtained which was removed by vacuum filtration and the

liquid was added with dilute acid to drop the pH to 3 and left overnight for lignin precipitation.

3.4. Optimization of production media

Modified Olga medium was used for optimization and production of laccase from *Ganoderma lucidum* (Koroljova-Skorobogat'ko et al., 1998). Four different parameters were varied for optimization of the media and they were: i) Temperature, ii)pH, iii)copper sulphate and iv)presence of lignin. In each experiment culture 100 ml conical flasks containing 50 ml modified Olga media were taken and kept in shaker incubator with 160-200 rpm shaking. 5 discs of size of 7 mm diameter from 5days old culture of actively growing *G. lucidum* mycelium were taken as samples for 50 ml media.

Temperature optimization was done in the range of 20 to 50 ^{o}C at 10 ^{o}C interval. pH optimization was done from pH 3 to 8 at the interval of 0.5 pH using 0.1M sodium acetate, and copper sulphate concentration was optimized using 10 mM to 50 mM concentration of copper sulphate with the concentration difference of 10mM and activity of laccase was noted after every 48 hours. Blank was set without using copper sulphate in the media. Lignin (5g/L) was used to see its effect in laccase production.

3.5. Laccase activity assay

Laccase activity was measured at room temperature using ABTS solution as the substrate of 1 mM concentration prepared in 0.1 M of sodium acetate buffer of pH 5. Reaction volume consisted of 350 µL of enzyme and 350 µL of 1 mM ABTS prepared in the same buffer to give total volume of 700 µL while for enzyme characterization, where dialyzed enzyme was used, reaction volume consisted of 10 µL of dialyzed enzyme with 350 µL of ABTS solution and 340 µL of buffer. Reaction was started by adding enzyme to the substrate and oxidation of ABTS was monitored by determining the increase in absorbance at 420 nm for 90 seconds. The amount of enzyme required to oxidize 1 µmol ABTS per minute forming $ABTS^{o+}$ was taken as 1 unit. (ε_{420} = 36000 M^{-1} cm^{-1} and path length l = 1cm) (Bourbonnais et al., 1995).

3.6. Purification of laccase

100 ml conical flasks containing 50 ml modified and optimized Olga media was taken and kept in shaker incubator with 160-200 rpm shaking. 5 discs with size of 7 mm diameter from 5days old culture of actively growing *G. lucidium* mycelium were taken as samples. Laccase produced from production media was purified in three different steps. The cultures in the flasks were filtered through Whatman No. 1 filter paper by vacuum filtration to separate mycelial mat and culture filtrate. Thus obtained crude enzyme was centrifuged at 4000 rpm for 10 min and the supernatant was used for further purification process. (Han *et al.*, 2005).

Ammoinum sulphate concentration was optimized from 40% to 80%. Suitable concentration of copper sulphate was used to get the precipitated enzyme with high activity. The protein precipitate was dissolved in least volume of 0.1 M (pH 5.0) sodium acetate buffer. Thus obtained enzyme was subjected to dialysis over night over the same buffer of pH 5 to desalt the enzyme. Finally the desalted enzyme solution was applied to a DEAE sepharose anion exchange column chromatography pre-equilibrated with pH 7.0 sodium acetate buffer solution (Zhongyang Ding, 2011). Bed volume was taken 5ml and it was washed with 10 times bed volume with distilled water then by 5 times bed volume with washing buffer (sodium acetate buffer 0.1M pH 8). The column was then loaded with sample and then washed with 3 times bed volume of linear gradient of 0.05 M to 1.0 M NaCl in the sodium acetate buffer at flow rate of 2 ml/min. Final washing was done with 5 times bed volume of 1 M NaCl buffer to elute out all proteins. Total 54 fractions were collected. Fractions containing high laccase activity were pooled out and run in SDS PAGE and native PAGE.

3.7. Protein estimation

Protein content was estimated using Bradford method (Bradford, 1976). After laccase production and after every step of purification protein estimation was done using BSA as standard protein. Each time standard graph was plotted using 10 µg/mL to 50 µg/mL of BSA solution and protein content was determined.

3.8. Gel electrophoresis

Molecular weight of laccase was determined through SDS PAGE gel electrophoresis using standard molecular weight markers (Genei India Pvt. Ltd.) according to laemmli (Laemmli, 1970). 10% polyacrylamide gel was prepared and the protein was run at 25 mA for 3 hours at RT. EZ-Visi Blue protein staining solution was used to stain the proteins (Hildén et al., 2007). Fixation was done after washing with distilled water for 5 min with shaking. Then it was washed with fixative solution with shaking for 10 min and again washed with distilled water 3 times, 10 minutes each, with shaking and observed for the bands.

Native PAGE was run without SDS at RT under 25 mA for 2 hours and 30 minutes in 10% polyacrylamide gel. The gel was stained with 5 mM ABTS solution and 1% guaiacol solutions for 15 min for color development.

3.9. Characterization of laccase enzyme

3.9.1. Optimization of pH

Optimum pH for laccase was determined by performing enzymatic assays from pH 2-8 using ABTS as substrate. pH levels were adjusted using 0.1 M sodium acetate buffer. Assay was carried at room temperature.

3.9.2. Optimization of tempertature

Optimum temperature for laccase was determined by performing enzymatic assays from temperatures 20 – 70 ^{o}C using 0.1M sodium acetate buffer of pH 5.

3.9.3. pH tolerability

pH stability of laccase was determined according to More et. al. (More et al., 2011). To investigate the effect of pH on enzyme stability, the purified enzyme was incubated at room temperature for 240 min in buffers of pH from 2-8 using ABTS as substrate.

3.9.4. Thermostability

Thermostability was determined according to More et. al. (More et al., 2011). The thermostability of the enzyme was determined by measuring laccase activity after every 30 min for 180 min. The assay was carried out at optimal pH using ABTS as substrate over a temperature range of 30 to 80°C.

3.9.5. Kinetic study

Michaelis–Menten kinetics was used to determine the kinetic constants (K_m and V_{max}) for purified laccase using ABTS as the substrate of concentration ranging from 0.01 to 10 mM (More et al., 2011).

4. RESULTS

Laccase production by fungi has been found to be highly affected by various culture conditions, such as carbohydrate and nitrogen source, presence or absence of lignin source, different concentrations of metal ions and presence or absence of microelements. Laccases are generally produced in low concentrations by fungi, but we can get higher concentrations by adding various supplements to liquid growth media. Therefore optimization of liquid media for higher production of enzymes is a necessary step.

4.1. Screening for laccase activities

In the screening step oxidation of tannic acid, 1-naphthol and guaiacol was seen after 2 days of culture. The oxidative polymerization of guaiacol was seen forming reddish brown zones in the medium, tannic acid was oxidized to brown color (Kiiskinen et al., 2004) and 1-naphthol was oxidized to a deep purple complex (More et al., 2011) giving a visual confirmation for the presence of the enzyme. *G. luciduim* and *Shittake* gave visual color formation indicating secretion of extracellular laccase in the media while color development was not observed in case of G*. japonicum*. *G. luciduim* formed larger zone of coloration in comparision to *Shittake*, so it was taken for the further work.

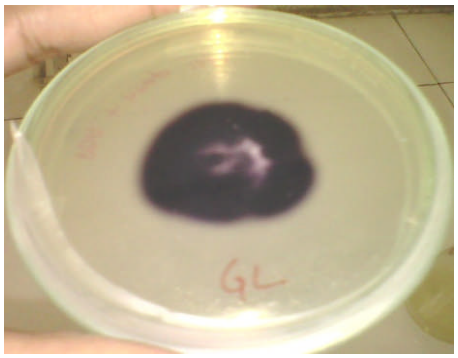

Fig. 4.1.a. *Ganoderma lucidium* Day 3 in 1-naphthol

Fig. 4.1.b. *Shittake* Day 3 in 1-naphthol

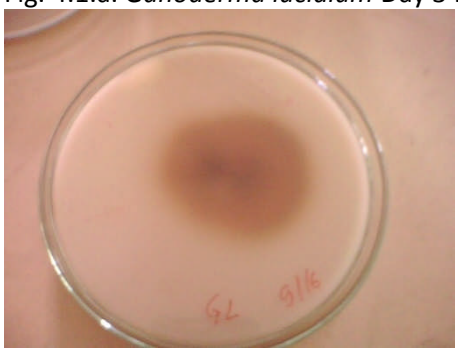

Fig. 4.1.c. *Ganoderma lucidium* Day 4 in tannic acid

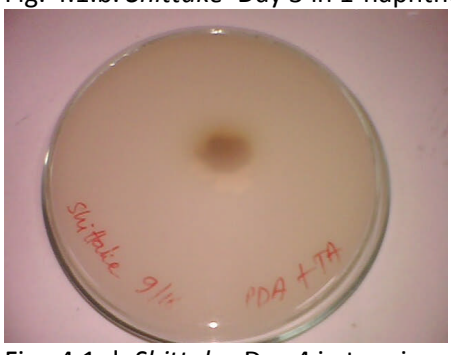

Fig. 4.1.d. *Shittake* Day 4 in tannic acid

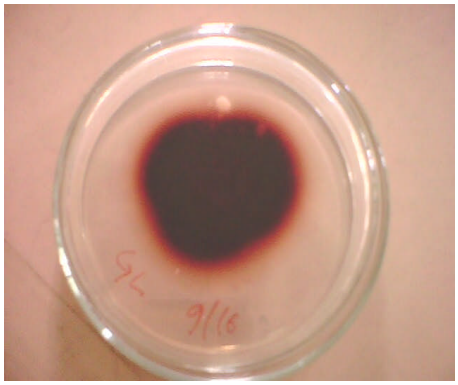

Fig. 4.1.e. *Ganoderma lucidium* Day 4 in guaiacol

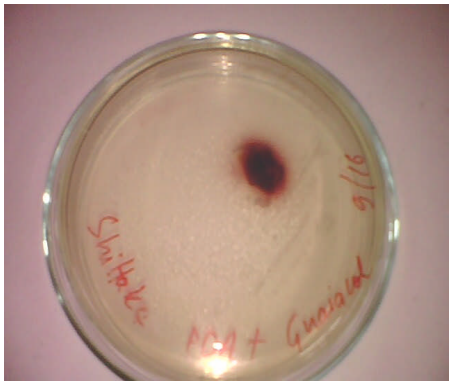

Fig. 4.1.f. *Shittake* Day 4 in guaiacol

4.2. Optimization of Production media

4.2.1. Temperature optimization

The temperature optimization was done in the range from 20 to 50°C, and the optimal temperature for high activity laccase production was determined to be 30°C (Figure.4.2.a) as highest laccase activity of 88.703 U/mL was demonstrated by enzyme produced at 30°C. Enzyme activity declined when the temperature was increased from 30 to 50°C.

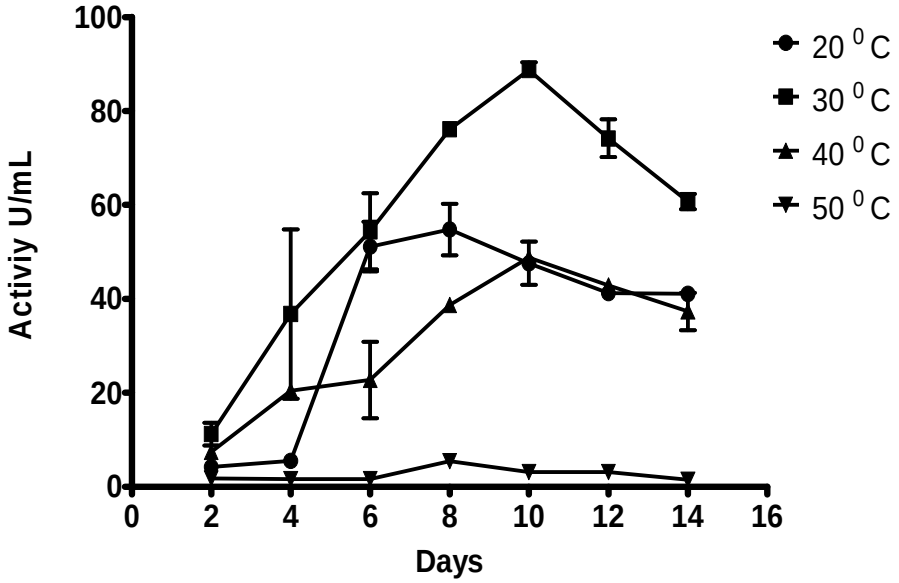

Fig. 4.2.a. Temperature optimization of laccase production.

4.2.2. pH optimization

The effects of pH values on laccase production were tested from pH range of 3.0 to 8.0 (Figure .4.2.b). Laccase exhibited the highest activity at pH 5.0 of 91.8254 U/mL at day 10. Enzyme activity decreased sharply as the pH value increased from 5.0 towards the neutral range.

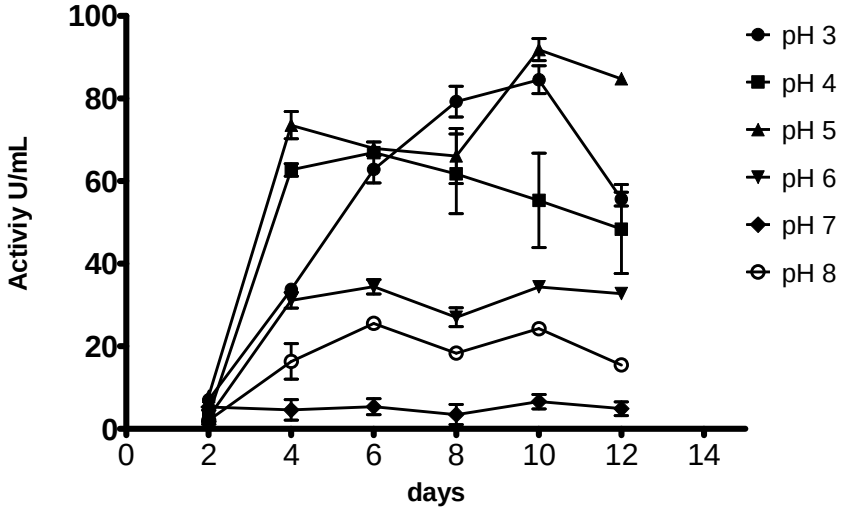

Fig. 4.2.b. pH optimization of laccase production.

4.2.3. Effect of copper sulphate

Five different concentrations of Copper sulphate, 10 µM, 20 µM, 30 µM, 40 µM and 50µM, were taken to see the effect of copper sulphate in production of laccase from *Ganoderma* sp. Among them, 30 µM supported the maximum laccase production on 10th day (Figure 4.3) with activity upto 93.1217 U/mL which was 113.7% more as compared to blank.

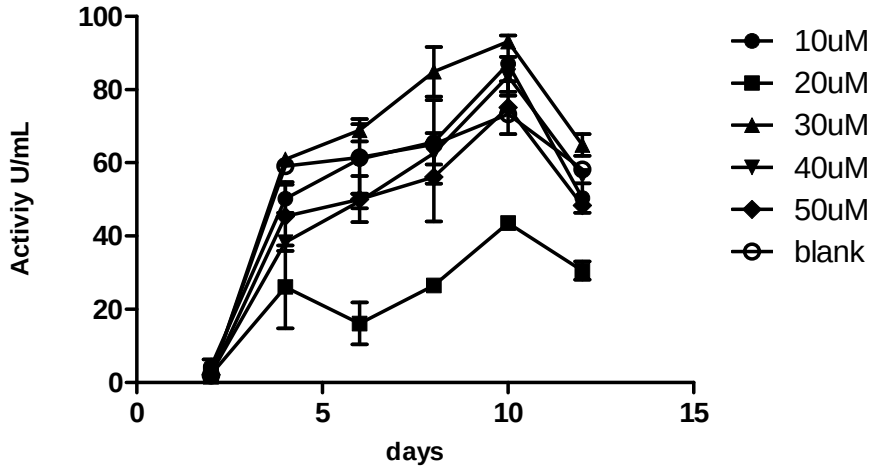

Fig. 4.3. Optimization of copper sulphate in laccase production.

4.2.4. Effect of lignin

To see the effect of lignin in laccase production two different sets of experiment were performed. One set was supplied with isolated rice straw lignin in 5 g/L concentration while the other set was without lignin. Presence of lignin effectively increased the extracellular laccase production (Figure 4.4) by 110.6 % as compared to the media containing glucose only.

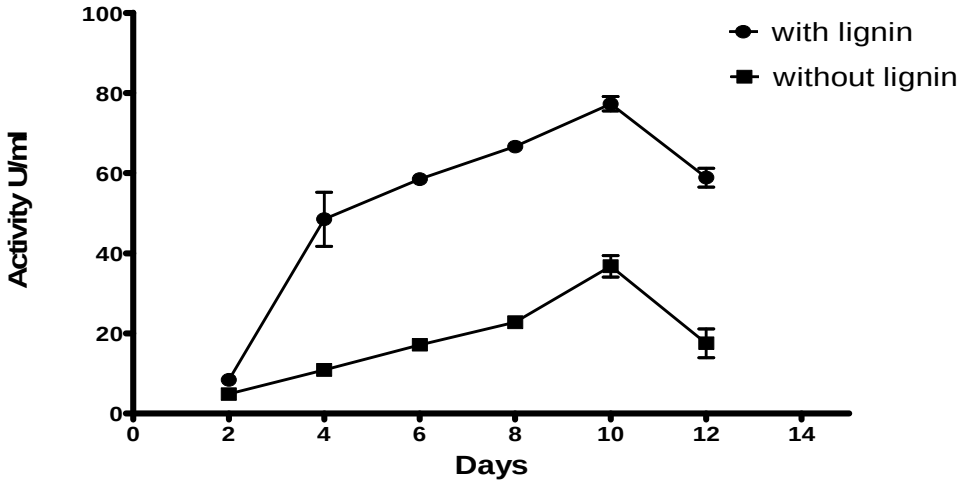

Fig. 4.4. Effect of lignin in laccase production.

4.3. Purification of laccase

Purification was done by vacuum filtration, ammonium sulphate precipitation and ion exchange chromatography. Precipitate of 70% ammonium sulphate gave maximum activity of laccase as shown in fig. 4.5 and so enzyme was precipitated with 70% ammonium sulphate and the enzyme was dissolved in least volume of sodium acetate buffer. Dialysis bag was used to desalt the enzyme.

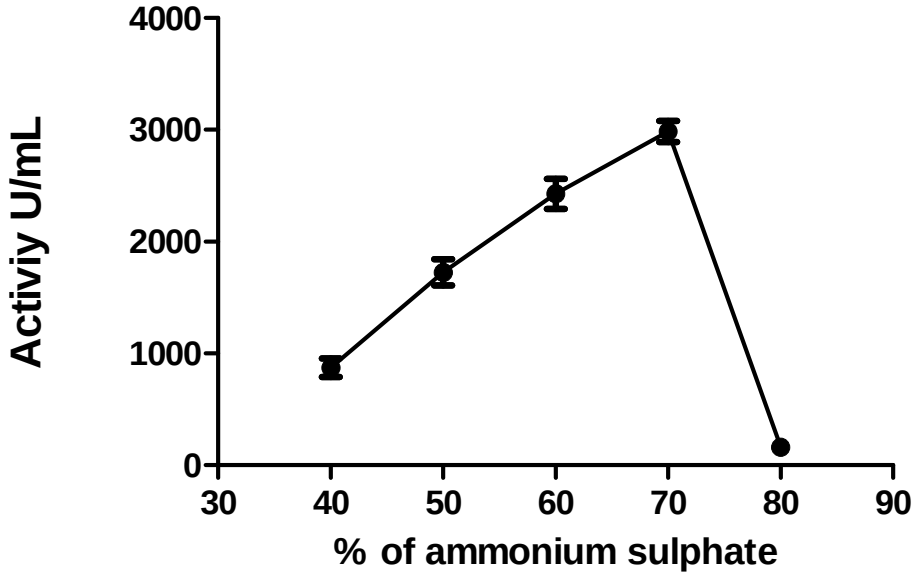

Fig. 4.5 Optimization of concentration of ammonium sulphate for protein precipitation.

4.4. Gel electrophoresis

The desalted enzyme was further purified through DEAE sepharose anion exchange column chromatography. Total 54 fractions were obtained and laccase assay was done

to each fraction. First peak was obtained from fraction number 9, 10, 11 and 12 which had maximum activity of 2378.64 U/mL in 10th fraction eluted out by 0.1 M NaCl. Second peak was observed in the 16th and 17th fractions with maximum activity of 1937.037 U/mL (Figure. 4.6). The purified laccase was analyzed by native-PAGE and SDS-PAGE. The purified protein demonstrated a single band in SDS-PAGE analysis, with a molecular weight of 43 kDa (Figure 4.7.a). But three bands were observed after incubating the native-PAGE gel with the laccase substrate ABTS and guaiacol indicating the possibility of three isoforms of laccase in the crude enzyme extract (Figure 4.7.b-c).

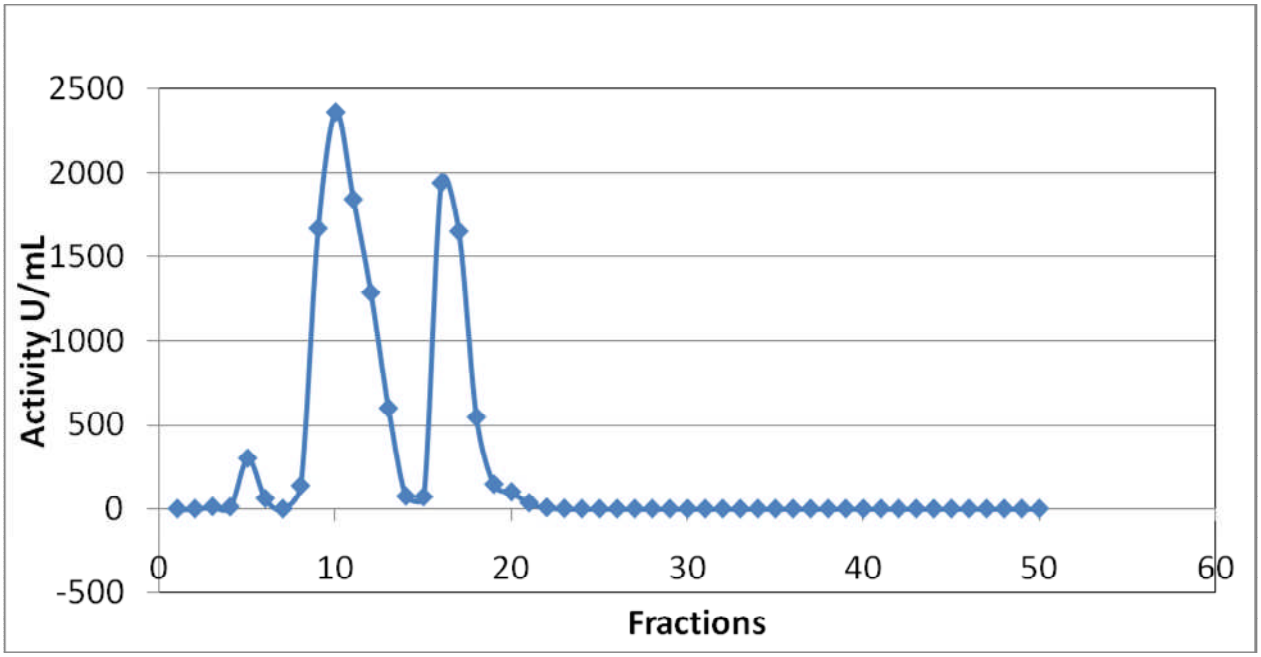

Fig. 4.6. DEAE-sepharose anion exchange chromatography of ammonium sulphate salt precipitated laccase enzyme.

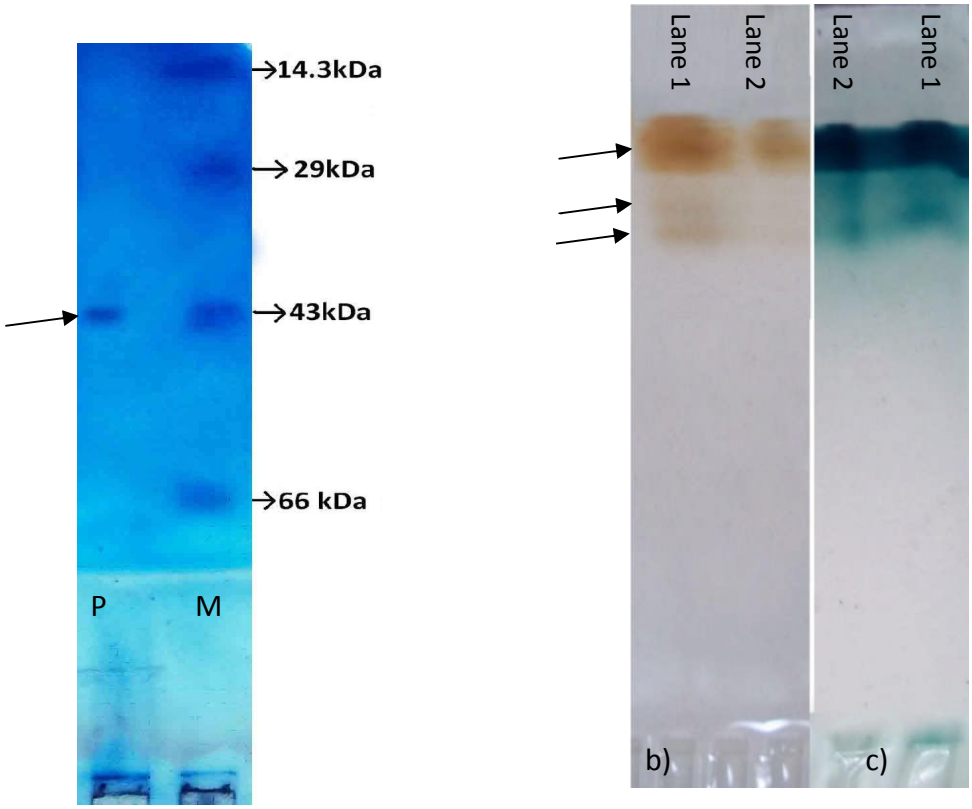

Fig.4.7. a) SDS PAGE of laccase after dialysis, M=marker, P=purified protein. b) native PAGE using guaiacol, c) native PAGE using ABTS lane 1=enzyme obtained after dialysis, lane 2=enzyme purified by ion exchange chromatography.

4.5. Characterization of purified laccase

4.5.1. Temperature optima

The influence of temperature on laccase activity was determined at temperatures ranging from 20 to 80 °C, and the optimal temperature for laccase was determined to be 30 °C (Figure 4.8.a). Enzyme activity declined when the temperature was increased from 30 to 80 °C. It decreased by 34.6 % at 80 °c of the maximum activity observed at 30 °C.

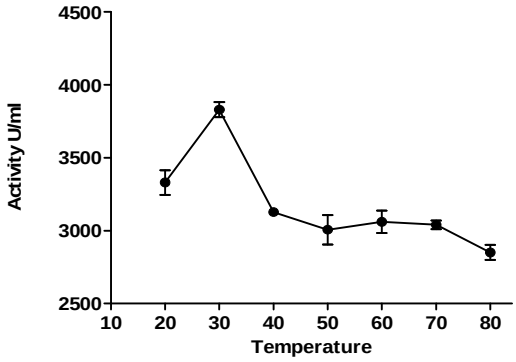

Fig. 4.8.a. Optimum temperature for laccase assay

4.5.2. Thermostability

The stability of the enzyme with respect to temperature was also studied (Figure 4.8.b). After 1 hour, laccase activity at 70 ^{o}C decreased by 35.7 % of the initial optimal activity while after 180 min at 70 ^{o}c laccase activity decreased by 94.84%.

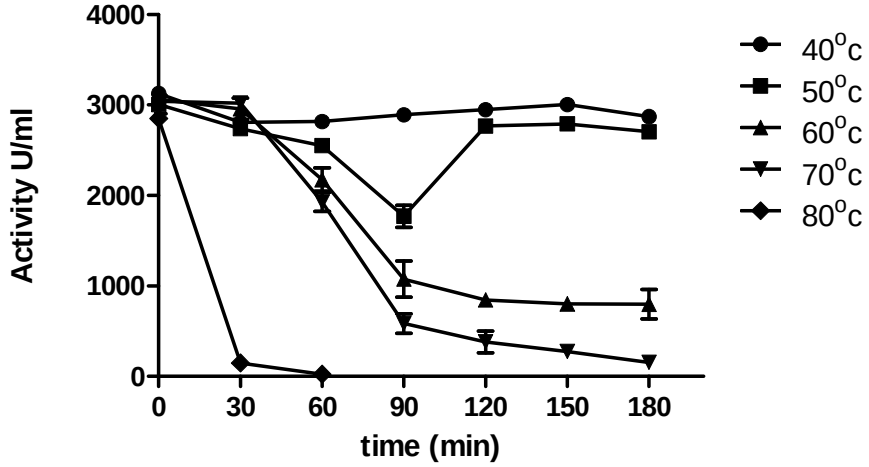

Fig. 4.8.b. Thermostability of laccase.

4.5.3. pH optima

laccase was observed to be active in wide range of pH. The optimum pH for the maximum laccase activity was observed at pH 5 when ABTS was used as substrate with enzyme activity upto 3326.85 U/mL (Figure 4.9.a). At pH values larger than 5, the enzyme activity decreased gradually and it decreased by 50.25% of optimal activity at pH 8. The activity of laccase did not decrease significantly at pH 3-7.

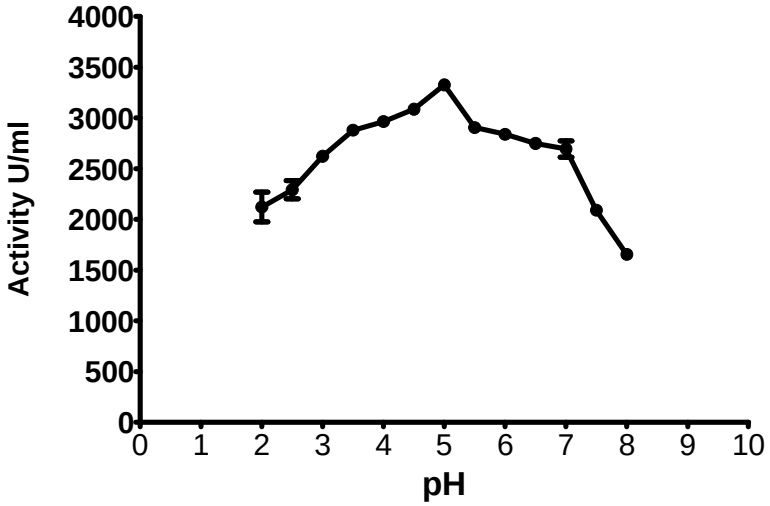

Fig. 4.9.a. Optimum pH of laccase assay.

4.5.4. pH tolerability

The effect of pH on laccase enzyme in course of time was also studied from pH 2 to 8. laccase activity decreased highly at pH 2 and 8. After 180 min at pH 8 laccase activity decreased by 92.86% of its optimal activity (Figure 4.9.b). But at pH 3-7, the enzyme was active and stable even upto 3 hours.

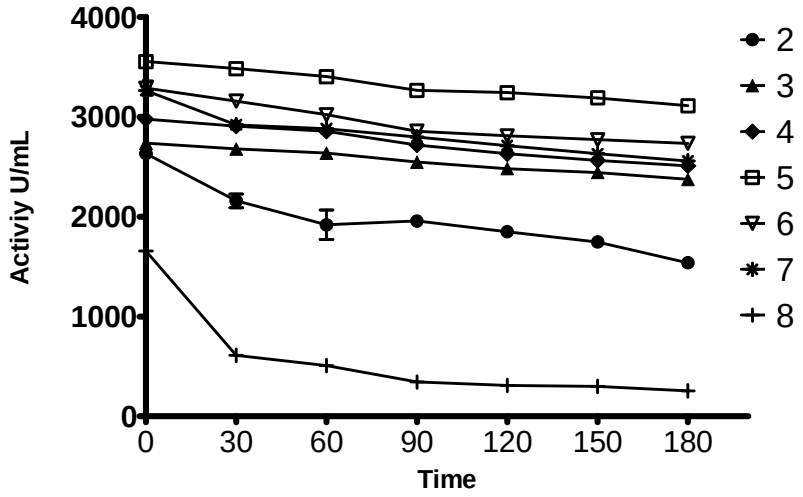

Fig. 4.9.b. pH tolerability of laccase.

4.6. Kinetic study

The kinetic parameters of purified laccase were characterized using the Michaelis constant $k_{m,}$ maximum velocity (V_{max}) and catalytic constant k_{cat} . K_m (affinity constant) was found to be 0.11 mM and V_{max} (maximum enzyme velocity) was found to be 35.71 µmol/min from Lineweaver–Burk plot using excel while Michaelis-Menten curve was plotted using Graph pad prism version 5.0 (Figure .4.10 a,b). Similarly, k_{cat} was found to be 246.21 min^{-1}.

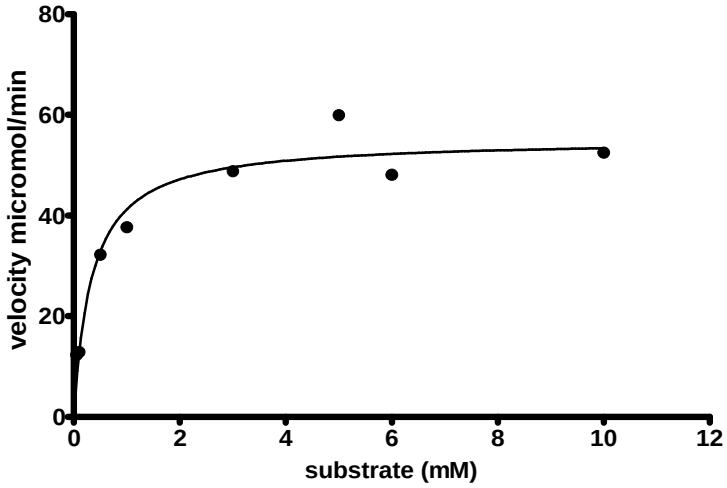

Fig. 4.10. a. Michaelis-Menten plot of laccase using ABTS as substrate.

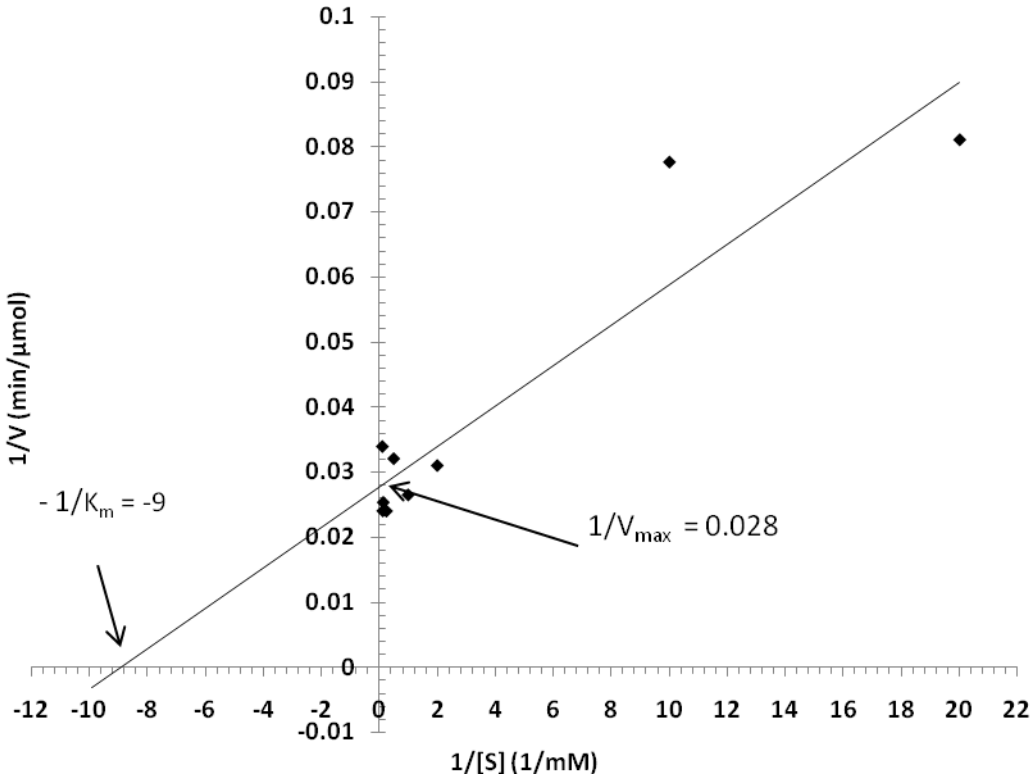

Fig. 4.10. b. Lineweaver–Burk plot of laccase using ABTS as substrate.

5. DISCUSSION

The major component of farming and agricultural waste is ligninocellulose, which is composed of cellulose, hemicellulose and lignin (Sánchez, 2009). Lignin is the major barrier that encases cellulose and hemicellulose from enzymatic attack. WRF can attack the lignin to obtain energy from cellulose by secreting ligninolytic enzymes, like laccases. *Ganoderma* is a important member of WRF family. Laccase produced from this organism has been used in study of olive mill wastewater decolourization and dephenolization, decolorization of reactive dyes, ethanol production, pentachlorophenol removal, improvement of feed for monogastric animals and many more (Kumarasamy Murugesan, 2006; Jeon et al., 2008; Sharma et al., 2013).

In this study, *G. lucidum* laccase production and activity was determined using submerged culture fermentation. It has been reported that the carbon source is the most important factor in laccase production, and that the addition of suitable amounts of other sugars to the culture media has a benign influence on laccase synthesis (Teerapatsakul et al., 2007). In this work 0.4% concentration of glucose was used, while others have used 1% (Kumar et al., 2011) as literature review suggests that low concentrations of glucose are the optimal carbon source for *G. lucidum* laccase production. At high concentrations, sugars may satisfy the nutrient demands of *G. lucidum* for biomass growth, without necessitating secretion of laccase to degrade lignin in order to obtain energy from cellulose. *Trametes pubescens* laccase synthesis is also repressed when glucose exceeds a certain concentration (Galhaup et al., 2002). Additionally, it has been reported that high concentrations of glucose can trigger the synthesis of extracellular polysaccharides which can interfere with the extraction of laccase from the culture broth (Eggert et al., 1996). It was also observed in this study that presence of lignin enhances laccase production which is in accordance with other works (Zhongyang Ding, 2011). Increased laccase activity in culture media with lignin as compared to the culture media containing only glucose might be due to a) synthesis of inducible laccase isozymes and b) combinatorial effect of laccase and HOBT (a heterocyclic compound with N-OH moiety which can be oxidized by laccase to its nitroxide radical). These redicals act as oxidant of lignin which depolymerizes lignin and give rise to phenolics (ferulic acid, vanillic acid, 3–4 dihydroxy benzoic acid) as well as non phenolic structural polymers. The increase in laccase yield from *Ganoderma* sp. rckk-02 was observed by Sharma et al with increase in lignin concentration. Similarly veratryl alcohol, syringic acid and 2,5-xylidine are most frequently used aromativ substances for enhancement of laccase production.(D'Souza et al., 1999; Sharma et al., 2013).

Peptone was used as nitrogen source which is known to enhance laccase synthesis (Kumar et al., 2011). Due to the complex composition of peptones, they provide a wide range of benefits to the cells and cell performance.

Copper is the most frequently applied substance to enhance laccase production in fungi. It is part of the active center of laccases, and is thus crucial for the synthesis of a catalytically active laccase protein. Addition of copper enhances the levels of gene transcription for laccase enzyme. Some of the genes are expressed constitutively and many are induced by nitrogen deficiency, by chemicals, like copper (Collins and Dobson, 2005). The promoter region of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals (Baldrian et al., 2003). It has been demonstrated that the *Pleurotus* laccase genes poxc and poxa1b are transcriptionally induced by copper and several putative metal responsive elements have been found in the promoter region of these genes (Calmieri et al., 2000). The suppression of fungal laccase production at higher concentration of a metal ion like Cu^{2+} could be due to activation of the white rot fungal defense mechanism. Production of oxalic acid along with which repression of laccase synthesis was concomitantly observed in such exposure (Baldrian, 2003). In addition to copper, distinct organic inducers with structural similarities or relationships with lignin are often applied, (Vishwanath et al., 2008; Couto et al., 2002).

The pH optima of laccases are different as they are highly dependent on the substrate used. It has been found that optimum pH can range from 3-7 for fungal laccase. When ABTS has been used as substrate the pH optima are more acidic and are found in the range of 3-5 (Madhavi and Lele, 2009). In this study also the pH optimum curve showed that optimum pH for laccase is 5 and the enzyme was active till very wide range of pH suggesting its possibility for many biotechnological processes. The decrease in activity at higher pH is most probably due to the binding of a hydroxide anion to the type 2/3 copper centers of laccase, which inhibits the binding of oxygen and therefore inhibits the activity (Xu, 1997; Patrick et al., 2009).

The optimum temperature for laccase production was found to be 30 ^{o}C which is supported by the fact that *G. lucidum* is a mesophilic fungi. Laccase from *Ganoderma lucidum* has been reported with highest activity at 25 ^{o}C elsewhere (Ko et al., 2001). But the enzyme was active over higher temperature range also. After 1 hour, laccase activity at 70 ^{o}C decreased only by 28.42% indicating its thermostable nature.

Many fungal laccase enzymes have been purified and their molecular properties has been studied (Table 2). V_{max} varies with the source of laccase (50–300 M/s). The kinetic constants differ in their dependence on pH but Km is pH-independent for substrate. Larger the velocity higher will be the amount of substrate binding which is a desirable

quality for an enzyme. Similarly lower the k_m value higher is the affinity towards the substrate.

The *Ganoderma lucidum* laccase had K_m value of 3.7 mM for ABTS (Ko et al., 2001) and 0.114M (Zhongyang Ding, 2011). The K_m and V_{max} values of recombinant laccase of *Ganoderma lucidum* heterologously expressed in *Pichia pastoris* for ABTS were 0.521 mM and 19.65 mM min^{-1}, respectively. In this work K_m and V_{max} were found to be 0.11 mM and 35.71 µmol/min respectively and k_{cat} was found to be 246.21 min^{-1}. The K_m reported in this work is lower than other reported values suggesting this enzyme has higher affinity towards non-phenolic substrate like ABTS while the other studies have reported lower affinity of laccase towards non-phenolic substrate (Zhongyang Ding, 2011).

Fungal laccases have a number of different isoforms with a molecular mass ranging from 40 to 80 kDa (Eggert et al., 1996), and this variation could be attributed to the different ecological origins of each species or different culture conditions, in particular presence of inducer in the media. The fungus *P. pulmon*arius produced three laccase isoforms, two of which (lcc1 and lcc2) are constitutive and the isoform lcc3 was detected only when the fungus is cultured in the presence of inducers (O. V. Morozova, 2007). In this study ion exchange chromatography profile showed two major peaks. Native-PAGE analysis suggested that *Ganoderma* laccase has three isoforms which is similar to the result obtained by Ko et al., 2001. Molecular weight of the laccase was found to be 43 kDa from SDS-PAGE which is in agreement with Murugesan et al., 2007. But the isoforms were not visible in SDS PAGE which may be due to low concentration of protein. It has been reported that *G. lucidum* produces three isoforms (Ko et al., 2001) or two isoforms of 40 to 68 kDa (D'Souza et al., 1999). *Trametes multicolor* is reported to secrete 5 isozymes of laccase (Leitner et al., 2002). Similarly the fungus *P. ostreatus* secretes eight different laccase isozymes induced by copper ions in the culture medium and regulated on the level of gene transcription (O. V. Morozova, 2007).

6. SUMMARY

Laccases (EC 1.10.3.2), *p*-benzenediol:oxygen oxidoreductase, are mono or multimeric copper containing oxidases that catalyse the one-electron oxidation of a vast number of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water. The ability of laccases to oxidise phenolic compounds and reduce molecular oxygen to water has led to intensive study of these enzymes. The ideal laccases for industrial use would exhibit stability at high temperature and pH conditions.

This study demonstrates that *G. lucidum* is an important white rot fungi which secrets extracellular laccase as the ligninolytic enzyme. The laccase production was optimized in modified Olga medium for enhanced laccase production and thus produced laccase was purified and characterized. The production of laccase was stimulated by the presence of lignin and copper sulphate in the medium. Presence of lignin in the media increased the extracellular laccase production by 110.6 % as compared to the media containing glucose only and 30mM of copper sulphate stimulated laccase production by 113.7%. Highest enzyme activity during enzyme production was reported as 93.1217 U/mL under optimized condition. Optimum temperature was found to be 30 ^{o}C and optimum pH was found to be 5. Laccase was purified by ammonium sulphate salt precipitation and by anion exchange chromatography through DEAE sepharose gel column. Native PAGE and ion exchange chromatography indicated the presence of three isoforms of laccase but single band was observed in SDS-PAGE with molecular weight of 43 kDa. Laccase showed highest activity at 30 ^{o}C and at pH 5 respectively. *Ganoderma lucidum* laccase is a thermostable laccase as laccase activity at 70 ^{o}c decreased only by 28.42% after 1 hour incubation. V_{max} and K_m were found to be 0.11 mM and 35.71 µmol/min respectively and k_{cat} was found to be 246.21 min^{-1} for ABTS as substrate. The isolated thermostable laccase can be used for various biotechnological and industrial processes.

Thus produced enzyme was effective over wide pH range and higher temperature suggesting its possible applications in process where pH range is wide and temperatures are higher. Kinetic study of the enzyme indicated its higher affinity towards non-phenolic substrate suggesting that it could be used for oxidation of non phenolic substrates as well.

One of the limitations to the large-scale application of the enzyme is low volume production of highly active enzyme. These problems can be solved by screening for natural hypersecretory strains or using recombinant organisms. Environmental factors also influence the ability of fungi to produce high titres of laccase, and different strains react differently to these conditions. Hence it is essential to select a strain capable of producing high concentrations of a suitable enzyme and then optimize culture

conditions for enhanced laccase production by the selected organism. It is therefore not surprising that this enzyme being studied since the nineteenth century is yet being studied intensively and remains a topic of intense research today also.

Table. 5. Summary of laccase purification from *Ganoderma lucidium.*

Enzyme	Total activity (U)	Protein concentration (µg/mL)	Specific activity (U/mg)	Purification fold
Crude	97.2236	105.66	0.92016	1
Dialyzed	3115.74	35.875	86.8499	94.38
Ion exchanged	2662.04	13.25	200.908	218.34

7. CONCLUSION

Fungal laccases are considered as ideal green catalysts because of their requirement of oxygen as electron donor, and their only by-product water. They have broad substrate specificity, including direct bioelectrocatalysis which broadens their use for a number of biotechnological applications. Furthermore, the inventions of laccase-mediator systems have expanded the substrate range of laccases leading them to their successful applications in bioremediation, paper pulp bleaching, finishing of textiles, bio-fuel cells and more (Kunamneni et al., 2008). Delignification is another important application of laccases which have helped in overcoming the obstacle presented by lignin during conversion of lignocellulosic biomass into biofuels. Though there are physical and chemical methods of delignification, enzymes are more beneficial as they act as biocatalyst carrying the reactions in milder form saving both energy and cost and are biodegradable. Moreover, chemical delignification produces furan derivatives, weak acids and phenolic compounds after the breakdown of lignin which have inhibitory effects (Chunxia Lu, 2010). However, the unstable nature of enzymes, and the high cost of enzyme isolation and purification still discourages their extensive use. In spite of these drawbacks, the research on enzyme applications is in steady development and many industrial laccase are already in the market.

The present work was done to study the biochemical and kinetic properties of laccase isolated and purified from *Ganoderma lucidum*. Biochemical properties of enzyme help to know the dependence and effect of various parameters on the enzyme activity which are crucial for understanding the mechanism of catalysis of enzyme. A good finding of this work is that the enzyme was effective in broad pH range and at higher temperatures which suggests that the enzyme can be used over wide range of pH and even at higher temperatures without significant loss of enzyme activity. It had low k_m value for ABTS (non-phenolic substrate) which is another good finding of this work.

Recommendations:

a. To clone the laccase coding gene into suitable host.
b. To immobilize the laccase enzyme and study its efficacy.
c. To study polymerizing ability of laccase with L-DOPA and D-DOPA for melanin synthesis.
d. To study depolymerization of dye and waste water.
e. To study effect of possible inhibitor of laccase.

8. APPENDIX

Media composition of Modified olga medium

Components	Concentration
Glucose	4 g/L
Peptone	3 g/L
Potassium di-hydrogen phosphate (KH_2PO_4)	0.6 g/L
Di-potassim hydrogen phosphate (K_2HPO_4)	0.4 g/L
Zinc sulphate	1 mg/L
Iron sulphate	5mg/L
Manganese sulphate	0.5 g/L
Magnesium sulphate	0.5 g/L
Copper sulphate	0.5mg/L
Lignin	5 g/L

Table 6. Lignin estimation in rice straw stem and rice straw leaf

sample		Dry filter paper (DF)	Dry filter paper + lignin (DFL)	Dry Lignin (DL)	Wet filter paper (WF)	Wet filter paper + lignin (WFL)	Wet lignin (WL)	Lignin (WL-DL)= m	Acid insoluble lignin (AIL) = (m/M) ×1000	Acid soluble lignin (ASL)	TOAL L(mg/g)	% dry weight	mean
Rice stem	1	0.57	0.579	0.009	0.585	0.608	0.023	0.014	140	24.044	164.044	16.404	19.42
	2	0.574	0.596	0.022	0.589	0.626	0.037	0.015	150	23.094	173.094	17.309	
	3	0.574	0.593	0.019	0.587	0.628	0.041	0.022	220	25.704	245.704	24.570	
Rice leaf	1	0.551	0.578	0.027	0.57	0.619	0.049	0.022	220	31.425	251.425	25.142	25.8
	2	0.571	0.572	0.001	0.59	0.611	0.021	0.02	200	24.096	224.096	22.409	
	3	0.566	0.575	0.009	0.58	0.616	0.036	0.027	270	29.791	299.791	29.979	

Acid insoluble lignin (ASL) = $\frac{m}{M} \times 100$ mg/g, Acid soluble lignin (AIL) = $\frac{A.D.V}{abM} \times 1000$ mg/g,

Total lignin = ASL + AIL

Where m= increase in weight, M = Weight of sample = 0.1g, A = absorption at 205nm, D = dilution factor, V = volume of filtrate in ml (0.029L), a= Extinction coefficient of enzyme in g/Lcm = 110 g/Lcm (TAPPI CM 250), b = cuvete length path = 1cm

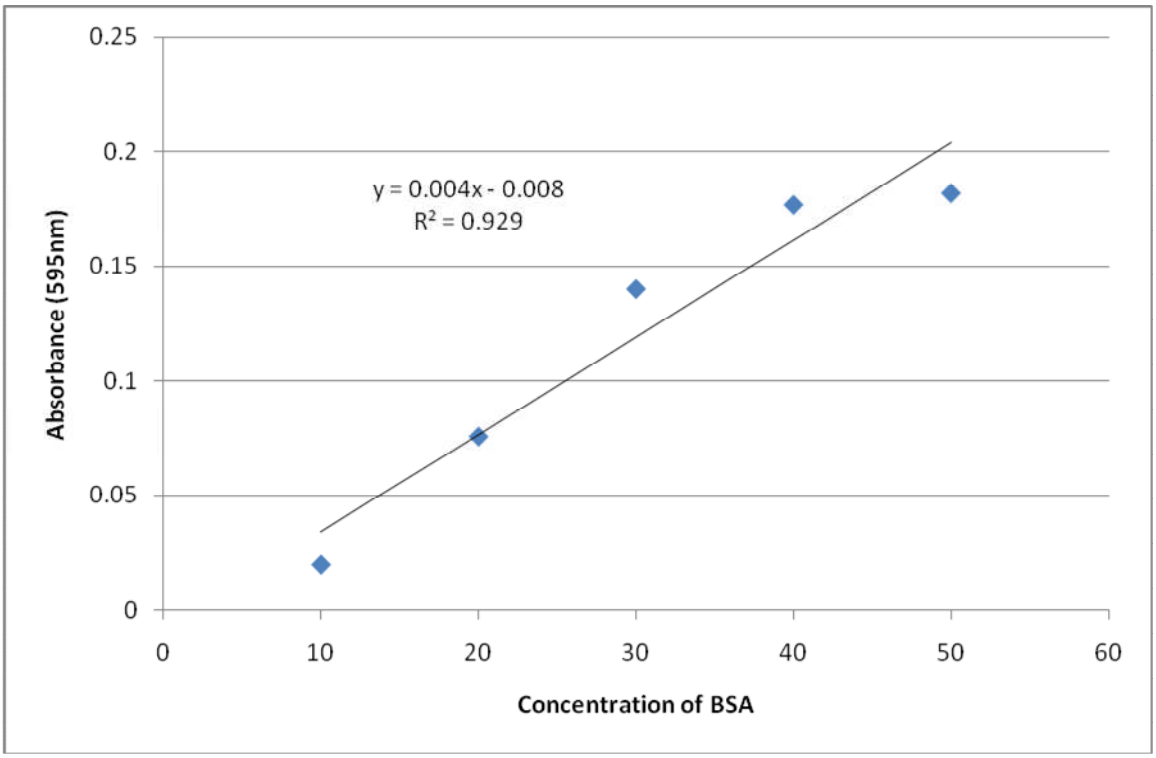

Fig. 5. Standard protein estimation graph

Components of SDS-PAGE

30% acrylamide solution preparation (for 100ml)

1gm bisacrylamide (neurotoxic) mixed with 29 gm acrylamide in 50 ml TDW and final volume

Maintained to 100 ml.

Lower tirs pH 8.8 (for 100ml)

1 1.5 M Tris base (18.17gm) dissolved in distilled water

pH = 8.8

Upper tirs pH 6.8 (for 50 ml)

0.5 M tris base (3.03gm) dissolved in distilled water

pH 6.8

Loading buffer ph 6.8 for 10 ml

upper tirs	1.25 ml	
10%SDS	3	
Glycerol	4.75	
2-mercaptoethanol/B-mercaptoethanol	0.5ml	
0.1% bromophenol blue	0.5ml	-store at 4 ^{0}c

Resolving gel

10% (For 10 ml)

Water	4
30%acrylamide	3.3
1.5 Tris (8.8)lower tris	2.5
10%SDS	0.1
10% APS(Ammonium per sulphate)	0.1
TEMED	0.006

Stacking gel (for 5% - 4 ml)

Water	2.7
30%acrylamide	0.67
1.5 Tris (8.8)lower tris	0.5
10%SDS	0.04
10% APS(Ammonium per sulphate)	0.04
TEMED	0.006

Running buffer for 1000ml

39mM tris	(ie 4.724g)
48mM glycine	(ie 3.603g)
0.37gm of 0.1%sds	

Fixative solution:
5% acetic acid solution or 30% methanol, 10% acetic acid solution).

ABTS solution (Mol. Wt. of ABTS = 548.68 g)
27.4 g ABTS dissolved in 50 ml and 137 g ABTS dissolved in 50 ml of sodium acetate buffer of pH 5 to make 1 mM ABTS and 5mM solution and kept at 4 ^{o}c for further use.

1% Guaiacol solution

510 µl of 98% Guaiacol was mixed with 49.490ml of 10% glycerol and mixed well

Sodium acetate buffer (0.1M pH 5)

1.05 ml of 100% acetic acid (sp. Gravity 1.04) and 4.32 g of 100% sodium acetate were mixed to make final volume 500 ml and pH 5

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