OPTIMIZED PRODUCTION OF LACCASE ENZYME FROM POTENT MICROBE AND CHARACTERIZATION OF PURIFIED ENZYME

M.Sc. Thesis

2017 A.D.

Submitted to

Central Department of Biotechnology (CDBT)
Institute of Science and Technology, Tribhuvan University
Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement for the Master of Science in Biotechnology

Submitted by

Mukesh Yadav

T.U. Regd. No.: 5-2-15-3410-2009

Supervisor
Mrs. Jarina Joshi
Assistant Professor
Central Department of Biotechnology (CDBT)
Institute of Science and Technology, Tribhuvan University
Kirtipur, Kathmandu, Nepal

ACKNOWLEDGEMENT

First and foremost I would like to express my thanks and utmost gratitude to my thesis supervisor, Mrs. Jarina Joshi, faculty of Central Department of Biotechnology, T.U. for her exemplary guidance and support to explore the fascinating world of microorganisms particularly about fungi. I am extremely grateful for her constant encouragement, keen interest, careful and valuable supervision that contributed a lot in completing my thesis.

I would express my gratitude to **Prof. Dr. Krishna Das Manandhar**, Head of the Department and faculty members **Prof. Dr. Mohan Kharel, Prof. Dr. Tribikram Bhattarai, Prof. Dr. Tilak R. Shrestha, Prof. Dr. Ganga Kharel, Dr. Smita Shrestha, Mr. Balhari Paudel** and all the staff of Central department of Biotechnology. The equal thanks are provided to Lab assistants for their efforts and assisting me in time to time.

I would like to especially thank **Dr. Jay Kant Raut** (Senior scientific officer, NAST), for providing culture and whole dried fungi body for my thesis work.

My unlimited thanks goes to my dearest friends viz:, **Sandesh Maharjan, Rupesh Pokharel, Dilli Raman Devkota, Ankit Jaiswal, Sujan Bishwokarma, Kapil Adhikari, Bhagwat Majhi, Gauri Thapa, Krisha Sthapit, Archana Maharjan, Nutan Thakur, Rosy Chaulagain, Garima Bista, Binod Neupane, Puskar Thapa, Medha KC, Bijay Kumar Karki, Sunita koirala**, the seniors and juniors of Biotechnology, who have directly or indirectly helped me to carry out my work.

I want to pay my special regards and thanks to my senior **Mitesh Shrestha** for his continuous support and tolerating my endless queries during my thesis period.

Words fail to express my gratitude towards my beloved parents, and maternal uncle, whose affection, unfailing encouragement and source of inspiration have enabled me to owe this coveted achievement.

The one above all of us, the omnipresent God, for answering my prayers and giving me the strength to trudge on, thank you so much my Dear Lord.

Mukesh Yadav

LIST OF ABBREVIATIONS

ABTS	=	2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt
BLAST	=	Basic Local Alignment Sequence Tool
bp	=	Base pair
BSA	=	Bovine Serum Albumin
DNA	=	Deoxy-ribose Nucleic Acid
DO	=	Dissolved Oxygen
EC	=	Enzyme Commision
EDTA	=	Ethylenediaminetetra-acetate
EPR	=	Electronic Paramagnetic Resonance
EtBr	=	Ethidium Bromide
FDM	=	Furan-2,5 dimethanol
HMF	=	Hydroxy Methyl Furfural
HOBT	=	Hydroxybenzotriazole
ISP	=	International *Streptomyces* project
Lac	=	Laccase
Lig	=	Lignin
LiP	=	Lignin Peroxide
LME	=	Lignin Modifying Enzymes
MnP	=	Manganese Peroxidase
NEB	=	New England's Biolab
PAGE	=	Poly Acrylamide Gel Electrophoresis
PAH	=	Polycyclic Aromatic Hydrocarbons
PCR	=	Polymerase Chain Reaction
PDA	=	Potato Dextrose Agar
PDB	=	Potato Dextrose Broth
RNA	=	Ribo-nucleic Acid

RPM	=	Revolution Per Minute
SD	=	Standard deviation
SDS	=	Sodium Dodecyl Sulphate
TAE	=	Tris acetic acid EDTA
TE	=	Tris EDTA
tris	=	Tris-hydroxymethyl-aminomethane
WHO	=	World Health Organization
WRF	=	White Rot Fungi
ε	=	Molar extinction coefficient($\varepsilon_{420} = 36000M^{-1}cm^{-1}$ for ABTS)

TABLE OF CONTENTS

CHAPTERS **PAGE NO**

ACKNOWLEDGEMENT I

LIST OF ABBREVIATIONS.. II

LIST OF FIGURES VIII

ABSTRACT 1

CHAPTER I: INTRODUCTION 2

1.1 Background 2

1.2 Rationale 3

1.3 Research Hypothesis 4

1.4 General Objective 4

1.5 Specific Objectives 4

CHAPTER II: LITERATURE REVIEW 5

2.1 Composition of lignocellulosic material 5

2.1.1 The Plant Cell Layers 5

2.2. Lignin Degrading Organism 10

2.2.1 Actinomycetes as sources of novel laccases 10

2.2.2 White-rot Fungi 10

2.2.3 Ganoderma lucidum 11

2.2.4 Brown-rot Fungi 12

2.2.5. Aerobic Bacteria 12

2.2.6. Anaerobic Bacteria 13

2.3. Classification of Polyphenol oxidase according to Substrate Specificity ... 13

2.3.1 Laccase 14

2.3.2. Molecular structure of Laccase 15

2.3.3. Mechanism of Catalysis by Laccase 19

2.4. Production of fungal laccases 22

2.4.1. Induction of laccase production 22
2.4.2. Influence of pH on laccase production 23
2.4.3. Influence of temperature on laccase production 23
2.4.4. Inhibition of laccase production 23
2.4.5. Effect of Metal ions on laccase production 24
2.4.6. Effect of surfactant on laccase production 24
2.4.7. Fermentation of ascomycetes in liquid cultures 24
2.5. Important parameters of fermentation on fungal morphology and on production yields of enzymes 26
2.5.1. Inoculum 26
2.5.2. Oxygen 26
2.5.3. Vessel and impeller 27
2.5.4. Mode of cultivation 29
2.6. Heterologous expression 30
2.7. Isoforms of laccase 32
2.8. Substrates for Detection of Laccase Activity 33
2.9. Laccase as a Lignin modifying enzymes 34
2.10. Unusual properties of laccase 36
2.11. Application of Laccase 36
2.11.1 Industrial Application of Laccase 37
2.11.2 Delignification and Pulp bleaching 37
2.11.3 Bioremediation 37
2.11.4 Food Industry 38
2.11.5 Organic Synthesis 39
2.11.6 Pharmceutical Sector 39
2.11.7 Nanobiotechnology 40
2.11.8 Industrial Wastewater Treatment 40
2.11.9 Laccase based biosensor 41
2.11.10 Cosmetic Industry 41
2.11.11 Alternative applications 42
CHAPTER III: MATERIAL AND METHODOLOGY 44
3.1 Settings of Laboratory 44

3.2 Sample collection 44
3.3 Isolation and Culture conditions 44
3.4 Screening of laccase producing microbes 44
3.5 Morphology of Isolate ‘G1’ 45
3.6 Isolation of gDNA from selected organism 45
3.7 PCR amplification of gDNA 45
3.8 Optimized production of Laccase 46
3.8.1 Laccase activity assay 46
3.8.2 pH and temperature optimization 46
3.8.3 Agitation optimization 47
3.8.4 Dissolved Oxygen (DO) optimization 47
3.8.5 Effect of Inducers 47
3.8.6 Effect of Inhibitors 48
3.8.8 Effect of surfactants 48
3.9 Optimized production of laccase 48
3.10 Purification of laccase 48
3.10.1 Ammonium Sulfate precipitation: 48
3.10.2 Acetone precipitation: 49
3.11 Protein estimation 50
3.12 Gel electrophoresis 50
3.13 Characterization of Laccase enzyme 50
3.13.1 Optimum pH and optimum temperature 50
3.13.2 pH tolerability and thermo-stability of laccases 50
3.14 Kinetic study 50
CHAPTER IV: RESULTS 51
4.1 Isolation and screening of laccase producing microbes 51
4.2 Morphology of Isolate ‘G1’ 52
4.3 gDNA extraction and PCR amplification of Isolate G1 53
4.4 Production media optimization 56
4.4.1 pH optimization 56
4.4.2 Temperature optimization 57
4.4.3 Agitation optimization 58

4.4.4 Dissolved Oxygen (DO) optimization ... 58
4.4.5 Effects of Inducers on Laccase production ... 59
4.4.6 Effects of Inhibitors on Laccase productions ... 60
4.4.7 Effects of Metal ions on Laccase production ... 60
4.5 Purification of crude laccase from fermentation broth ... 62
4.5.1 Ammonium sulfate precipiatation ... 62
4.5.2 Acetone precipitation ... 62
4.6 Characterization of purified laccase ... 62
4.6.1 Temperature optima and thermal stability ... 62
4.6.2 pH optima and pH tolerability ... 64
4.6.3 SDS gel electrophoresis ... 65
CHAPTER V: DISCUSSION ... 67
CHAPTER VI: SUMMARY ... 71
CHAPTER VII: CONCLUSION AND RECOMMENDATION73
CHAPTER VIII: REFERENCES ... 74
Appendix ... 95

LIST OF FIGURES

Fig 2.1.1: Representation of the plant cell walls; A) Wood cell structure showing the middle lamella (ML), the primary cell wall (P), and the secondary cell wall comprising of three layers (S1, S2, S3) (Côté, 1967); B) The primary cell wall with cellulose, hemicellulose and fibers, embedded in lignin (Rosgaard et al. 2005).

Fig 2.1.1a (i): Inter- and intra-molecular hydrogen bonds in cellulose; (ii) SEM image of the cellulose fibers, available athttp://www.personal.psu.edu/tjr5043/exten%20text/index.html

Fig 2.1.1.c.i: Schematic representation of three main monolignols forming a three-dimensional network of lignin.

Fig 2.1.1.c.ii: Examples of suggested lignin-carbohydrate bonds; 1) an ester linkage to xylan through 4-O-methyl glucuronic acid; 2) an ester linkage to xylan through arabnofuranose unit; 3) an ether linkage to galactoglucomannan through a galactopyranose unit.

Fig 2.1.1.c.iii: Adler's (1977) fragment of softwood model lignin. The most common linkages are bolded black.

Fig 2.2.3: *Ganoderma lucidum* fruiting bodies taken from NAST, a) dorsal surface b) ventral surface,

Fig 2.3.2a: Molecular Structure of Laccase

Fig. 2.3.2b: 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)

Fig 2.3.3a: Oxidation of phenolic subunits of lignin by laccase

Fig 2.3.3b: Oxidation of non-phenolic lignin model compounds of lignin by laccase

Fig.2.3.3c: Role of mediator in laccase mediated catalysis (reproduced from (Banci *et al.,* 1999).

Fig.2.3.3d: Oxidation of ABTS

Fig. 2.5.3: Impeller types: pitched blade impeller (left), rushton turbine or disk stirrer (middle), paddle impeller or blade stirrer (right).

Fig.4.2: Isolate ”G1” growing on PDB with 1% kraft ignin, b) Cotton blue staining of isolate “G1”

Fig 4.3a: Gel-electrophoresis (1% agarose) of PCR product.Lane-2: 100 bp NEB ladder, Lane-3: PCR product.

Fig 4.3b: Blast result showing percentage identity of Isolate G1 with other fungal species

Fig 4.3c: Phylogenetic tree of Isolate “G1”

Fig 4.4.1: Effect of media pH on laccase production by Isolate “G1”

Fig 4.4.2: Effect of Temperature on laccase production by Isolate “G1”

Fig 4.4.3: Effect of Agitation rate on laccase production by isolate “G1”

Fig 4.4.4: Effect of Dissolved Oxygen rate on laccase production by isolate ‘G1’

Fig 4.4.5: Effect of inducers on laccase production by isolate ‘G1’

Fig 4.4.6: Effect of different concentrations of Inhibitors (mM) on laccase production.

Fig 4.4.7: Effect of different concentration of metal ions (mM) on laccase production.

Fig 4.4.8: Effect of different concentrations of surfactants on Laccase production

Fig 4.6.1.a: Characterization of optimum temperature

Fig 4.6.1.b: Characterization of temperature tolerability

Fig 4.6.2.a: Characterization of optimum pH

Fig 4.6.2.b: Characterization of pH tolerability

Fig 4.6.3: SDS PAGE of Laccase enzyme. L1- BSA, L2 & L3- purified laccase (G1), L5 & L6- crude laccase (G1) and L8- Protein Ladder 3.5-205 KDa GeNei

Fig 4.6.4: Lineweaver-Burk plot of laccase using ABTS as substrate

LIST OF TABLES

Table 2.1.1b: The major hemicellulose components

Table. 2.6: Laccase production in heterologous hosts.

Table. 2.7: Number of isoforms of laccase produced in different fungus

Table 2.8 Substrate used for detection of laccase activity

Table 2.11.11 Commercial preparations based on laccases for industrial processes

Table 3.7: Condition for PCR amplification of gDNA

Table 4.1 List of selected strains for research work.

Table 6: Summary of purification of laccase from Isolate 'G1'

ABSTRACT

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is an extracellular enzyme that belongs to the blue multi-copper oxidases group. The broad specificity of enzyme has the ability to oxidize wide range of aromatic compounds especially phenolic compounds through radical-catalyzed mechanism involving four electrons reduction of oxygen molecule into water. Laccase producing microbes were isolated using laccase screening test in guaiacol, 1-naphthol and tannic acid. Among the isolated microbes, isolate "G1" isolated from whole dried fungus body collected from Phulchoki hill of Nepal was found to be best laccase producing microbe. Genus name was specified by sequencing PCR amplicon using 18S rRNA primer as *Pestalotiopsis spp.* Laccase production efficiency was optimized using PDB media containing 1% kraft lignin. The optimized pH and temperature were found to be 5 and 30°C respectively. For economical and industrial point of view further optimization for agitation and DO (Dissolved Oxygen) was done in Electrolab fermenter and best agitation and DO were found to be 200 RPM and 40%. Effect of different concentrations of each inducers, inhibitors and metal ions on laccase production were also obtained. Highest laccase activity were found for inducer 1mM gallic acid (69.22±10.84 U/ml) and metal ion 1mM magnesium sulphate (50.52±24.75 U/ml). 0.1mM cysteine inhibited more than others. 15 ppm Tween 80 (54.40±14.4 U/ml) showed as good surfactant. Laccase obtained from fermentation broth were purified by ammonium sulphate and acetone precipitation method and found to be 882.0 U/ml and 956.66 U/ml respectively. Acetone precipitation was found to be best. Optimum pH and temperature was found to be 6 and 60°C respectively for optimum laccase activity. Laccase was highly stable at pH 5 and 40°C. SDS PAGE showed the molecular weight of laccase as 43 kDa. Laccase produced by *Pestalotiopsis sp.* was thermo-stable. V_{max} and K_m were found to be 100 μmol/min and 0.11 mM respectively for ABTS as substrate.

Key words: Laccase, Lignin, *Pestalotiopsis spp.*, Guaiacol, 1-naphthol and tannic acid.

CHAPTER I: INTRODUCTION

1.1 Background

The biofuel production from the lignocellulosic material is an alternative to the rising demands for oil in the transportation sector, especially in the USA and Asia (India, Nepal, China). The need for higher ethanol production is because of an increase number of vehicles per citizen, booming infrastructure, high prices for the depleting fossil oil, and desire of the politicians to become independent of the foreign oil suppliers (Edenhofer, 2011).

For economic production, the biofuel production should use lignocellulosic materials to produce ethanol. However, the nature of the lignocellulose is complex and resistant to the enzymatic attack. Therefore, the lignocellulosic biomass needs to be subjected to pretreatment. Pretreatment is a process where the structure of lignin, cellulose and hemicellulose is altered, in order to increase the surface area of the material, leading the same to an easier access to the cellulose and hemicellulose fibers. These fibers can, in turn, be hydrolyzed by cellulases to monosaccharides. Glucose, and now also xylose, are the two most important carbon sources for microbial fermentation where the metabolic end product is ethanol.

The main obstacle to the cellulolytic conversion of cellulose to glucose is the presence of lignin and lignin-derived phenolics that are released after pretreatment and which tend to retard the enzymatic conversion (Palonen *et al.,* 2004; Selig *et al.,* 2007). Despite the significant progress, recently been made with respect to improving the cellulolytic enzyme blends and minimizing the cellulolytic enzyme adsorption to lignin by development of integrated pretreatment systems for removing the lignin from pretreated biomass, there is a surprising scarcity of work on enzymatic modifications of lignin and lignin-derived compounds to improve lignocellulose processes (Koo *et al.,* 2012; Yang *et al.,* 2012).

In nature, there exist microorganisms that can efficiently degrade lignin in wood. These microorganisms, namely *Pestalotiopsis sp.* fungi which are proven to be the best lignin degraders, can completely devoid wood of lignin. Their enzymes could therefore be of a big value for further improvements of the cellulolytic enzymes blends for the biofuel production and make the cellulose-to-glucose conversion less laborious.

Enzyme is one of the most promising products from industrial biotechnology. In many biocatalyst processes, enzyme acts as a catalyst to speed up specific chemical reactions. Enzyme is made from sequence of amino acids with different three-dimensional structures which gives the molecule unique properties. Several enzymes have broad substrate

specificity which has been exploited in several biotechnological applications. In the current days, modernization has resulted in more problems related to the environmental problems. Large number of pollutants keeps increasing as a result of industrial activities. The used of enzyme in solving environmental problems was due to costs effectiveness which are characterized by higher catalytic ability, wide range of substrate, less toxicity, mild reaction conditions, etc. (Alcalde *et al.,* 2006).

Since nineteenth century, laccase has been the subject of study and interest in biotechnology mainly due their ability to oxidize a wide range of aromatic compounds and non-aromatic compounds by a radical-catalysed reaction mechanism. From previous studies, laccase has been found to degrade phenolic compound. The wide range of substrate specificity and strong oxidative activity has made laccase enzyme become more important compare to other oxidative enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP). Due to its numerous advantages, the mass production of the enzyme in order to fulfill the needs of industrial sector is one of the main thrust for many biotechnological companies. One way to produce laccase is through the fermentation process using microorganism such as white rot fungi. Renewable and environmental friendly materials have been used as nutrients and substrate during enzyme production. One of the limiting factors that influence the large-scale fungal laccase productions is the lack of efficient system at production scale. Optimization of fermentation parameters is required for maximum laccase productivity. This can be archived by using statistical experimental design which can provide us the knowledge and better explanation of important parameters involved in the process.
This paper studied the optimized production of laccase from potent microbe and characterization of laccase enzyme. Its applications ranges from detoxification to pulp bleaching, dye decoularization, bioremediation, industrial waste water treatment etc.

1.2 Rationale

Lignocellulosic biomass has great importance in biofuel production, paper and pulp industry, 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 pulp bleaching, prevention of wine discoloration, waste decolorization and detoxification, bioremediation, biosensors, biofuel cells etc. Laccase has been the subject of research since 19th ntury and still potent laccase is being searched continously. 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 till now isolated 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.

1.3 Research Hypothesis

The hypothesis of this study is that fungal species of Nepal may have adapted in different way and some of 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 to optimize the production of laccase enzyme from potent microbe and characterization of purified enzyme.

1.5 Specific Objectives

The specific objectives are as follows

- Isolation and screening of potent laccase producing microbes,
- Molecular characterization of potent microbe,
- Optimization of laccase production from the isolated microbes.
- Partial purification and characterization of laccase.

CHAPTER II: LITERATURE REVIEW

2.1 Composition of lignocellulosic material

2.1.1 The Plant Cell Layers

The plant cell wall consists of several layers (Fig. 2.1.1 A), which include; middle lamella (ML),primary wall (P), inner layer of the secondary wall (S1), middle layer of the secondary wall (S2), inner layer of the secondary wall (S3), and wart layer (W) (Sjöström, 1993). These walls provide mechanical strength, but also have ability to expand allowing the plant cells to grow and divide.

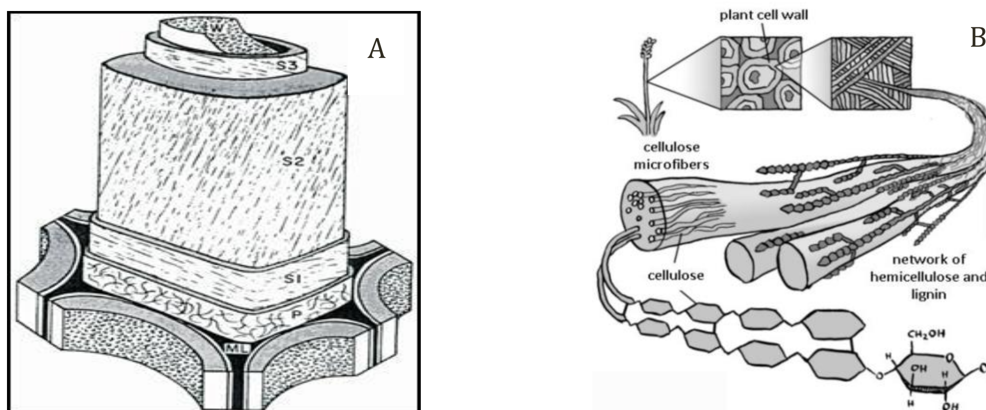


Figure 2.1.1: Representation of the plant cell walls; A) Wood cell structure showing the middle lamella (ML), the primary cell wall (P), and the secondary cell wall comprising of three layers (S1, S2, S3) (Côté, 1967); B) The primary cell wall with cellulose, hemicellulose and fibers, embedded in lignin (Rosgaard et al. 2005).

The *middle lamella*'s function is to cement two adjoining cells of the cell wall together. Early in the growth development of the plant, it mainly contains pectic substances which eventually become highly lignified (Alberts *et al.,* 2008). The *primary wall* is generally thin (0.1-0.2μm), flexible, and extensible layer formed while the cell is growing. It mainly consists of cellulose, hemicelluloses and protein and is completely embedded in lignin (Fig. 2.1.1 B). The *secondary wall* is a thick layer formed inside the primary wall, after the cell is fully grown. It is built of three layers namely S1, S2, and S3 (as described above), which consist mostly of cellulose microfibrils wounded around the plant cell wall in a neatly organized matrix of hemicellulose and lignin with different orientations in each layer (Sjöström, 1993; Krogh, 2008). The warty layer is a thin amorphous membrane located in the inner surface of the cell wall in all softwoods and hardwoods, containing deposits of still unknown, but characteristic for each species, composition (Sjöström,1993).

2.1.1.a Cellulose

Cellulose is a linear polymer of D-glucose monomers, linked by β-1,4-glucosidic bonds in which every second residue is rotated 180° around its longitudinal direction (Fig. 2.1.1 a). The linearity of the cellulose chain is stabilized through inter- and intramolecular hydrogen bonds (Fig. 2.1.1 a) (Gardner and Blackwell, 1974). Typically, 36 cellulose chains, with 10,000 D-glucose molecules each, having the same direction but a different starting and ending point, assemble in one Microfibrils (5-15 nm in diameter) (Krogh, 2008). The microfibril is greatly stabilized through inter and intramolecular hydrogen bonds, which prevents cellulose from the microbial degradation and penetration by as small molecules as water (Lynd *et al.,* 2002). From the structural point of view, micro fibrils are heterogeneous in structure and comprise of highly ordered (crystalline) regions, which are alternated by less ordered (amorphous) regions (Sjöström, 1993). The amorphous regions of cellulose also have weaker intrermolecular hydrogen bonds. Microfibrils build up fibrils and finally fibers (Fig. 2.1.1 b). In addition to crystalline and amorphous regions, cellulose fibers contain various types of irregularities, such as kinks, twists of microfibrils or voids such as surface micropores, large pits, and capillaries (Lynd *et al.,* 2002). In general, cellulose gives rigidity, but to confer the rigidity, e.g. to a stem, there is a need for other materials that can stick and glue the polysaccharides together. The stickiness is caused by hemicellulose and the glue is lignin (Krogh 2008).

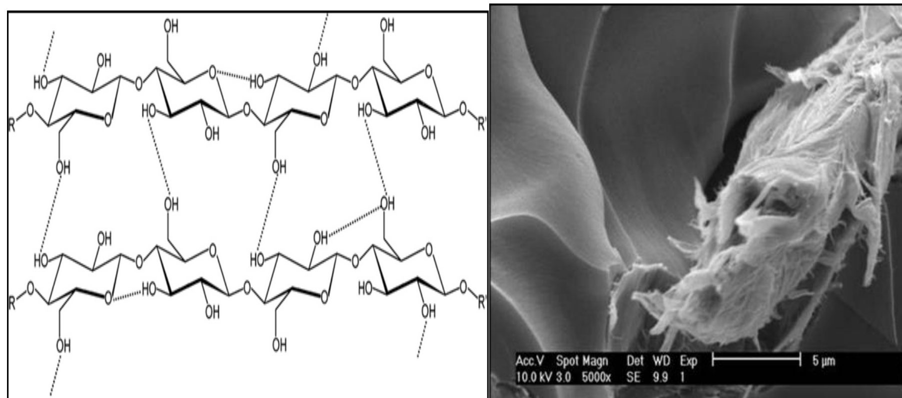


Figure 2.1.1a (i) Inter- and intra-molecular hydrogen bonds in cellulose; (ii) SEM image of the cellulose fibers, available athttp://www.personal.psu.edu/tjr5043/extened%20text/index.html

2.1.1.b Hemicellulose

Hemicellulose, in contrast to cellulose, is a heterogeneous polysaccharide and functions as an interlock to the cellulose microfibrils. Hemicellulose can be a branched chain polysaccharide of both different hexoses and pentoses. The hexose monomers are mainly D-glucose, D-mannose, D-galactose, and the pentose monomers are mainly D-xylose and L-arabinose (Krogh, 2008). Another monomeric component that is also present in the hemicellulose is uronic acid (Sjöström, 1993). The hemicelluloses from different types of wood, e.g. softwood and hardwood, differ both in structure and amount. In hardwood (angiosperm tree), e.g. birch, beech, poplar, aspen or oak, the predominant hemicellulose type is glucuronoxylan (O-acetyl-4-Omethylglucurono- β-D-xylan). On the other hand, softwood (gymnosperm tree) e.g. spruce, pine or fir is mostly (20%) composed of galactoglucomannan (Sjöström, 1993). In the grass family (poaceae), e.g. rice, wheat, oat and switch grass is mainly glucoarabinoxylan as in hardwood, but in contrast the substituting glucouronic acid is not methylated (Carpita, 1996).

Table 2.1.1b: The major hemicellulose components

Hemicellulose type	Occurrence	Amount (% of wood)	Units	Composition Molar ratio	Linkage
Gallactoglucomannan	Softwood	5-8	β- D-Manp β-D-Glcp Acetyl β-D-Manp	3 1 1 1 4	1→4 1→4 1→6
(Galacto)glucomannan	Softwood	10-15	β—D-Glcp α-D-Galp Acetyl β-D-Xylp	1 0.1 1 10	1→4 1→4 1→6 1→4
Arabinoglucuronoxylan	Softwood	7-10	4-O-Me-α-D-GlcpA α-L-Araf β-D-Xylp	2 1.3 6	1→2 1→3 1→3 1→6
Glucuronoxylan	Hardwood	15-30	4-O-Me-α-D-GlcpA Acetyl β-D-Manp β-D-Glcp	10 1 7 1-2	1→4 1→2 1→4
Glucomannan	Hardwood	2-5	β-D-Glcp	1	1→4

β- D-Manp: β-mannopyranose, β-D-Glcp: β-D-Glucopyranose, α-D-Galp: α-D-Galactopyranose, β-D-Xylp:β-D-Xylopyranose, 4-O-Me-α-D-: 4-O-Methyl-α-D-Glucuronic Acid, α-L-Araf:α-arabino-furanose.

2.1.1.c Lignin

Lignin derives from a Latin word *lignum* which means wood. Lignin is an integral part of secondary cell walls and its main function is to strengthen and conduct the water in the plant stems. Due to its hydrophobic nature and crosslinking with polysaccharides, lignin is a barrier for water to access the cell walls. Lignin is a heterogeneous biopolymer build of three phenylpropane units (Fig. 2.1..1.c.i); guaiacyl (from the precursor coniferyl alcohol), syringyl (from the precursor sinapyl alcohol), and *p*-hydroxyphenyl (from the precursor *p*-coumaryl alcohol).

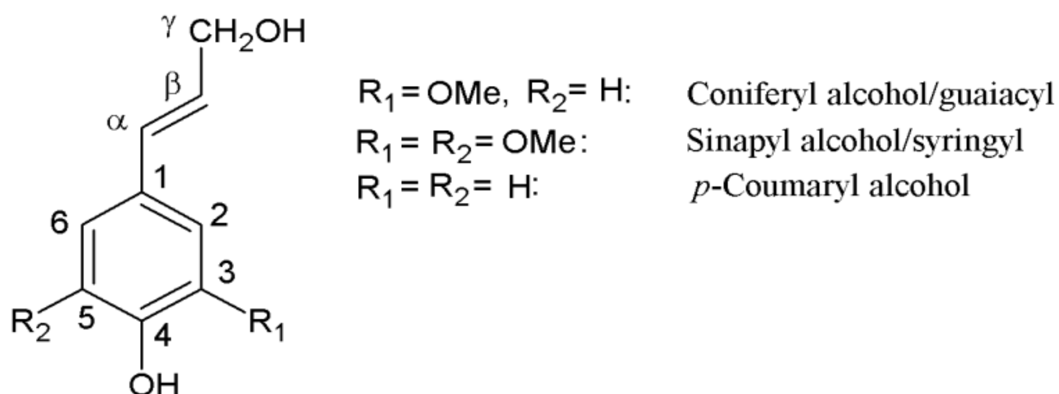


Figure 2.1.1.c.i: Schematic representation of three main monolignols forming a three-dimensional network of lignin.

Those three building blocks of lignin are generated from D-glucose through complex reactions catalyzed by various enzymes (Sjöström, 1993) and their amount differs significantly between hardwood, softwood and grasses. For example, there are 98% of guaiacyl units in softwood, *Cycas revoluta,* and 88% in *Podocarpus macrophyllus.* Softwood is almost completely devoid of syringyl units and instead contains *p*-hydroxyphenyl units (Gross, 1980). In contrast to softwood, hardwood contains a higher amount of syringyl units, whose content can vary from 40% (beech) to 84% (Eucalyptus globulus) (Choi *et al.* 2001; Pinto et al. 2005). The syringyl unit has two methoxy groups on the phenyl ring, whereas guaiacyl has one that confers different chemical properties and reactivity. One theory is that guaiacyl lignin keeps the lignin-cellulose more densely packed than syringyl lignin, which might be the reason for restricted hydrolysis of cellulose (Krogh, 2008). The radical coupling of the monomeric phenylropane units yield in a complex irregular matrix where the single phenylpropane units are joined together by variety of linkages. The model lignin presented by Adler (1977) consists of 16 phenylpropane units and it represents only a fragment of the lignin matrix (Sjöström, 1993). The high variability in the molecular structure of lignin is to a large degree due to the different resonance structures of the phenoxy radicals (Durbeej *et al.* 2003). The linkage type in lignin differs significantly in softwoods and hardwoods (Table 2.1.1b), however, the most common linkages are β-O-4, β-5, β-β, β-1, 5-5, and 5-O-4 (Fig. 2.1.4; Table 2.1.3).

Lignin has also been reported to bind to hemicellulose and probably also cellulose in the so-called lingo-cellulose- or lignin-carbohydratecomplex (Fenegel and Wegener, 1984; Sjöström, 1993) which might be responsible for structural enforcement of lignocellulose structure. The covalent binding between lignin and hemicellulose can be either of an ester or ether type (also glycosidic bonds are possible).

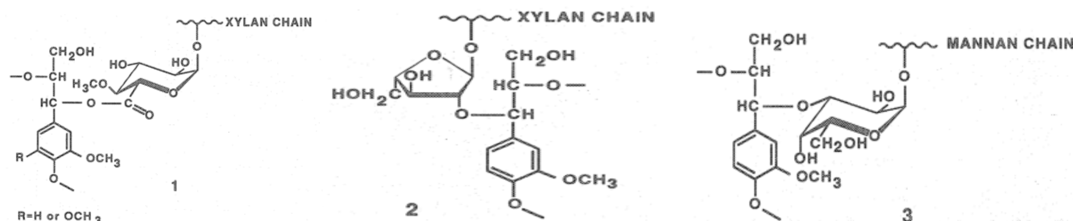


Figure 2.1.1.c.ii: Examples of suggested lignin-carbohydrate bonds; 1) an ester linkage to xylan through 4-O-methyl glucuronic acid; 2) an ester linkage to xylan through arabnofuranose unit; 3) an ether linkage to galactoglucomannan through a galactopyranose unit.

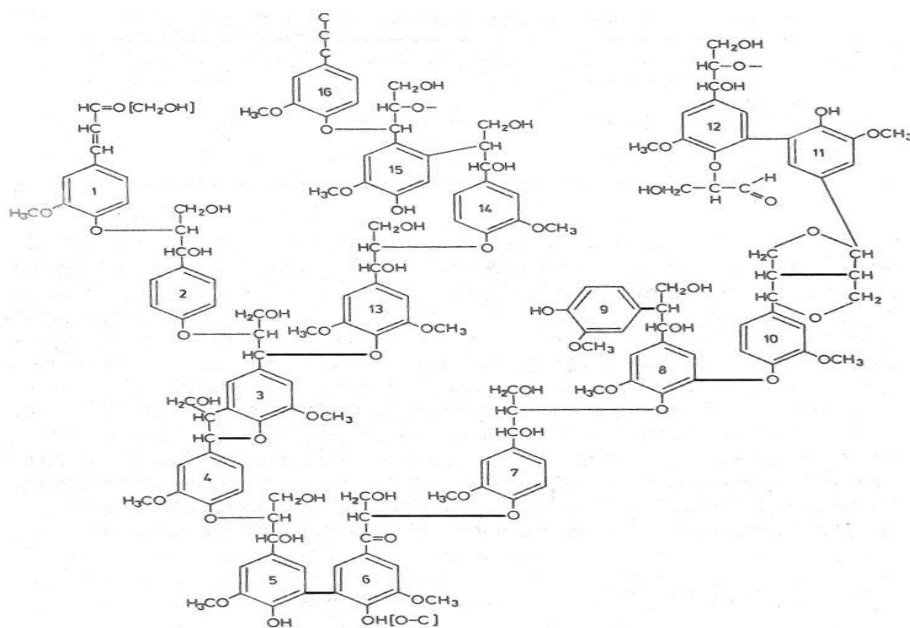


Figure 2.1.1.c.iii: Adler's (1977) fragment of softwood model lignin. The most common linkages are bolded black.

2.2. Lignin Degrading Organism

2.2.1 Actinomycetes as sources of novel laccases

Laccases from novel strains may possess different properties from those of the known laccases, and thus, the same starting compounds may be converted to different products by laccases with different properties. Comparisons between known and novel laccases may also lead to insights revealing, for example, differences in catalytic mechanisms or selectivity. Further, new laccases may not catalyse entirely novel bioconversion reactions but may still 6possess superior properties in terms of application, and these could then be used to replace existing enzymes with the aim of improving target bioconversion reactions. Laccases from plants and fungi (both eukaryotes) have been extensively studied. However, laccases from prokaryotes, including the actinomycetes bacteria, have only recently been discovered and are currently the subject of much research interest. New applications can be derived from such bacteria, and comparisons with fungi can be used to identify advantages of using actinomycetes and their laccases.

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 comparison 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 represses enzyme synthesis and hence fungi can degrade low concentration of polymer as well.

2.2.2 White-rot Fungi

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 (WRF) lignin either selectively or non-selectively (i.e. Simultaneous decay) which may be controlled bynutritional factors. Organisms like

Ceriporiopsis 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 superoxie 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.

2.2.3 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. They can grow on both coniferous and hardwood species. It has been found to produce antitumor and hypoglycemic polysaccharides, and immune-modulatory 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); Ding et al 2012); however, the molecular weight of laccase was determined to be 43 kDa by other investigators (Murugesan *et al.,* 2007).

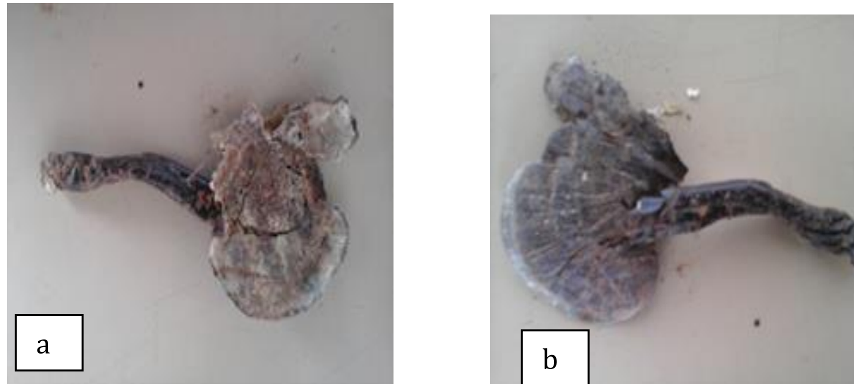


Fig. 2.2.3.*Ganoderma lucidum* fruiting bodies taken from Nepal Academy of Science and Technology (NAST); a) ventral surface, b) dorsal surface,

2.2.4 Brown-rot Fungi

Serpula lacrymans, Gloephyllum trabeum, Polyporus ostreiformis and *Meruliporia incrassate* are brown rot 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). 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 whereas 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).

2.2.5. 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).

2.2.6. 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 mono-aromatic 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 lignin carbohydrate-complex and a variable amount is digested, although the biochemical pathways are unknown (Susmel and Stefanon, 1993).

2.3. Classification of Polyphenol oxidase according to Substrate Specificity

Laccases (EC 1.10.3.2) is a blue copper protein, but it also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature that they are able to oxidize aromatic compounds with molecular oxygen as the electron acceptor (Mayer 1987).
Polyphenol oxidases are associated with three types of activities (Mayer 1987).

a) Catechol oxidase or o-diphenol: oxygen oxidoreductase (EC 1.10.3.1)
b) Laccase or p-diphenol: oxygen oxidoreductase(EC 1.10.3.2)
c) Cresolase or monophenol monooxygenase(EC 1. 18.14.1)

The different enzymes can therefore be differentiated on the basis of substrate specificity (Walker and McCallion 1980). There is, however, difficulty in defining laccase according to substrate specificity, because laccase has an overlapping range of substrate with tyrosinase. Catechol oxidases or tyrosinases have o-diphenol as well as cresolase activity(oxidation of L-tyrosine). Laccases have both ortho and paradiphenol activity, usually with more affinity towards the second group. Only tyrosinases possess cresolase activity, and only laccases have the ability to oxidize syringaldazine (Thurston 1994; Eggert et al. 19996). Another difficulty in defining laccase according to substrate is that the substrate range varies from one organism to another. *Neurospora crassa* laccase (German et al.1988) only effectively oxidizes para and ortho-diphenols with the exception of phloroglucinol, while laccase from *Pyricularia oryzae* preferred phloroglucinol as a substrate other substituted monophenols. Laccases from

Cerrena unicolor and *Trametes versicolor* oxidize meta-substituted phenols, but to varying degrees. Laccase from *Cerrena unicolor* oxidizes para-substituted phenols to the greatest extent (Filazzola et al. 1999), while *Trametes versicolor* laccase ortho-substituted phenols to the greatest extent (Jolivalt et al. 1999). An immobilize commercial laccase was able to degrade meta, ortho and para-substituted methoxyphenols, chlorophenols and cresols but the substituted phenols from these three types of phenols are oxidized in different orders and to different extents (Lante et al. 2000). Reports suggest that many different reactions have been catalyzed by laccases from different fungi. A comparative study concerning properties of fungal laccases indicated that all the laccases in the study had the ability to oxidize methoxyphenolic acids, but to different degrees and the oxidation efficiencies were also dependent on pH (Bollag and Leonowicz 1984). Phenolic compounds that were oxidized very slowly by laccase have recently been used to increase the storage stability of laccase activity for *Trametes versicolor* (Mai et al. 2000). The increassed stability of laccase could have technological importance, as there are so many potential applications of laccase.

2.3.1 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 favored 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 ascomycetes 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).

2.3.2. 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), S*treptomyces 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 coA laccase from *Bacillus subtilis* endospore (Enguita *et al.,* 2003; Enguita *et al.,* 2004)

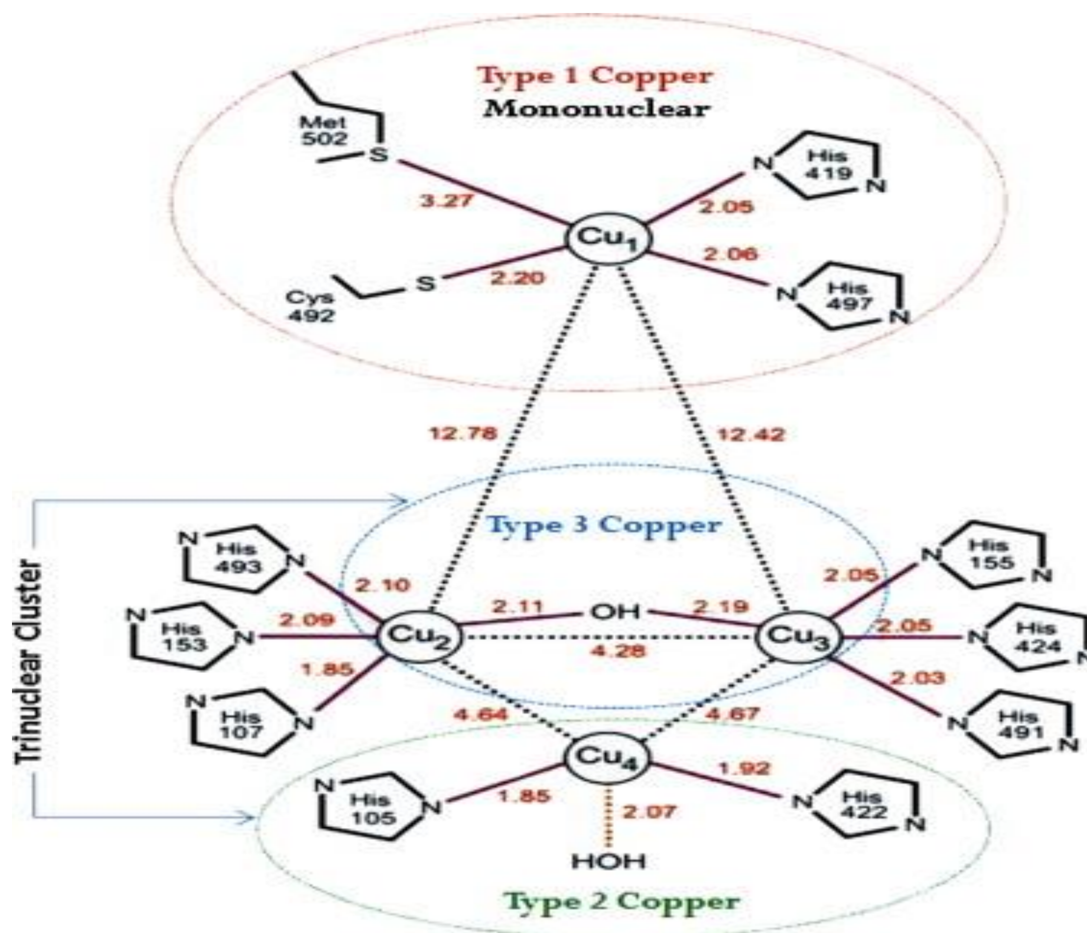


Fig 2.3.2a: Molecular Structure of Laccase

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 (Yoshtake *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). 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 600-610nm. The T1 and T3 Cu are linked mainly by His-Cys-

His tripeptide, whose Cys ligates the T1 Cu and His ligate two T3 Cu, and the T2/T3 Cu is 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 $N\delta 1$ nitrogen of two histidines. In other blue proteins like ascorbate oxidase, azurin and plastocyanin there is additional axial ligand in the T1 center 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 amino acids 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: Solomon et. al., 1996). 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.

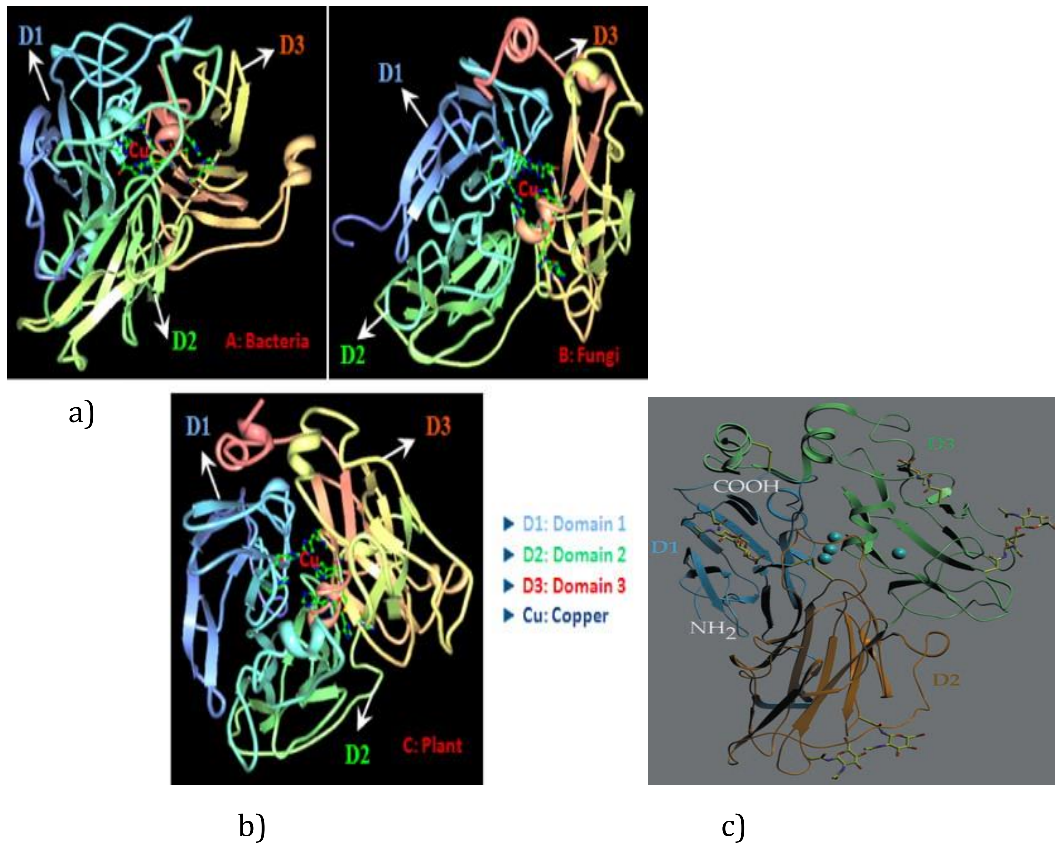


Fig. 2.3.2b: Three dimensional structure of (a) bacterial laccase (***Bacillus subtilis***), (b) plant laccase (***Populus trichocarpa***; and (c) fungi laccase (***Tramates versicolor***). Bacterial laccases have larger binding cavity as compared to those of plant and fungi. ▸ Differences in residues of copper binding site are the basis for their diverse functions (source : (Dwivedi *et al.,* 2011)).

2.3.3. 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 tri-nuclear 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.

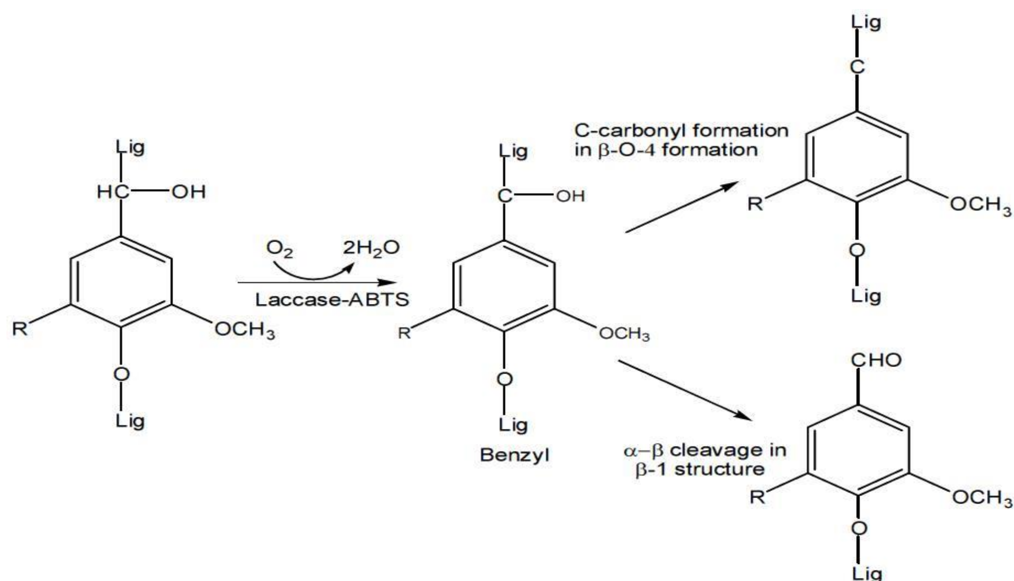


Fig 2.3.3a. Oxidation of phenolic subunits of lignin by laccase

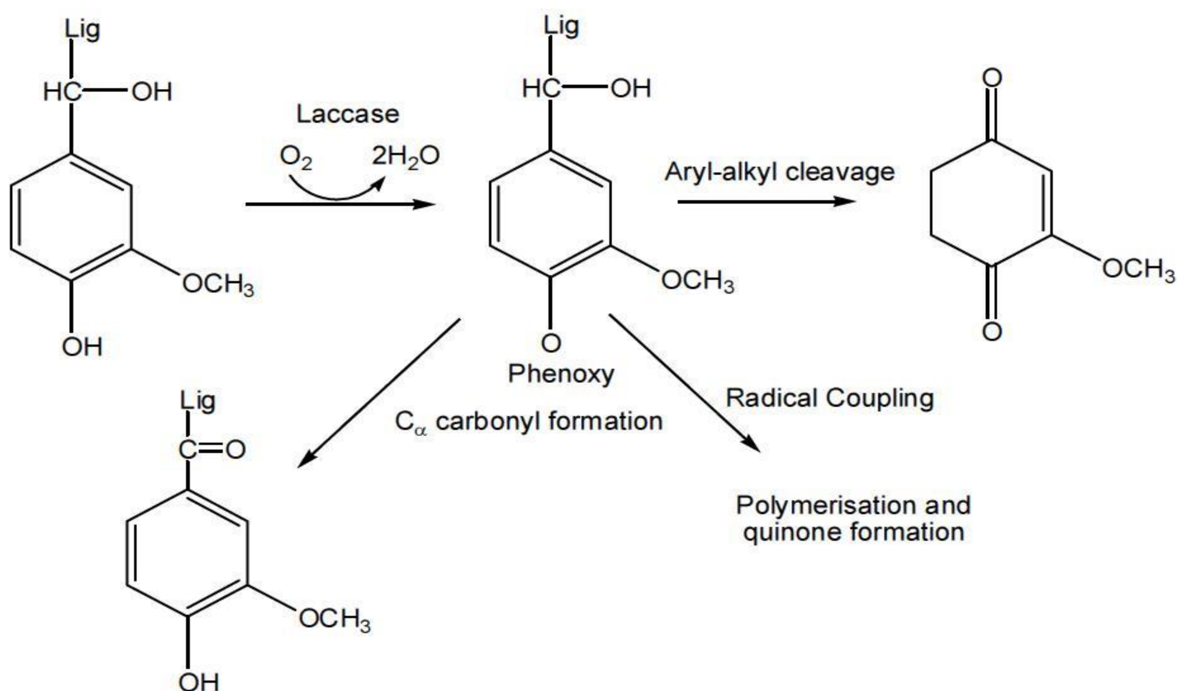


Fig 2.3.3b. Oxidation of non-phenolic lignin model compounds of lignin by laccase

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 form 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 benzyl-alcohols 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).

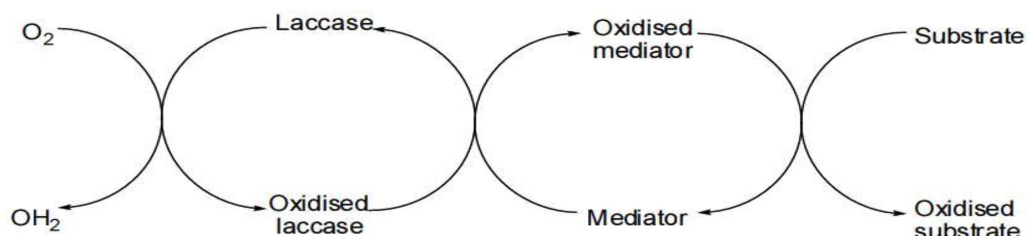


Fig.2.3.3c.. Role of mediator in laccase mediated catalysis (reproduced from (Banci *et al.,* 1999).

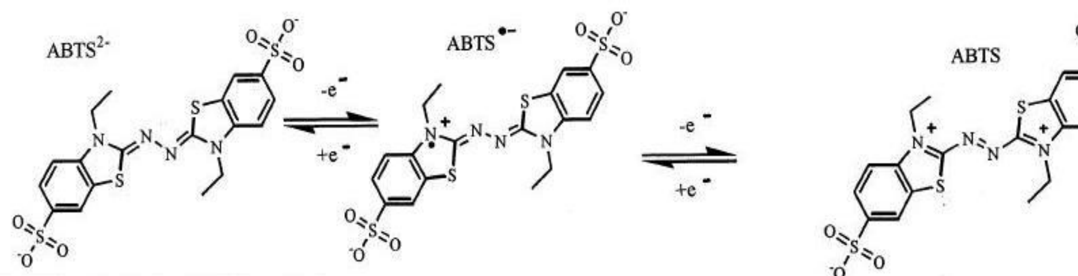


Fig.2.3.3d : Oxidation of ABTS

2.4. Production of fungal laccases

2.4.1. 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.

Cu^{++} 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).

2.4.2. 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 centers. 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).

2.4.3. 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 immobilize 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-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).

2.4.4. Inhibition of laccase production

In general, laccases responds similarly to several inhibitors of enzyme activity (Bollang and Leonowicz, 1984). 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^{++}), fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, deferral and cationic quaternary ammonium detergents, the reactions with which may involve amino acid residue modifications, conformational changes or Cu chelation (Bollang and Leonowicz 1984).

2.4.5. Effect of Metal ions on laccase production

The addition of low concentrations of copper to the cultivation media of laccase producing fungi (Assavanig *et al.,* 1992) or cadmium (Baldrian and Gabriel, 2002) stimulates laccase production. Addition of 150 μM copper sulphate to the cultivation media resulted in a fifty-fold increase in laccase activity compared to a basal medium (Palmieri et al. 2000). Palmieri et al. found that the copper ions may increase laccase production. As Cu+2 forms an integral prosthetic group within the enzyme (Baldrian and Gabriel, 2002; Dittmer *et al.,* 1997), or by increasing laccase mRNA translation (Collins and Dobson, 1997).

2.4.6. Effect of surfactant on laccase production

The addition of detergents, e.g. Tween 20 or 80 has resulted in higher yields of ligninolytic enzymes in certain fungi. There is evidence that these detergents result in higher permeability of oxygen and extracellular enzyme transport through the cell membranes of fungi (Leštan *et al.,* 1994; Rothschild *et al.,* 1995). Effective induction of laccase from *Pleurotus floridae* with anionic and cationic surfactants has been demonstrated (Dombrovskaya and Kostyshin, 1996).

2.4.7. Fermentation of ascomycetes in liquid cultures

Filamentous fungi, to which many of the basidiomycetes and ascomycetes belong, can grow in liquid culture either in a free form of loose mycelial hyphae or in an aggregated form, called mycelia aggregates or mycelial pellets (Braun and Vecht-Lifshitz 1991, Paul and Thomas 1998, Cui et al. 1998). Normally, on the laboratory scale, flasks are used for the liquid cultivation of fungi either as standing or shaken cultures. On the technical and industrial scale, the microorganisms are grown in vessels called bioreactors (fermenters) which can differ in their mode of mixing. Accordingly, they are referred to as airlift reactors, stirring fermenters or bubble columns. The advantage of the agitated systems over the standing culture is the higher aeration rate and, therefore, better availability of oxygen. In standing cultures, mycelial mats do form at the upper surface of the liquid medium due to a lack of mixing, which would bring the mycelium into the liquid. In comparison, in agitated cultures different mycelial forms can occur. Several factors have an influence on the type of growth in the agitated cultures, such as strain, growth rate, inoculum size, cell-wall

composition, nutrition, C/N-ratio, Cu^{2+} concentrations, pH, temperature, dissolved oxygen, solid particles inside the culture broth, fermentation time, surface-active agents, aeration, agitation or stirring speed, respectively shear forces, bioreactor construction and parameters related to the fermenter design, such as foaming (Braun and Vecht-Lifshitz 1991, Cui et al. 1998, Papagianni 2004, Fazenda et al. 2008). Some factors such as inoculum, oxygen, vessel and impeller type, as well as modes of cultivation will be discussed in the following regarding their effect on fungal biomass, the morphology of the cultivated fungi and yields of different metabolic products (enzymes, acids).

Generally, the production of secondary metabolites (i.e. citric acid, enzymes, and antibiotics) and recombinant production of proteins (i.e. glucoamylase, human interleukin 6) is of great interest. Liquid cultivation studies on fungi concentrate on ascomycetes capable of high product yields for natural production of secondary metabolites and recombinant proteins (Radzio and Kück 1997, Punt et al. 2002, Papagianni 2004, Grimm et al. 2005). Thus, most of the research on fungal morphology was done for filamentous ascomycetes, such as Aspergillus and Penicillium species either in liquid cultivation in a laboratory scale in shake flask cultures or in a more technical scale in fermenters (Vanhoutte et al. 1995, Cui et al. 1997, Agger et al. 1998, McIntyre et al. 2001, Kelly et al. 2006, Lecault et al. 2007).

Due to the low growth rates and their special requirements for liquid cultivation, basidiomycetes are rarely described in the literature in terms of morphology in liquid cultures. Nevertheless, also basidiomycetes are applied in liquid cultures for production of glucans, peroxidases or laccases (Fenice et al. 2003, Boh and Berovic 2007). Herein, the probably most important parameters on fungal morphology are the stirring speed of the impeller in the stirred vessel reactor, the aeration amount, the pH of the medium and the cultivation temperature (Braun and Vecht-Lifshitz 1991, Cui et al. 1997, Papagianni 2004, Fazenda et al. 2008).

Generally, shake flask cultures are used for optimization of culture conditions, such as media composition, cultivation temperature and pH, prior to technical scale cultivation. The information given in the available literature references only on pellet sizes and, so far, effects by the morphology of the fungi were in the studies mostly neglected (Gehrig et al. 1998, Fang and Zhong 2002, Žmak et al. 2006). An area in which basidiomycetes play an important role is the solid-state fermentation (SSF). Most basidiomycetes possess a complex enzymatic system for the degradation of lignocellulosic waste materials which may serve as solid substrates. Therefore, SSF provides filamentous organisms with a more natural growth environment and higher oxygen availability (for advantages and disadvantages of SSF). With regard to recombinant protein production, liquid cultivation might be more favorable due to a more easy process of protein purification compared to SSF with lignocellulosic materials, where phenolic substances and other degradation products contaminate the culture broth. This chapter intends to give a literature overview on liquid cultivation of ascomycetes and

basidiomycetes fungi regarding laccase production in bioreactors, morphological structures of the mycelium and parameters affecting the filamentous structure.

2.5. Important parameters of fermentation on fungal morphology and on production yields of enzymes

2.5.1. Inoculum

Cultivation of any microorganism starts with an inoculum. The type and amount of this inoculum for liquid cultures can vary, especially when filamentous organisms growing in multicellular hyphal forms are used. Submerged cultures of filamentous fungi can be inoculated with spores if available (Vecht-Lifshitz et al. 1990, Tucker and Thomas 1992) or with mycelium, which might be available in loose filamentous form (Tavares et al. 2005) or pelleted form (Carmichael and Pickard 1989, Thiruchelvam and Ramsay 2007). These forms, normally derived from a pre-culture, can be used directly without homogenization or after smashing the mycelial agglomerates into smaller fragments. When spores are used, the inoculum level depends on the spore stock preparation, germination of the spores (time and viability) and altering conditions influencing the germination of the spores, like medium, pH and aeration (Tucker and Thomas 1992, Paul et al. 1993).

Advantages of spore inoculi are the countability of the inoculum and, therefore, the reproducibility. On the other hand, the lag phase is longer in cultures where spores are used and a higher contamination danger exists. As most basidiomycetes do not produce asexual spores, filamentous inoculum are usually used. Exceptions, where asexual spores from basidiomycetes were used in liquid cultivation, are *Coprinopsis cinerea*, *Phanerochaete chrysosporium* (Leisola et al. 1985, Jiménez-Tobon et al. 2003, Žmak et al. 2006) and *Punctularia atropurpurascens* (Böker 1990).

2.5.2. Oxygen

Oxygen is a limiting factor for growth. When the fungus grows in a pelleted form, a reduction of O_2 inside the pellet can occur which leads to oxygen starvation and, therefore, to cell death and lower yields of enzymes or other metabolites (Michel et al. 1992). To balance this problem, a reduction of the pellet size or an increase of oxygen concentration can be applied. The latter one can be achieved when using pure oxygen instead of compressed air. Kirk et al. (1978) obtained higher lignin degradation activity on synthetic 14C-lignin at 100% oxygen saturation compared to air saturation (21% O2) in liquid *Phanerochaete chrysosporium* cultures in basal medium (defined medium with glucose as a C-course). Similar observations were made by Dosoretz et al. (1990) who reported an increase in ligninolytic enzyme yield in submerged *P. chrysosporium* cultures when aerating with pure

oxygen. However, there was a concomitant higher proteolytic activity and, in consequence, a faster degradation rate of the ligninolytic activity. In their oxygenated cultures, Dosoretz et al. (1990) observed that the *P. chrysosporium* pellets had a dark colour and a smoother surface compared to the pellets from cultures with "normal" aeration showing hairy-white pellets. To clarify the factor for higher ligninolytic enzyme yields in oxygenated cultures of *P. chrysosporium*, Michel et al. (1990) determined the oxygen concentration within fungal pellets. The authors used pellets derived from liquid shaken cultures, which were flushed daily with pure oxygen. The obtained data were used for generating a mathematic model, which allowed predictions of respiration kinetics in liquid cultures on the basis of pellet size and oxygen concentration inside the medium. The authors stated by applying their model on different studies with P. *chrysosporium* that oxygen limitations inside *P. chrysosporium* pellets can be compensated when using pure oxygen.

2.5.3. Vessel and impeller

The aeration of fungal cultures depends not only on the flow rate of compressed air, pure oxygen or other gases, but also on the quality of mixing of the broth. This dispersion is guaranteed by the design of the bioreactor and the used impellers, which are also affecting the mycelia morphology (Cui et al. 1998). On the other side, high biomass concentrations in the bioreactor vessel lead to non-Newtonian fluids (the viscosity of the fluid is not stable), lower oxygen supply and bad heat transfer within the bioreactor. Therefore, an adequate type of mixing is required for optimal fermentation conditions. However, with higher stirring and agitation rates an increased shear stress towards the hyphae, either in pelleted or filamentous form, emerges and, thus, is critical on fungal morphology.

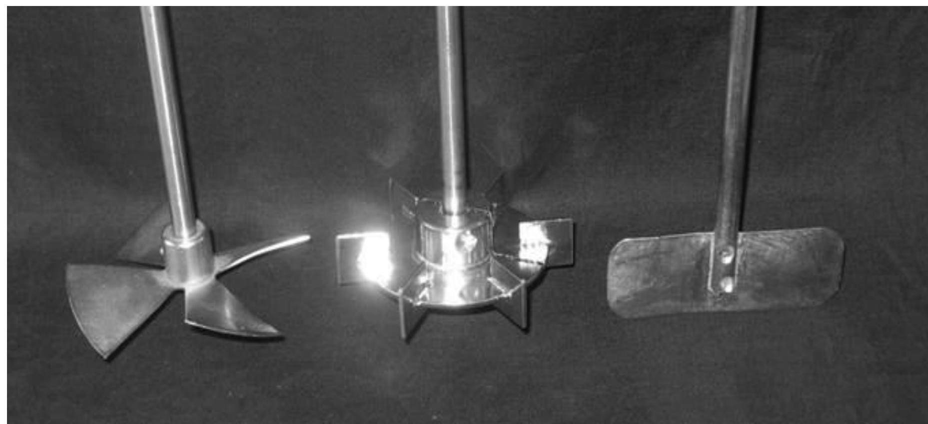


Fig. 2.5.3 Impeller types: pitched blade impeller (left), rushton turbine or disk stirrer (middle), paddle impeller or blade stirrer (right).

Next to the agitation speed, the impeller (Fig. 2.5.3) has a direct influence on the filamentous growth form of the organism: Paddle impellers and rushton turbines showed less damage to

freely dispersed *Penicillium chrysogenum* hyphae compared to pitched blade impellers (Jüsten et al. 1996). In bioreactor cultures of the basidiomycete *P. ostreatus*, a rushton turbine was compared with two other impeller types: a helical ribbon and an intermig impeller (see http://www.ekato.eu/en/products/ekato-rmt/products/ekato-impellers/ for the different impeller types) (Márquez-Rocha et al. 1999). At similar agitation speeds of the tested impellers, the size of fungal pellets varied in diameter with 2.3, 3.5 and 5.1 mm for cultures mixed with helical ribbon impeller, rushton turbine and intermig impeller, respectively. On the other side, the specific growth rate of *P. ostreatus* increased with decreasing pellet diameter (Márquez-Rocha et al. 1999). So far, this is the only study with different impeller types in basidiomycete culture, but a more general comparison between impeller and other type of agitation exists. In a stirred vessel of the type BioFloIIC (New Brunswick Scientific) with rushton turbines, the white-rot fungus *T. versicolor* grows in a pellet form with variable diameters of 0.5 to 3 mm, whereas a self-assembled magnetic stir bar reactor (MSBR) gave smaller pellets of more unique size (~1 mm) (Thiruchelvam and Ramsay 2007). This shows that impeller types have an direct impact on the fungal morphology, beside their main aim of mixing the culture broth.

Almost no studies on pellet morphology are available on large scale for ascomycetes and basidiomycetes, but lab scale studies can give an overview. Generally, it seems that with higher agitation rate of flask cultures the pellet diameter decreases, with diameters of 6.6 mm at 100 rpm, 1.0 to 3.5 mm at 100-200 rpm and below 1.0 mm with agitation rates higher than 200 rpm. In a study with the white rot fungus *Lentinus tigrinus*, pellet morphology was not discussed, but the mode of agitation (stirred vessel versus airlift-reactor) had an influence on the yield of enzyme. In the stirred vessel, an agitation rate of 250 and 500 rpm gave best results for laccase yields of 4.6 and 4.0 U/ml, respectively. The used aeration rate of 1.0 vvm gave highest laccase yields, whereas higher aeration increased fungal biomass, but decreased laccase activity (Fenice et al. 2003). In contrast, *L. tigrinus* showed higher MnP yields in bubble columns compared to stirred vessels (Quaratino et al. 2006). Lower shear forces and better oxygen supply in the bubble columns might be the reason for higher enzyme yields, but it shows also that probably for each fungus a new approach is needed for defining best fermentation conditions.

When looking more closely at the pellet morphology, it is seen that the inner structure of the pellets might be influenced by agitation rate or stirrer speed. Evidence of this comes from shaken cultures of *P. chrysosporium* (Michel et al. 1990, 1992), in which pellet morphology depends on the agitation rate of the shaking flask. Cross sections of pellets obtained at lower speed (100 rpm, pellet Ø 6.6 mm) showed a hollow centre with an outer shell of about 1 mm. Higher agitation rates of 150 and 200 rpm resulted in smaller pellets between 1-2 mm in diameter and, concomitantly, there was an increased enzymatic yield with an optimal extra-cellular LiP activity of 341 and 376 U/l, respectively (Michel et al. 1990).

Also the working volume, which is defined as the volume of medium per vessel volume and given in percentage, has an impact on fungal morphology in flask cultures, whereas larger culture volumes seems favorable over small ones as stated by Leisola et al. (1985), who used a culture broth to flask factor of 0.3.

2.5.4. Mode of cultivation

The process of submerged cultivation can be classified into discontinuous (batch and fed-batch) and continuous cultures. In batch and fed-batch cultures the product (metabolite or biomass) is harvested at the end of the process, whereas in continuous mode the culture is kept at a steady state and harvest takes place continuously. Most of the cultivations are done in batch and sometimes in fed-batch mode, but very rarely continuously. The complexity of the problems for fermentation of filamentous organisms is even higher in continuous cultures than within normal batch operations (Papagianni 2004). An example for a continuous cultivation system is given by Yao et al. (1995), who cultivated the basidiomycete *Arthromyces ramosus* for peroxidase production. The authors used a stainless steel mesh with a specific size to retain the mycelium of *A. ramosus* inside the stirred vessel bioreactor just above its elutriation point, where the mycelial biomass would be washed out from the fermenter. A comparison between two mesh sizes (1 mm and 2 mm) and a normal continuous culture, where the culture broth was abstracted from the bioreactor without a mesh, revealed that the mesh with a size of ~ 2 mm was most adequate for *A. ramosus* cultures. In the continuous culture with a mesh size of 2 mm, the production rate of the peroxidase increased up to 3.2 times compared to the fed-batch culture and this rate was kept for over 100 h (Yao et al. 1995). The fine mesh (1 mm) retained almost all mycelia and, therefore, leads to high biomass yields but low productivity. In continuous cultures without application of a mesh, the mycelium was washed out from the fermenter. By altering the mesh size, this technique could be applied to other filamentous organisms, having a different morphological pattern in submerged agitated cultures.

The overview from literature data given here showed that several factors in liquid fermentations have an influence on fungal morphology and, therefore, on the productivity of enzymes of interest. Some factors, such as vessel and impeller type, are defined by the construction of the bioreactor. Some cultivation parameters, such as the inoculums size, are defined before the submerged fermentation start. Others, such as oxygen concentration, pH and temperature, change during cultivation. Generally, these latter parameters can be automatically controlled in bioreactors in contrast to shake flask cultures, where controllable parameters are normally restricted to temperature and agitation. Control of fermentation parameters requires standard methods for observation of the filamentous growth, which can help to clarify the relation between fungal morphology and growth conditions. Most of the available studies focus on the morphology of filamentous ascomycetes and only some studies

reveal an insight into the filamentous growth of basidiomycetes in submerged cultures. More studies on the fermentation of basidiomycetes are needed to understand their morphological behavior in shake flask cultures, stirred bioreactors or other fermentation vessels (Fazenda *et al.,* 2008).

2.6. Heterologous expression

One of the serious problems 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 oryzae* (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 pastoris* (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. 2.6: Laccase production in heterologous hosts. (Kunamneni *et al.,* 2008)
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 fermenter.

Laccase gene	**Production host**	**Laccase Production (mg l^{-1})**
Ceriporiopsis subvermispora lcs-1	*Aspergillus nidulans* *Aspergillus niger*	1.5 1.5
Coprinus cinereus lcc1	*Aspergillus oryzae*	135
Melanocarpus albomyces lac1	*Trichoderma reesei*	920
Myceliophthora thermophila lcc1	*Aspergillus oryzae* *Saccharomyces cerevisiae*	19 18
Phlebia radiata lac1	*Trichoderma reesei*	20
Pleurotus sajor-caju lac4	*Pichia pastoris*	4.9
Pycnoporus cinnabarinus lac1	*Pichia pastoris* *Aspergillus niger* *Aspergillus oryzae* *Schizophyllum commune*	8 70 80 1200

2.7. Isoforms of laccase

Laccase producing organisms produce different isoforms of enzyme depending upon the species and culture conditions in which they 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 2.7.

Table. 2.7 Number of isoforms of laccase produced in different fungus (Madhavi and Lele, 2009)

Organism	Maximum number of isoenzymes	Molecular mass(kDa)
Trametes multicolor	5	63
Trametes trogii	2	70
Ganoderma lucidum	3	65-68
Pycnoporus cinnabarinus	1	81
Coriolus hirsutus	1	73
Ceriporiopsis subvermispora	2	71 68
Trichoderma	1	71
Trametes sanguine M85-2	1	62
Ganoderma lucidum	2	40 68
Chaetomium thermophiliu	1	77
Chalara paradoxa CH 32	1	67
Pleurotus ostreatus	1	59
Coriolus hirsutus	1	55
Polyporus versicolor	2	60-65
Botrytis cinerea 61-34	1	74
Monocillium indicum	1	72
Neurospora crassa	1	65
Podospora anserine	3	70 80 390

2.8. Substrates for Detection of Laccase Activity

A special care needs to be undertaken when detecting laccase activity in the fungal crude protein extracts due to the reasons mentioned below:

i) nature of laccase substrates, most of them are phenol homolog that are or might be carcinogenic,
ii) presence of oxidases that produce H_2O_2 which may auto-oxidize laccase substrates,
iii) presence of peroxidases (lignin and manganese peroxidase), that catalyze H_2O_2 – dependent oxidation of laccase substrates,
iv) ability of laccase substrates to be oxidized by catalyze, that instead of removing H_2O_2, acts as a peroxidase and uses H_2O_2 to oxidize laccase substrates,

Table 2.8 Substrate used for detection of laccase activity

Substrate	Advantages	Disadvantages
ABTS 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	• Non toxic • H_2O soluble (at a concentration of 10-50μM) • Immediate oxidation reaction after addition of laccase • Suited for laccase detection in the crude protein extract in the absence of catalyses, peroxidases and H_2O_2 • Not reactive with tyrosinase	Oxidized by catalyse, during removal of H_2O_2 Not suited for laccase detection in the in crude protein extracts inpresence of catalyses and H_2O_2 or peroxidase, and high level of H_2O_2 Auto-oxidation with high H_2O_2
Syringaldazine	• Soluble in methanolic or 95% ethanol • Rapid color formation at all pH levels • Suited for laccase detection in the crude protein extract in the presence of catalyses, and low level of H_2O_2 • Not reactive with tyrosinase • Not reactive with H_2O_2 alone	Toxic Color tends to fade rapidly outside the pH 3 to 7 Not uited for laccase detection in the crude protein extract in the presence of lignin peroxidase and H_2O_2(high level) and Mn^{++}, Manganese peroxidase and H_2O_2
MBTH/DMAB 3-methyl-2-benzothiazolinone hydrazine/(dimethyl amino)benzoic acid	• H_2O soluble • High rate of reaction (only slightly lower than in case of ABTS) • Color stable, fades slowly over several hours	Toxic Requires treatment with catalyse prior to laccase detection (oxidation by lignin and manganese peroxidase at low H_2O_2 concentrations) In presence of Mn^{++} and 0.05mM H_2O_2 is used for manganese peroxidase activity

2.9. Laccase as a Lignin modifying enzymes

The degradation of lignin in the wood is seen upon as a complex process involving different classes of enzymes. The purpose of this thesis is to introduce the known enzymes whose specificity of action may contribute to the depolymerization of wood, rather than their detailed description which can be found in a number of review papers (Bourbonnais and Paice; Martinez et al. 2005; Kersten and Cullen, 2007; Wang, 2009).

Lignin digestion is considered as an “enzymatic combustion” process, involving several oxidoreductases such as laccases, ligninolytic peroxidases and peroxide generating oxidases. The enzymes involved in lignin digestion are furthermore classified in four major groups, based on their EC number; lignin peroxidase (LiP; EC 1.11.1.14), manganese-dependent peroxidase (MnP; EC 1.11.1.13), versatile peroxidases (VP; EC 1.11.1.16), and laccases (EC 1.10.3.2). Based on their sequence and catalytic properties, the extracellular fungal LiP, MnP, and VP belong to class-II peroxidases (Wong, 2009). Moreover, the “enzymatic combustion” process is further enhanced by synergistic action of several accessory enzymes, which might include glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (veratryl alcohol oxidase; EC 1.1.3.7), pyranose 2-oxidase (glucose 1-oxidase; EC 1.1.3.4), cellobiose/quinone oxidoreductase (EC 1.1.5.1), and cellobiose dehydrogenase (EC 1.1.99.18) (Martinez et al. 2005; Kersten and Cullen, 2007; Wang, 2009).

For a long time laccase was not considered as an enzyme that could contribute to the ligninolysis due to the two aspects; its redox potential was too low, meaning that a laccase could only oxidize phenolic lignin structures, which contain less than 10% of the woody materials, and *P. chrysosporuim* that could delignify wood was suspected not to express laccase. Only research by Bourbonnais and Paice (1990) revealed that redox potential of laccase can be modulated by addition of an artificial laccase substrate, ABTS, which enabled a laccase catalyzed oxidation of non-phenolic lignin compounds (Eggert et al. 1997). Additionally Eggert (1996) demonstrated that this small redox tuning molecule can be also of natural origin, such as *Pycnoporus cinnabarinus* metabolite, 3-HAA (3-hydroxyanthranilate) (Eggert et al. 1996). The role of laccase in the wood decay was therefore contradictory and it was rather difficult to understand its exact contribution to the lignin-in-wood degradation. However in the course of study and during the literature search I came across a number of research papers that claim that laccase is essential for lignin degradation. The arguments for that hypothesis and additional features of laccase that make it an important enzyme during wood decay are presented below:

a) Laccase catalyzes oxidative reactions of lignin with atmospheric oxygen as an electron acceptor (in contrast to lignin peroxidase and manganese peroxidase that are dependent on a continuous supply of H_2O_2) (Martinez et al. 2005)

b) The combination of laccase with either LiP and/or MnP, in white-rot fungi, is a much more common combination of phenyloxidases than the LiP/MnP pattern found in *Phanerochaete chrysosporium* (Peláez et al. 1995; Tour et al. 1995; Eggert et al. 1997)
c) In some fungi, e.g. *Pycnoporus cinnabarinus* – a very efficient lignin degrader expressing only laccase and no LiP and/or MnP, the knock-out of the laccase gene unables the fungus to metabolize 14C ring-labeled DHP (dehydrogenative polymer). However, an addition of a purified laccase to the laccase-less mutant from *P. cinnabarinus* restores its lignin degradation ability (Eggert et al. 1997; Bermek et al. 1998)
d) Laccase is capable of oxidizing high molecular weight lignin compounds even though they are much too large to directly penetrate and donate electrons to the T1 copper site (Piontek et al. 2002;). The process is suspected to happen directly through a long range electron transfer process, since the T1 copper site is at least 7 Å away from the protein surface (Shleev et al. 2006)
e) Laccase in the presence (NHA; Palonen and Viikari 2004; HBT; Gutierrez et al. 2012) and absence (Jurado et al. 2009; Moilanen et al. 2011;) of the mediator is able to significantly detoxify the pretreated lignocellulosic materials (by up to 75%) and improve its glucose release yields (by up to 33%). The possible mechanism during laccase treatment of pretreated lignocellulosic materials is condensation and/or polymerization of low molecular weight compounds, which is seen upon as a possible role of laccase protection of the fungus mycelium from the toxic substances (Youn et al. 1995; Rochefort et al. 2002; Shleev et al. 2006)
f) Laccase is used for enzymatic detoxification of pretreated lignocellulosic hydrolysates from phenolic lignin degradation compounds, prior to fermentation processes and in turn yields higher ethanol yields than lignin peroxidase (Jönnson et al. 1998)
g) Laccase is a focus of much attention due to its wide range of applications in food industry (crosslinking of polysaccharides (Zaidel and Meyer, 2012), beverages, fruit juice processing, baking), paper industry (biobleaching and biopulping) bioremediation applications (waste water detoxification and decontamination), textile industry (dye decolorization, denim processing), personal and medical care applications, and biosensor and analytical applications (Madhavi and Lele, 2009)
h) Laccase is likely to contribute to the incipient decay of wood by brown-rot fungi *Postia placenta*, enabling it the access to polysaccharides (Wei et al. 2010).

2.10. Unusual properties of laccase

There are several reports of highly purified laccases that show different properties (Mayer and Staples, 2002; Giardina, *et al.,* 2009). There are purified laccases that are yellow or yellow-brown in colour, rather than blue (Leontievsky, *et al.,* 1997). Yellow and yellow-brown laccases, while having a copper content similar to the typical blue laccases, do not maintain their copper centres in the oxidized state in the resting enzyme under normal aerobic conditions (Leontievsky, *et al.,* 1997).

The fungus *Pleurotus ostreatus* produces a white laccase. This laccase contains only 1 copper atom per monomer instead of the usual 4, and furthermore, 2 zinc atoms and 1 iron atom are present per protein monomer (Palmieri, *et al.,* 1997). The suggestion that the enzyme belongs to the laccase family is supported by: The high degree of identity of the determined primary structures with the corresponding sequences of known laccases; the use of oxygen as the oxidative substrate; the lack of formation of H_2O_2 as a product in the catalysed reaction; and the standard pattern of substrate specificity displayed by this enzyme if compared with that of other known laccases. This laccase from *P. ostreatus* displays unique structural characteristics and is classified as a white laccase (Palmieri, *et al.,* 1997).

Laccases, and other four-copper oxidases, contain three domains: Domains one and three contain the copper sites, while domain two forms the substrate-binding cleft (Giardina, *et al.,* 2009; Molina- Guijarro, *et al.,* 2009). In contrast to this arrangement, the genome of *Streptomyces coelicolor*, an actinomycete, was found to encode a small, four-copper oxidase that lacks the second domain (Machczynski, *et al.,* 2004). The enzyme was named SLAC, for Small LACcase, because it lacks a domain and is thus much smaller than other laccases. It is the principal extracellular laccase produced by *S. coelicolor*. SLAC resembles the protein folds of *Coprinus cinereus* laccase, a fungal laccase, with >99% confidence (Machczynski, *et al.,* 2004). The T1 Cu site and the tri-nuclear cluster are intact. Despite lacking a domain, SLAC contains the normal complement of four copper ions, and is specific for the common range of laccase substrates. The enzyme is highly stable, and the maximum activity of SLAC at the unusually high pH of 9.4 suggests the suitability of the enzyme for industrial processes (Machczynski, *et al.,* 2004).

2.11. 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.

2.11.1 Industrial Application of Laccase

Due to its ability to oxidize various compounds at the expense of molecular oxygen, laccase has been used in various biotechnological applications. Compared to the conventional process, the reaction mechanism involving laccase exhibit more environmental friendly process with water as the only by-product. The used of laccase as biocatalyst in many conventional process which mainly using chemical process has significantly reduced the production cost. Furthermore the appearance of non-desired product due to the side reaction mechanisms from the conventional process can be avoided by using laccase as the enzyme has wide range of substrate.

2.11.2 Delignification and Pulp bleaching

In the delignification of lignocelluloses and their modification into new compounds like vanillin and guaiacol, 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)

2.11.3 Bioremediation

Bioremediation includes all procedures directed for biotransformation of a 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). Similarly 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).

2.11.4 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 laccase 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 scavenge oxygen can be of great importance in food and beverage industries.

2.11.5 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 described else where (Kunamneni *et al.,* 2008)

2.11.6 Pharmceutical 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)

2.11.7 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 [159,(Simkus *et al.,* 1996; Bauer *et al.,* 1999). Laccase catalysis have 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).

2.11.8 Industrial Wastewater Treatment

Biotechnological treatment of industrial effluents generated by printing and dye industry with laccase requires a large amount of enzyme. Treatment of the effluent with enzymes has attracted great attention in recent years (Daâssi *et al.,* 2012; Tišma *et al.,* 2012). Recently laccase from several microbial sources has been employed for the decolorization of various categories of dyes such as triphenylmethane, azo, indigoid, anthraquinone and heterocyclic/polymeric dyes (Couto and Herrera, 2006; Hadibarata *et al.,* 2012). The enzyme is also able to decolorize reactive dyes such as Remazol Brilliant Blue R (Soares *et al.,* 2001) and Remazol black-5 (Murugesan *et al.,* 2007). Decolorization of synthetic dyes by *Trametes modesta* most efficiently occur under acid conditions (pH 3-6) with the rate of decolorization

increase with temperature from 50 to 60 °C (Nyanhongo *et al.,* 2002). Laccase-based decolorization are potentially advantageous to the treatment of dye pollution since the enzyme can be produced in large amount and require less fastidious induction than either lignin peroxidase (LiP) or manganese peroxidase (MnP) (Pointing *et al.,* 2000).

Removal of phenolic or xenobiotic pollutants from wastewater using laccase can be accomplished in several ways. Ryan *et al.* (2005) has studied the removal of phenolic effluents using airlift reactor with maximum removal of phenol over biomass per day achieved at 0.033 gPhenol g-1Biomass day-1. It was reported that some phenols can inhibit the growth of white rot fungi (Buswell and 16 Eriksson, 1994). However adverse effects might be minimized by choosing appropriate dilutions and attaining appropriate stages of fungal growth. Using the appropriate flow rate of wastewater for remediation was shown to be important in experiments with dye-laden effluent (Romero-González *et al.,* 2006), where higher or lower rates led to laccase deactivation or inefficient system operation, respectively.

Wastewater treatment with laccase producing fungi growing directly is a relatively recent idea. The application of growing fungi on inert materials prior to exposure of the latter to effluent is one of the possibilities that have been tested in several studies (Rodríguez Couto *et al.,* 2004). Most experiments on the bioremediation of wastewater have been carried out with sterilized effluent. Nevertheless, Pedroza *et al.* (2007) successfully demonstrated that *Trametes versicolor* can treat wastewater from the pulp and paper industry under non-sterile conditions.

2.11.9 Laccase based biosensor

Laccase-based electrochemical biosensors have been developed which enable the amperometric determination of phenolic compounds in waste streams (Freire *et al.,* 2002; Torrecilla *et al.,* 2007) or the quantitative estimation of wide range of aromatic substances and reducing compounds (Zouari *et al.,* 1994). In food industry, laccase-based biosensor has been used to detect phenol or other enzymes in fruit juice and wine (Di Fusco *et al.,* 2010; Gil and Rebelo, 2010).

Oxygen determination using laccase have been investigated (Gardiol *et al.,* 1996). Electrochemical studies using laccase cathodes allow the electro-reduction of oxygen to water (Pita *et al.,* 2011) which eventually was used in bio-fuel cells (Jenkins *et al.,* 2012).

2.11.10 Cosmetic Industry

Laccase based hair dyes are less irritating 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 proteins for skin lightening have also been developed. (Rodríguez Couto and Toca Herrera, 2006).

2.11.11 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 2.11.11 Commercial preparations based on laccases for industrial processes. (source : (Osma *et al.,* 2010)

	Main application	Brand name	Manufacturer
Food industry	Brewing Color enhancement in tea, etc. Cork modification	Flavour star LACCASE Y120 Suberase	Advanced enzyme Technologies Ltd. (India) Amino Enzyme USA Co. Ltd. Novozymes (Denmark)
Paper industry	Pulp bleaching Paper pulp delignification	Lignozym-process Novozym 51003	Lignozym GmbH (Germany) Novozymes (Denmark)
Textile Industry	Denim bleaching Denim bleaching Denim finishing Denim bleaching Denim finishing	Bleach Cut 3-S Cololacc BB DeniLite Ecostone LC10 IndiStar Novoprime Base 268 Primagreen Ecofade LT100 ZyLite	Season Chemicals (China) Colotex Biotechnology Co. Ltd. (Hong Kong) Novozymes (Denmark) AB Enzymes GmbH (Germany) Genencor Inc. (Rochester, USA) Novozymes (Denmark) Genencor Inc. (Rochester, USA) ZyLite Zytex Pvt. Ltd. (India)

CHAPTER III: MATERIAL AND METHODOLOGY

3.1 Settings of Laboratory

This study was conducted in the laboratory of Central Department of Biotechnology, Tribhuvan University. ABTS and Guaiacol were purchased from Hi-media, India and all other chemicals were of reagent grade. All the experiments were done in triplicate.

3.2 Sample collection

Soil samples were collected from different places of Kathmandu valley and Rautahat district of Nepal. Previously isolated *streptomycetes* isolates were used for the screening of laccase activity. Both *Ganoderma spp.* (Whole body) and culture were obtained from Nepal Academy of Science and Technology (NAST), Nepal.

3.3 Isolation and Culture conditions

From each collected soil sample, 1g of soil sample were suspended in 100 ml of distilled water then incubated in an orbital shaker incubator at 28°C with shaking at 200 RPM for 30 minutes. Mixtures were allowed to settle and serial dilutions up to 10^{-5} were prepared using sterile water and agitated with the vortex at maximum speed. An aliquot of 0.1ml of each dilution from 10^{-2} to 10^{-5} were taken and spread evenly over the surface of starch casein agar medium containing 50 μg/ml cyclohexidine to inhibit bacterial and fungal contamination. Plates were incubated at 28°C and 37°C and monitored after 48, 72 and 96 h. Repeated streaking on starch casein agar plates led to purify bacterial colonies that showed an Actinomycetes like appearance. This isolated strains were preserved at 4°C (Williams and Davis, 1965) during two months and maintained for longer period by serial sub-culture.

Streptomycetes previously isolated from our laboratory were sub-cultured by using ISP-2 medium. At last, fungus was isolated from provided fungus body and culture of fungi in PDA. All the isolates were used for laccase screening.

3.4 Screening of laccase producing microbes

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 Actinomycetes and fungal strains and color change 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, Isolate G1 was selected for further work.

3.5 Morphology of Isolate 'G1'

Conidial morphology is the most widely used taxonomic character for identification of any fungi. Most species are divided into different groups based on the size of conidia. The length and width are good markers for the genus (Hu *et al.,* 2007).
A drop of Cotton blue reagent was placed on a clean dry glass slide. By using a nichrome inoculating wire, fungal culture was placed on the slide. It was covered with coverslip and waited for 5 minutes. Then it was observed under compound microscope.

3.6 Isolation of gDNA from selected organism

High quality of gDNA for PCR amplification was extracted from the best strain with highest laccase activity (Aljanabi and Martinez, 1997). Homogenization of fungal mycelia (tissues) was carried out in mortar and pestle containing 400 µl of sterilized salt homogenizing buffer (0.4M NaCl , 10mM Tris-HCl of pH 8.0 and 2 mM EDTA of pH 8), followed by addition of 40 µl of 20% Sodium dodecyl sulphate (SDS) (2% final concentration) and 8 µl of 20 mg/ml proteinase k (400mg/ml final concentration) were added and mixed well. Samples were incubated at 55-65°C for overnight. After that, 300 µl of 6M NaCl was added to the sample. Then the samples were vortexed for 30 seconds at high speed followed by spinning for 30 minutes at 10,000g. Then the supernatants were transferred to fresh Eppendroff tubes and equal volume of isoproponal was added to the sample. It was incubated at -20°C for 1 hour for proper precipitation. Finally, the sample was centrifuged at 4°C for 20 minutes at 10,000g. After removing the supernatant, the pellet was washed with 70% alcohol and dried for some time in laminar airflow. The dried pellet was re-suspended in 300-500 µl TE with RNAse and finally stored at -20°C for further use.

3.7 PCR amplification of gDNA

The gDNA was amplified by using 18S rRNA primers. The sequences of forward primer and reverse primer were 5'GGTCTTGTAATTGGAATGAG 3' and 5' CTTCCGTCAATTCCTTTAAG 3' (Rajesh K *et al.,* 2006). The PCR mixture was prepared in PCR tubes as (master mixture -10µl, $MgCl_2$-0.6µl, Forward primer (P_f)-1µl, Reverse primer (P_R-1µl) and nuclease free water 6.4µl). Then it was centrifuged and 1µl template was added in each tube. After that PCR mixture were kept in PCR machine previously set at following condition.

Table 3.7: Condition for PCR amplification of gDNA

Steps	Temperature	Time
1. Initial denaturation	95°C	2 min
2. Denaturation	95°C	30 sec
3. Annealing	51°C	30 sec
4. Extension	72°C	70 sec
5.	Repeated steps 2-4, 30 times	
6.Final extension	72°C	5 min
7. Hold	4°C	Forever

After completion of PCR, gDNA was run in 1% gel-electrophoresis at 50 V for 1 hour and then it was visualized in UV. It was sent to Xcelris Labs Limited, Ahmedabad, Gujarat,India for sequencing.

3.8 Optimized production of Laccase

PDA medium with 1% kraft lignin was used for optimization and production of laccase from best fungus. Different parameters were varied for optimization of the media. Tthey were: i) pH, ii) Temperature, iii) Agitation (in RPM) iii) DO (Dissolved oxygen at 200 RPM), (v) Inducers vi) Metal ions vii) Inhibitors.

3.8.1 Laccase activity assay

Laccase activity was measured at room temperature using ABTS solution as the substrate of 1mM concentration prepared in 0.1 M of sodium acetate buffer at pH 5. Reaction volume consisted of 350 µL of enzyme and 700 µL of 1mM ABTS prepared in the same buffer to give total volume of 1050 µL while for enzyme characterization, 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 45 seconds. The amount of enzyme required to oxidize 1 µmol ABTS per minute forming $ABTS^{o+}$ was taken as 1 unit. ($\varepsilon_{420} = 36000\ M^{-1}\ cm^{-1}$ and path length l = 1cm) (Bourbonnais *et al.,* 1995).

$$\text{Laccase activity}\ (\text{U ml}^{-1}) = \frac{\Delta \text{Abs}}{t} \times \frac{1}{\varepsilon} \times \frac{1}{l} \times \frac{\text{total assay volume}}{\text{enzyme volume}}$$

ΔAbs is the change of absorbance; t is the assay time (minute); ε is the extinction coefficient of ABTS ($36000\ M^{-1}\ Cm^{-1}$); l is the cuvette light path length (1cm).

100 ml conical flask containing 50 ml PDB media and 1% kraft lignin were taken. After that 100 μl of 5 days old culture of actively growing microorganism was inoculated. pH optimization was done from pH 4 to 10 at the interval of 1 pH using 0.1 M Sodium acetate buffer to maintain the pH. For temperature optimization, temperatures were taken in the range of 20° to 50°C at 5°C interval. Activity of laccase was noted daily. Each time enzyme blank (enzyme boiled at 100°C for half an hour and centrifuged at 10,000 RPM for 5 minutes to obtain clear enzyme broth) was set for spectrophotometry.

3.8.3 Agitation optimization

Bioreactor was cleaned with tap water and finally washed with distilled water. After that 2.5 L PDB media along with 1% Kraft lignin was added and then pH was maintained. It was autoclaved at 121°C for 15 minutes. After cooling to room temperature, all the ports were connected and bioreactor was run at 100 RPM. After few hours foam formation was occurred, so 1ml Silicone antifoaming was added. Everyday laccase activity was measured till 10 days by taking 1ml crude enzyme from bioreactor. In this way, at 150 RPM and 200 RPM bioreactor was also run. Above process was carried out in triplicate for better result

3.8.4 Dissolved Oxygen (DO) optimization

Again bioreactor containing 2.5 L PDB media with 1% kraft lignin at pH 5 was autoclaved and then cooled to room temperature. After connecting all the ports, bioreactor was run maintaining at 50 DO by changing the aeration rate. Similarly, 40 DO and 35 DO were also monitored. Above process was carried out in triplicate for better result.

3.8.5 Effect of Inducers

50 ml of 0.5, 1 and 2 mM concentrations of each Vanillin, Gallic acid, Catechol, Guaicol and control was taken in Falcon tube. In each tube 1% kraft lignin was added and all the tubes were autoclaved. After cooling the tubes, 1% 5 days old culture of Isolate G1 was inoculated and it was incubated at 30°C for 8 days. Laccase activity was measured after 8 days. Above process was carried out in triplicate for better result.

3.8.6 Effect of Inhibitors

For inhibitor optimization, 50 ml of 0.5, 1 and 2mM concentrations of each Sodium azide, cysteine and EDTA and control were taken. In each tube 1% kraft lignin was added and all the tubes were autoclaved. After cooling the tubes, 1% 5 days old culture of Isolate G1 was inoculated and it was incubated at 30°C for 8 days. Laccase activity was measured after 8 days. Above process was carried out in triplicate for better result.

3.8.7 Effect of metal ions

For metal ions optimization, 50 ml of 1 and 2mM concentrations of each Magnesium sulfate, Zinc sulfate, Ferric sulfate and Copper sulfate and a control were taken in falcon tubes. In each tube 1% kraft lignin was added and all the tubes were autoclaved. After cooling the tubes, 1% 5 days old culture of Isolate G1 was inoculated and it was incubated at 30°C for 8 days. Laccase activity was measured after 8 days. Above process was carried out in triplicate for better result.

3.8.8 Effect of surfactants

For surfactant optimization, 50ml of 5, 1 and 15 and 20 ppm concentrations of each Tween 20 and Tween 80 were in and control were taken. In each tube 1% kraft lignin was added and all the tubes were autoclaved. After cooling the tubes at room temperature, 1% 5 days old culture of Isolate 'G1' was inoculated and it was incubated at 30°C for 8 days. Laccase activity was measured after 8 days. Above process was carried out in triplicate for better result.

3.9 Optimized production of laccase

Laccase was produced using the optimized parameters *viz.* pH 5 at 30 ^{o}C with and 1g/L kraft lignin, at 200 RPM and 50 DO, and it was harvested when laccase activity reached maximum. It was then purified for characterization of the laccase.

3.10 Purification of laccase

3.10.1 Ammonium Sulfate precipitation:

One hundred millilitre enzyme broth from fermenter was taken in conical flask and filtered using whattman 1 filter paper. 40 % ice cold ammonium sulfate was added and incubated for 2 hour at 4°C. After that precipitate was discarded by centrifugation at 4000 RPM for 15 min. Supernatant was collected and then 70% ammonium sulfate was made. It was incubated overnight at 4°C. Next day precipitate was collected and dissolved in 0.1M Sodium acetate buffer of pH 5. It was further stored for characterization (Prabin *et al.,* 2016).

3.10.2 Acetone precipitation:

100 ml filtered broth and 500 ml cold acetone was taken in 1000 ml conical flask and it was incubated for 1 hour at -20°C. The sample was centrifuged at 4°C for 10 min at 15000RPM. The supernatant was discarded and pellet was air dried for 5 min. It was re-solubilized in 0.1M sodium acetate buffer (pH 5) for further use.

3.11 Protein estimation

Protein content estimation was done 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.

3.12 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 150 V for 1 hour 20 minutes at room temperature. Commassie Brilliant Blue G250 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.

3.13 Characterization of Laccase enzyme

3.13.1 Optimum pH and optimum temperature

Optimum temperature and optimum pH for laccase was determined by performing enzymatic assays from temperatures 20 – 70°C and from pH 2-8 using ABTS as substrate respectively. pH levels were adjusted using 0.1M sodium acetate buffer.

3.13.2 pH tolerability and thermo-stability of laccases

pH stability and thermo 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 150 min in Acetate buffer of pH from 2-10 using ABTS as the substrate. The thermo-stability of the enzyme was determined by measuring laccase activity after every 30 min for 120 min using ABTS as substrate at optimal pH over a temperature range of 30 to 80°C.

3.14 Kinetic study

Lineweaver-burk plot was used for kinetic parameters (Km, Vmax, and Kcat) for the partially purified laccase enzyme using varied concentrations of ABTS as substrate (More *et al.,* 2011).

CHAPTER IV: RESULTS

4.1 Isolation and screening of laccase producing microbes

Laccases are the versatile enzymes which catalyze oxidation reactions coupled to four-electron reduction of molecular oxygen to water. They are multi-copper enzymes which are widely distributed in higher plants and fungi. They are capable of degrading lignin, decolorize and detoxify the industrial effluents and help in wastewater treatment. Laccase production by different microbes have been found to be highly affected by various parameters such as presence or absence of lignin source and different concentrations of metal ions, presence or absence of microelements, aeration and agitation rate. Laccases are generally produced in low concentrations by microbes, 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.

Laccase producing actinomycetes and fungi were isolated and it was tested for laccase activities. In the initial screening oxidation of tannic acid, guaiacol and 1-naphthol were seen after 5 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 but there was no oxidation of 1-naphthol to a deep purple complex (More *et al.,* 2011) giving a visual confirmation for the presence of the enzyme.

Out of fifty three isolates, thirty were actinomycetes isolates from soil samples, twenty were previously isolated streptomycetes from our laboratory and three were fungi isolates. Among them, twelve isolates showed laccase positive in different screening media. Seven isolates gave laccase positive test in tannic acid and three in guaiacol. Isolates NXVI and G1 and gave laccase positive result in both tannic acid and guaiacol. On the basis of zone of color development in both media, Isolate G1 was selected for further work.

Table 4.1 Test of Laccase in different media

Sample code	Source of collection	Guiaicol	Tannic acid	1-Napthol
3	Isolated Actinomycetes	_	+	_
5	Isolated Actinomycetes	_	+	_
N6	Previously Isolated streptmyceteso	_	+	_
N7	Previously Isolated streptomycetes	_	+	_
N10	Previously Isolated streptomycetes	_	+	_
N11	Previously Isolated streptomycetes	_	+	_
N13	Previously Isolated streptomycetes	+	_	_
NXIV	Previously Isolated streptomycetes	_	+	_

NXVI	Previously Isolated streptomycetes	+	+	_
G1	Isolated from fungus	+	+	_
G2	Isolated from fungus	_	+	_
G3	Isolated from fungus	+	_	_

(+ = laccase produced, - = laccase not produced)

4.2 Morphology of Isolate ‘G1’

Colonies of isolate G1 on potato dextrose agar were white, cottony, margin nearly round; acervuli developed in mycelia and gave rise to black spore mass, punctate, discrete, and developed on the carnation leaves on potato dextrose agar, scattered, irregular.

Microscopy of Isolate G1 by Lactophenol cotton blue staining showed that the spores (conidia) are 4 to 6 celled conidia with deeply olivaceous central cells, brown to black in color, simple appendages arising from apex.

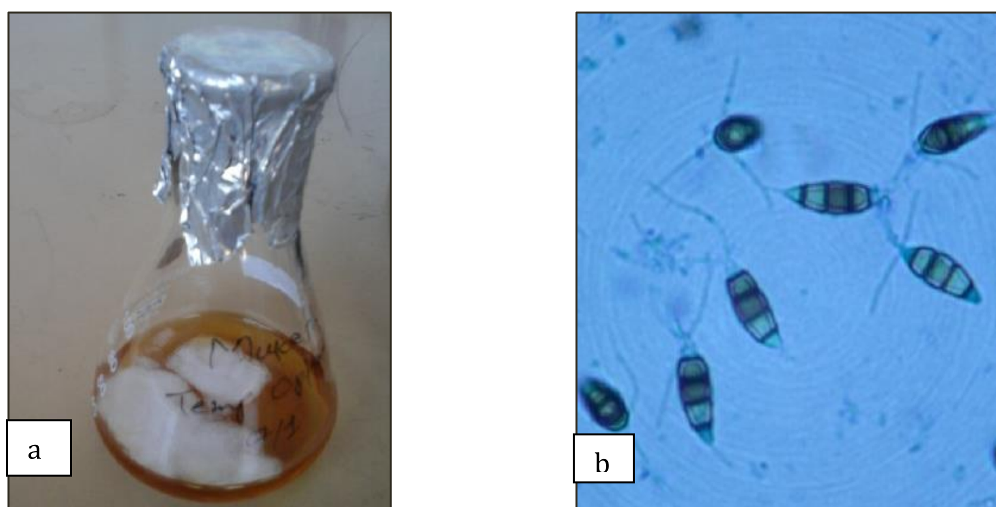


Fig 4.2: a) Isolate G1 growing on PDB with 1% kraft lignin b) Cotton blue staining of isolate ‘G1’

4.3 gDNA extraction and PCR amplification of Isolate G1

The genomic DNA was extracted and PCR performed successfully. The size of PCR product was found to be 658 bp. The sequence of Isolate G1 was obtained from Xcelris Lab, India and further BLAST was done to find the most probable genus of fungus.

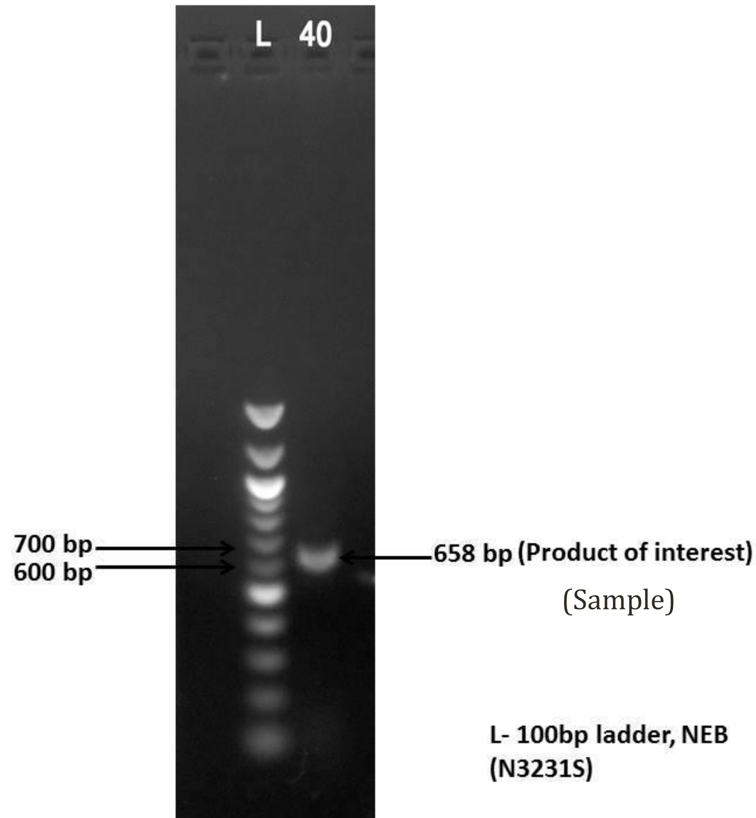


Fig4.3a: Gel-electrophoresis (1% agarose) of PCR product. Lane-2 100bp NEB ladder, Lane-3 PCR product.

BLAST ® » blastn suite » RID-NEUDACGF014

BLAST Results

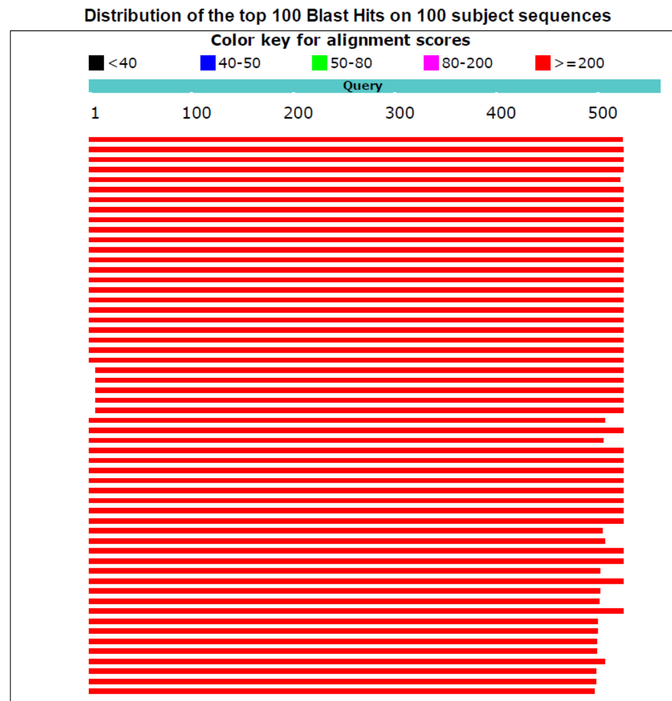
Job title: CTGCGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATT

RID NEUDACGF014 (Expires on 07-02 17:21 pm)

Query ID lcl|Query_26957
Description CTGCGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATT
Molecule type nucleic acid
Query Length 544

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.6.1+

Graphic Summary



Descriptions

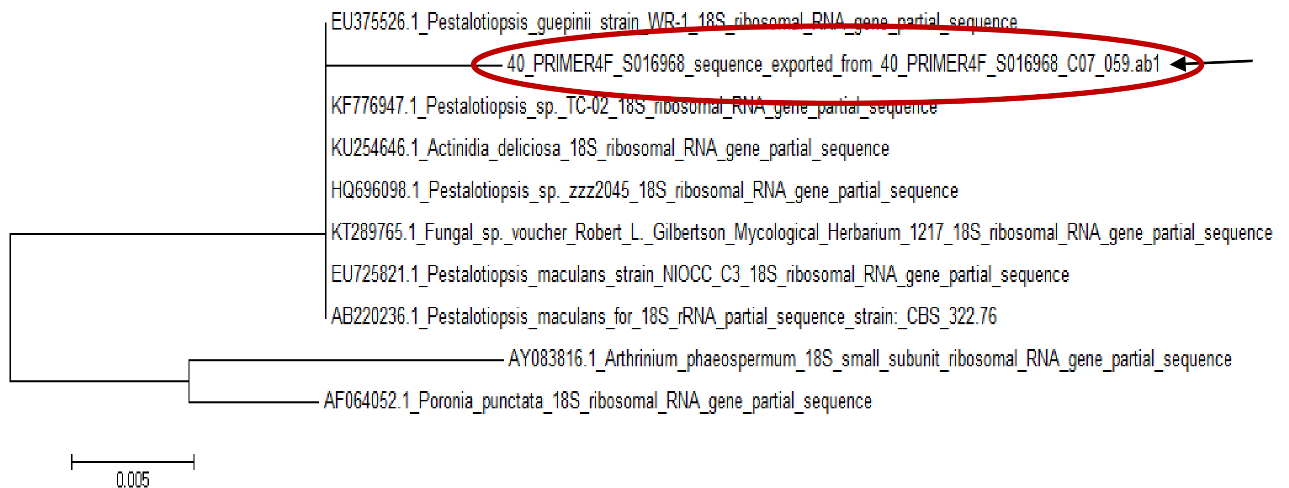
Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pestalotiopsis sp. zzz2045 18S ribosomal RNA gene, partial sequence	935	935	95%	0.0	100%	HQ696098.1
Actinidia deliciosa 18S ribosomal RNA gene, partial sequence	931	931	95%	0.0	99%	KU254646.1
Fungal sp. voucher Robert L. Gilbertson Mycological Herbarium 1217 18S ribosomal RNA gene, partial sequence	931	931	95%	0.0	99%	KT289765.1
Pestalotiopsis sp. TC-02 18S ribosomal RNA gene, partial sequence	931	931	95%	0.0	99%	KF776947.1
Pestalotiopsis maculans strain NIOCC C3 18S ribosomal RNA gene, partial sequence	931	931	94%	0.0	100%	EU725821.1

Fig 4.3b: Blast result showing percentage identity of Isolate ‘G1’ with other fungal species.

MEGA (Molecular Evolutionary Genetics Analysis) was used as a tool for conducting sequence alignment. Those aligned sequences were used to make phylogenetic trees. The analyses used to construct phylogenetic tree were conducted with the following specifications: Nucleotides were used as substitutions type, maximum likelihood was used as the statistical method, bootstrap method with 2000 bootstrap replications was used to test phylogeny.

Sequence similarity searches was performed for the fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information (NCBI) using the "Basic Local Alignment Search Tool" (BLAST) as the tool to infer functional and evolutionary relationships between sequences as well as help to identify members of gene families. The obtained sequence showed maximum similarity with *Pestalotiopsis spp.*



Figs 4.3c: Phylogenetic tree of Isolate "G1"

4.4 Production media optimization

4.4.1 pH optimization

The effect of pH is limited in case of laccase production (Thurston,1994) .The effect of pH values on laccase production were observed from pH range 4.0 to 10.0 (Fig 4.4.1). Laccase exhibited the highest activity at pH 5.0 of 19.78±2.31 U/mL at day 9. Enzyme activity decreased sharply as the pH value increased from 5.0 towards the neutral range. Laccase activity was seen less on other pH ranges (Fig. 4.4.1). The result was found to be significant at 5 % level of significance.

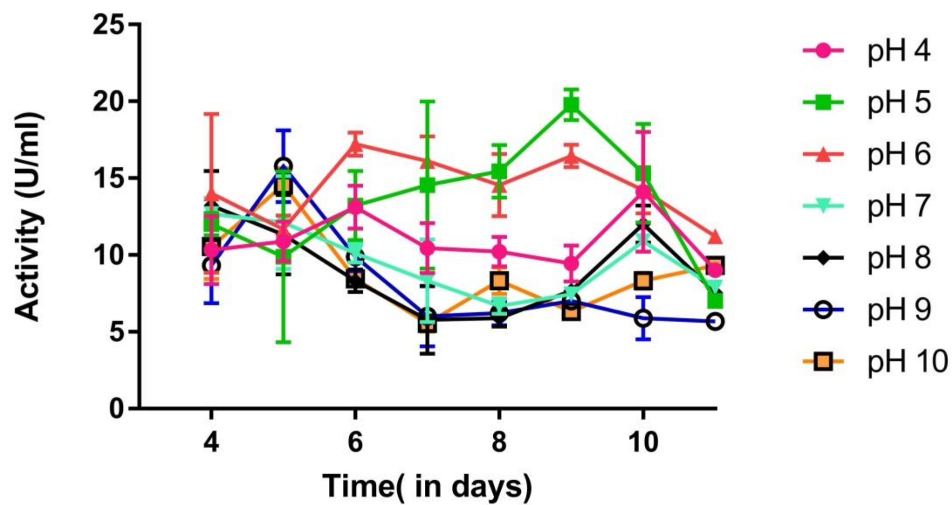


Fig 4.4.1: **Effect of media pH on laccase production by Isolate G1 ($p < 0.05$)**

4.4.2 Temperature optimization

The temperature optimization was done in the range from 20 to 50°C at the interval of 5 °C. Optimal temperature for highest laccase activity (23.06±0.43 U/ml) was found to be 30°C (Fig. 4.4.2). The result was found to be significant at 5 % level of significance.

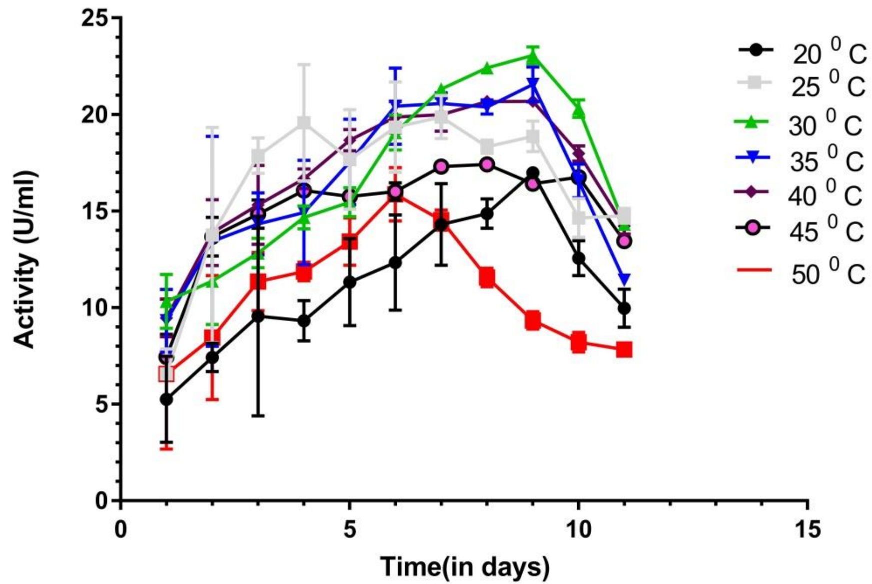


Fig. 4.4.2: Effect of Temperature on laccase production by isolate "G1"

4.4.3 Agitation optimization

The agitation optimization was done at 100, 150 and 200RPM. Laccase activity was measured daily till 11 days and it was found that maximum laccase activity 51.37±9.34 U/ml on 8th day at 200 RPM (figure 4.4.3). From this result, it can also be seen that number of days are reducing when agitation is carried out.

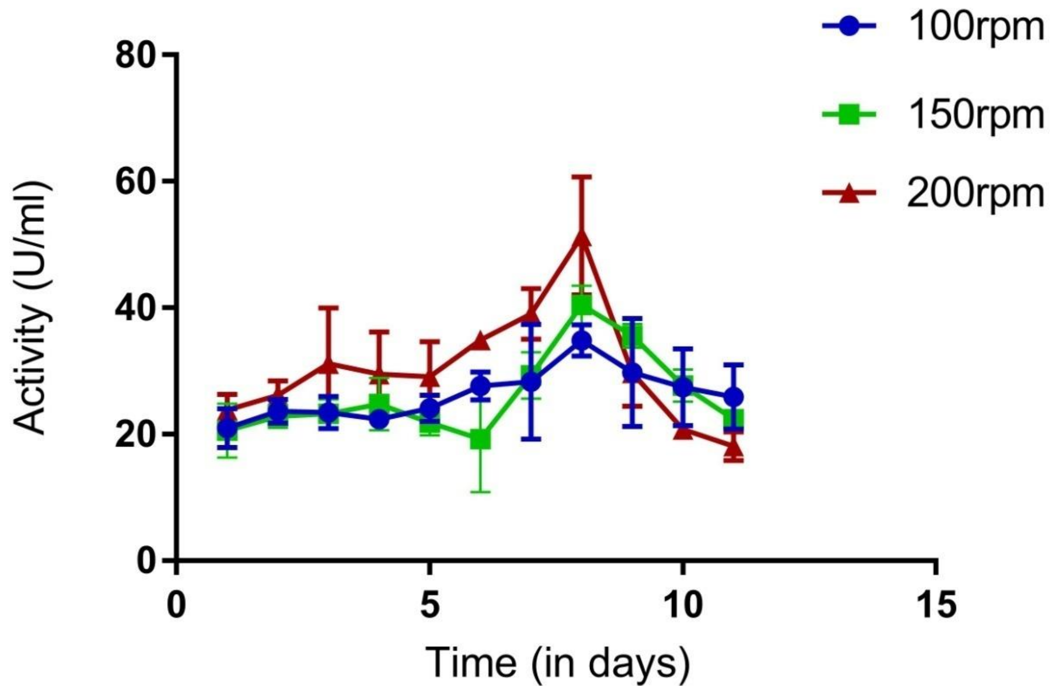


Fig 4.4.3 : Effect of Agitation rate on laccase production by isolate G1

4.4.4 Dissolved Oxygen (DO) optimization

Dissolved Oxygen is an important factor for higher laccase production. So 35, 40 and 50 DO were optimized for maximum laccase activity and it was found that 40 DO was best for maximum (57.33± 2.33 U/ml) laccase activity at 6th day (Fig. 4.34.4). After 6th day laccase activity gradually decreases. During DO optimization, number of days for maximum laccase activity also decreases.

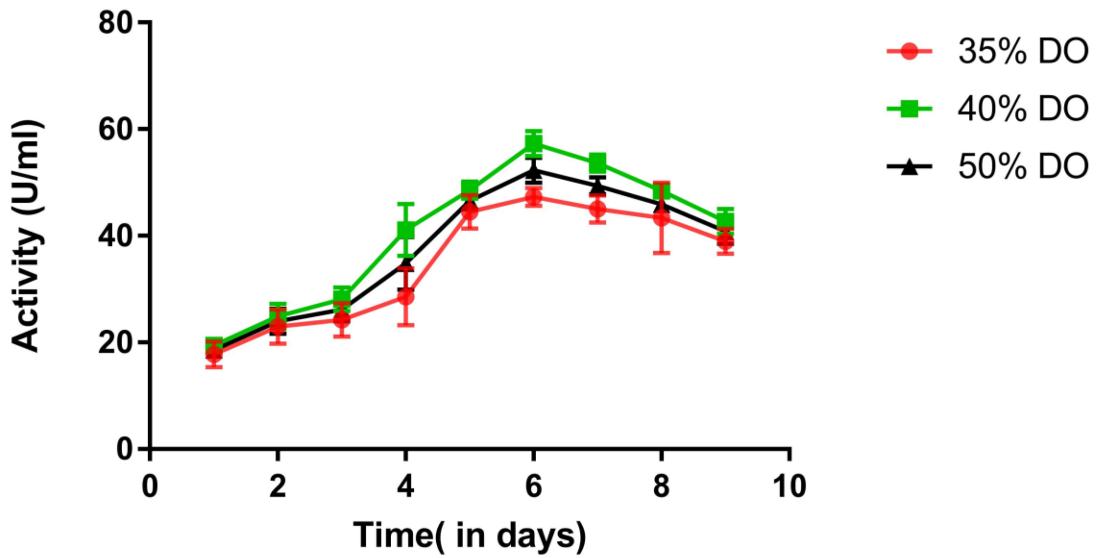


Fig 4.4..4 : Effect of Dissolved Oxygen rate on laccase production by isolate 'G1'

4.4.5 Effects of Inducers on Laccase production

The production of laccase by fungi was increased or induced when an aromatic compound (such as Vanillin, Guaiacol, Catechcol and Gallic acid) or Cu^{++} was added to the growth medium (Ikehata *et al.,* 2004). In my result, 1mM Gallic acid showed maximum lacccae activity(69.22± 10.84 U/ml) followed by 1mM Vanillin (67.88± 14.92 U/ml). The result was found to be significant at 5 % level of significance.

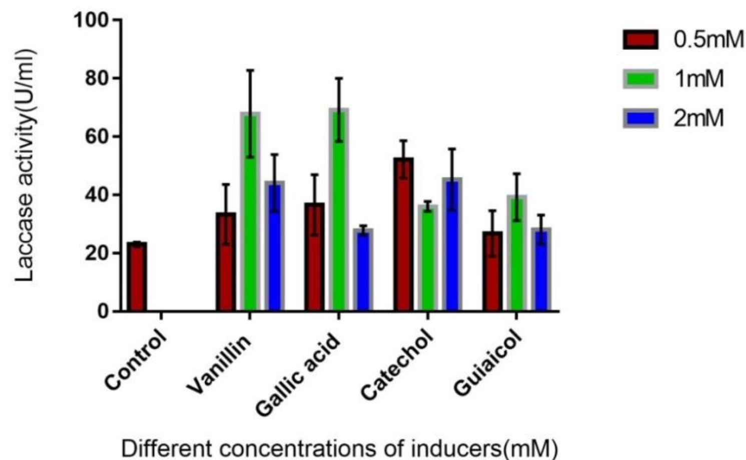


Fig 4.4.5: Effect of inducers on laccase production by isolate 'G1'($p < 0.05$)

4.4.6 Effects of Inhibitors on Laccase productions

Different concentrations of inhibitors acted differently. All concentrations of cysteine inhibited slightly but 0.5 and 2mM EDTA had no inhibition. All concentrations of sodium azide and 1mM EDTA gave opposite result. In spite of inhibition, Sodium azide and 1mM EDTA increases the laccase activity highly. The result was found to be significant at 5 % level of significance.

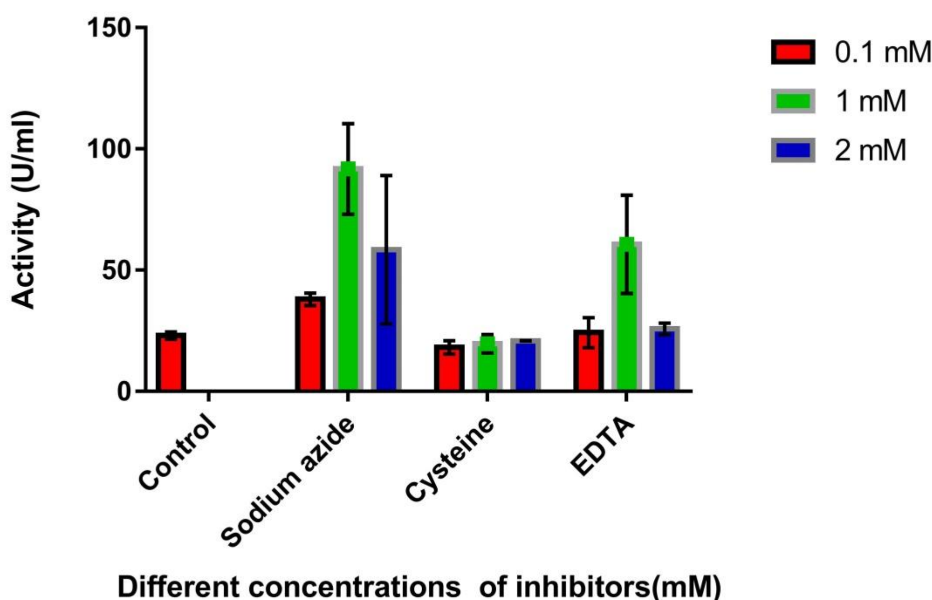


Fig 4.4.6: Effect of different concentrations of Inhibitors (mM) on laccase production. ($p < 0.05$)

4.4.7 Effects of Metal ions on Laccase production

Various metal ions were added in the form of their salts into the optimized condition (pH 5, temperature 30°C and 1% kraft lignin).The maximum laccase activity (50.52± 24.75 U/ml) was obtained with 1mM concentration of Magnesium sulfate after 9th days followed by 2 mM Magnesium sulfate (40.56 ± 15.89 U/ml) and then 1 mM Zinc sulfate (30.55 ± 1.56 U/ml) (Fig 4.4.7). There was slightly increase in laccase activity (29.88±8.65 U/ml) by Copper sulfate and Ferric nitrate (29.03 ± 5.68 U/ml). The result was found to be significant at 5 % level of significance. Different metal ions can enhance or inhibit the growth, causes morphological and physiological changes and may affect the reproduction of WRF (Wuyep *et al.,* 2003). Different strains and species of fungi differ in their sensitivity towards metals during their growth on lignocellulosic substrates (Sathiya- Moorthi *et al.,* 2007).

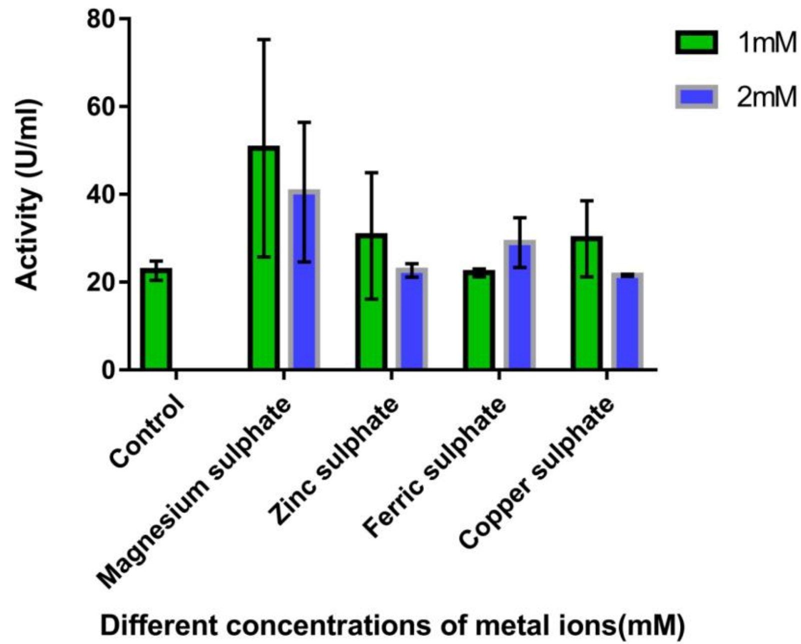


Fig 4.4.7: Effect of different concentration of metal ions (mM) on laccase production ($p < 0.05$)

4.4.8 Effects of Surfactant on Laccase production

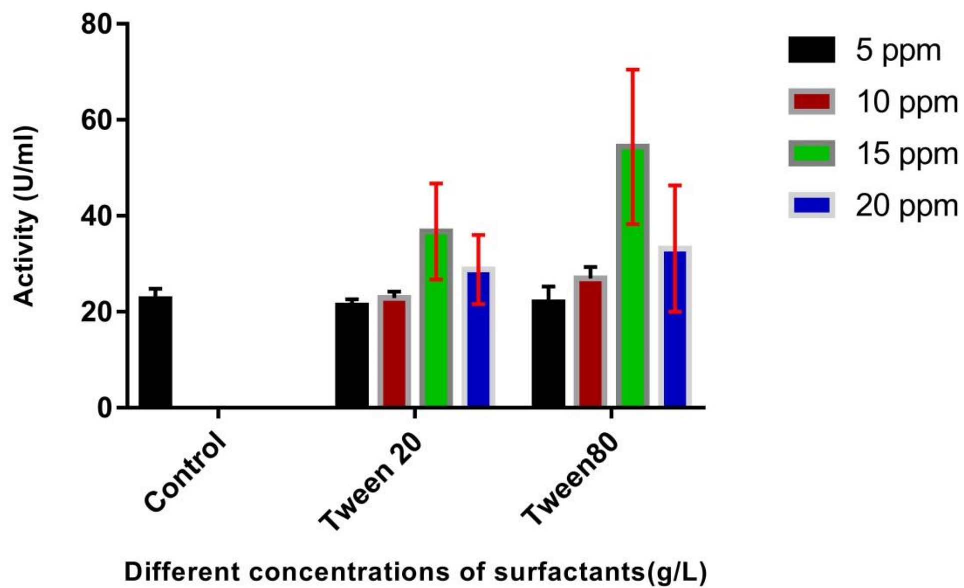


Fig 4.4.8: Effect of different concentrations of surfactants on Laccase production ($p < 0.05$)

Surfactants, especially Tween-80, can increase the bioavailability of less soluble substrates for the fungi and stimulate growth of fungal spores (Zheng and Obbard 2001). Tween-20 and Tween-80 were added to the medium at various concentrations of 5 – 20 ppm. Tween-80 with a concentration of 15 ppm stimulated higher laccase production (54.40±14.4 U/ml) as compared to control (22.62±2.61 U/ml) (Fig. 4.4.8). A trend of decreasing enzyme production at increasing concentrations above 15 ppm Tween-80 was observed. On the other hand, 15 ppm Tween-20 showed higher laccase activity but lesser than Tween-80. The result was found to be significant at 5 % level of significance. The specific mechanism by which surfactants enhance extracellular enzyme production in fungi has not been elucidated (Wang et al. 2008).

4.5 Purification of crude laccase from fermentation broth

Crude sample from the cultivation in submerged bioreactor was used for partial purification. In this study, the steps used for partial purification of laccase crude sample included ammonium sulfate precipitation and acetone precipitation. Firstly the crude sample was precipitated using ammonium sulfate in order to concentrate protein from liquid sample. In next step, protein precipitate was dialyzed using dialysis bag to get more pure enzyme and then activity of dialyzed enzyme was measured and it was further used for SDS to determine the molecular weight of laccase.

4.5.1 Ammonium sulfate precipiatation

Samples from the submerged bioreactor were used in fractionation using ammonium sulfate precipitation. Laccase activity and protein concentration of 70% ammonium sulfate saturation was 882.0 U/ml and 33.65µg/ml respectively.

4.5.2 Acetone precipitation

Sample from the submerged fermenter was taken and acetone precipitation was done in five times the volume of sample broth ice cold acetone. Laccase activity and protein concentration were 956.66 U/ml and 30.125 µg/ml.

4.6 Characterization of purified laccase

4.6.1 Temperature optima and thermal stability

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 60°C (Fig. 4.6.1.a). Enzyme activity declined when the temperature was increased from 60^{0}C to 80°C.

It decreased by 9.09% at 80°C of the maximum activity observed at 60°C.

The stability of the enzyme with respect to temperature was also studied. After 1 hour, laccase activity at 70°C decreased by 35.7 % of the initial optimal activity while after after 180 min at 70°C laccase activity decreased by 94.84%. The result was found to be significant at 5 % level of significance.

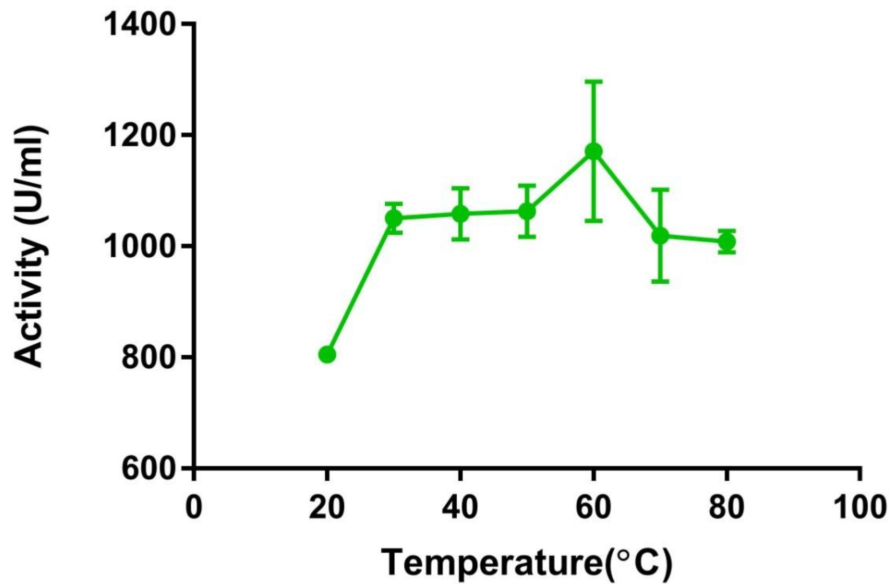


Fig 4.6.1.a: Optimum temperature for laccase activity ($p < 0.05$)

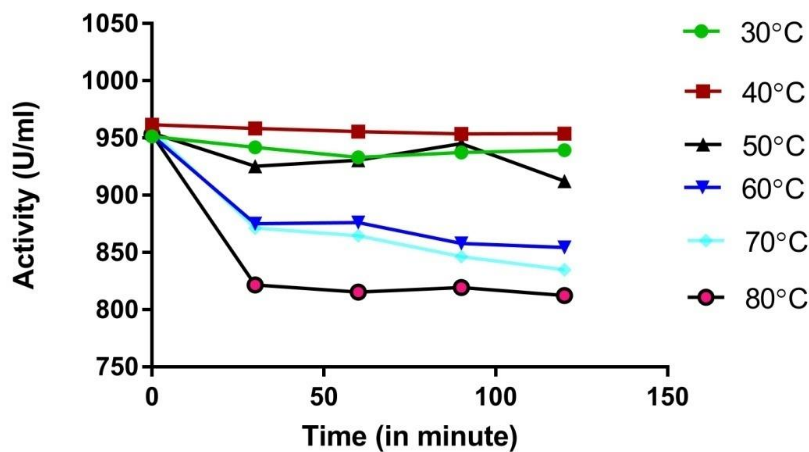


Fig 4.6.1.b.: Temperature tolerability of laccase activity ($p < 0.05$)

4.6.2 pH optima and pH tolerability

Laccase was observed to be active in wide range of pH. The optimum value of pH varies according to the substrate because different substrate causes different reaction for laccases. Many reports suggested that the bell shaped graph in case of laccase activity. At high pH, the potential difference between the phenolic substrate and T1 copper can increase the substrate oxidation while the hydroxide ion (OH^-) binds to the T2/T3 copper centre. These effects help us in determining the optimum pH value for laccase enzyme. The optimum pH for the maximum laccase activity was observed at pH 5 when ABTS was used as substrate with enzyme activity up to 1207.11±141 U/ml. At pH values larger than 5, the enzyme activity decreased gradually and it decreased by 44.07% of optimal activity at pH 10. The activity of laccase did not decrease significantly at pH 6-8. At pH 1 and 10 laccase activity decreased highly. Laccase was highly stable at pH 5 and both below and above pH 5 it decreases or increases randomly. The result was found to be significant at 5 % level of significance.

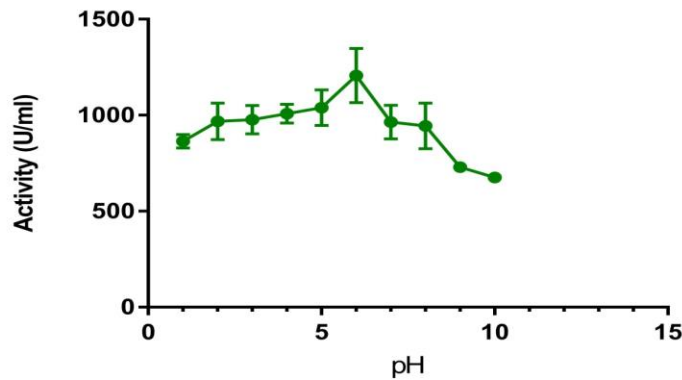


Fig 4.6.2.a.: Optimum pH for laccase activity ($p < 0.05$)

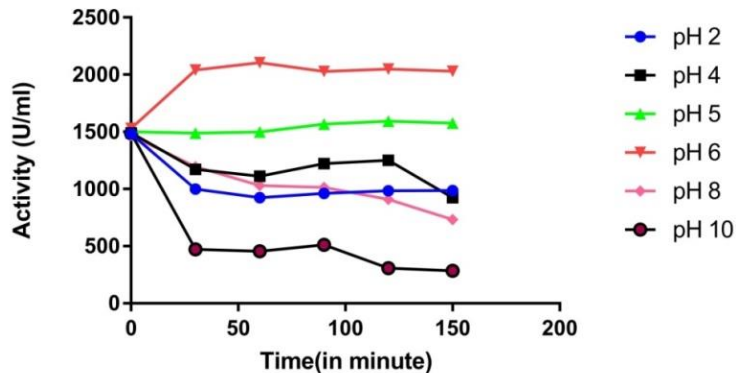


Fig 4.6.2.b.: pH tolerability of laccase activity ($p < 0.05$)

4.6.3 SDS gel electrophoresis

Samples from purification steps were loaded in Gel-electrophoresis. For molecular weight estimation, protein marker with broad range (Genie) was loaded in the gel electrophoresis. Fig. 4.6.3 showed the SDS page analysis on partially purified sample from laccase fragmentation. Crude sample, partially purified sample and a protein marker were loaded into the gel, L1 is the BSA, L2 and L3 are partially purified laccase L5 and L6 are crude laccase L8 is a protein marker.

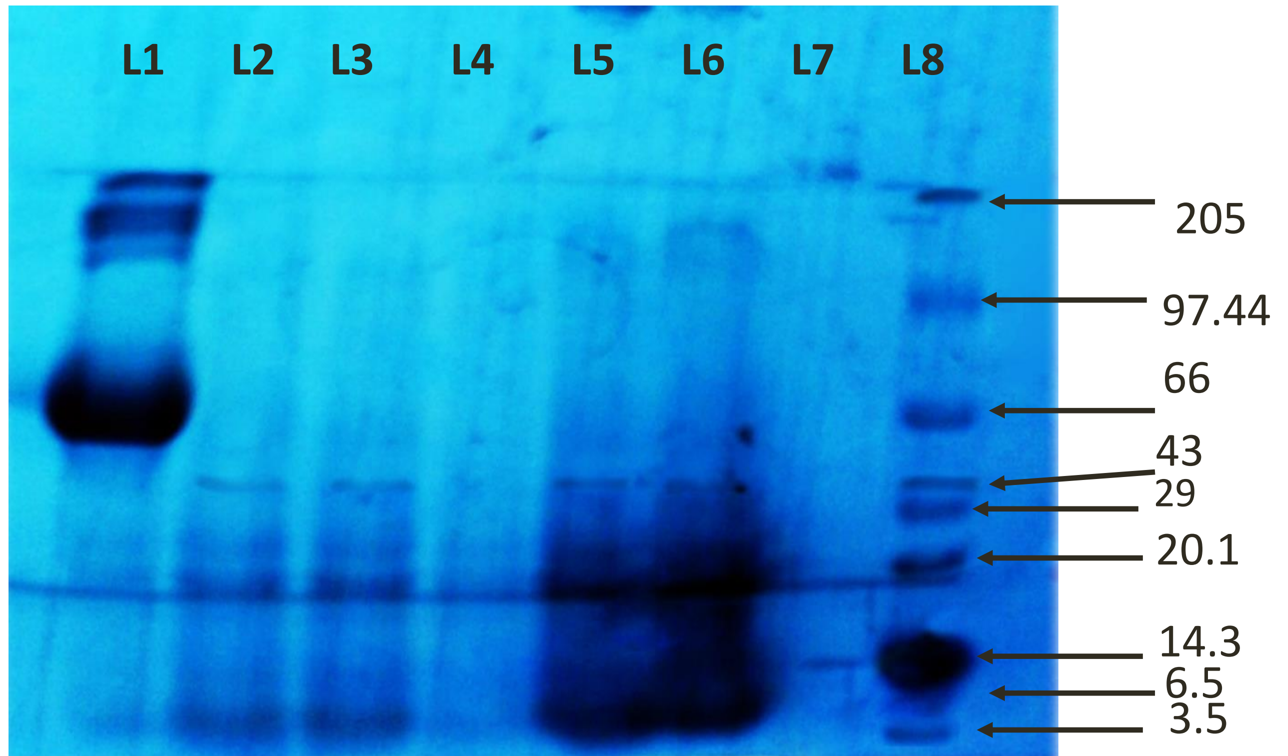


Fig 4.6.3: SDS PAGE of Laccase enzyme. L1- BSA, L2 & L3- purified laccase (G1), L5 & L6- crude laccase (G1) and L8- Protein Ladder 3.5-205 KDa GeNei

The protein bands seen in the marker and partially purified samples have similar molecular weight within 29 to 97 kDa. The size of protein was found to be 43 kDa. This could be the protein band for the laccase enzyme as it was reported that the molecular weight of laccase protein was about 43 kDa (Vite-vallejo *et al.,* 2009). Thus the visualized samples in the SDS page may contain identical laccase from Genei and other studies (Vite-vallejo et al. 2009). Appearance of extra bands less than 29 kDA in lines L2, L3, L5 and L6 suggested the presence of other unknown proteins which were eluted together with the probable laccase (43kDa).

The used of different precipitation methods was not sufficient to obtain high purity laccase that should be indicated by presence of single protein band in SDS page. Samples have to be further purified using other chromatographic methods based on the interaction between laccase and gel matrix through the charge properties i.e. Anion exchange chromatography (bahrin *et al.,* 2010 zhang *et al.,* 2010) and biorecognition i.e. Affinity chromatography (catala et. al., 2011).

4.6.4 Kinetic study

The kinetic parameters of purified laccase was characterized using the Michaelis constant (K_m), maximum velocity (V_{max}), catalytic constant (K_{cat}) and specificity constant (K_{cat}/K_m) using various concentrations of ABTS as a substrate. The Lineweaver-Burk plot showed that the K_m and V_{maxs} for purified laccase were 0.111 mM and 100 µmol/min.

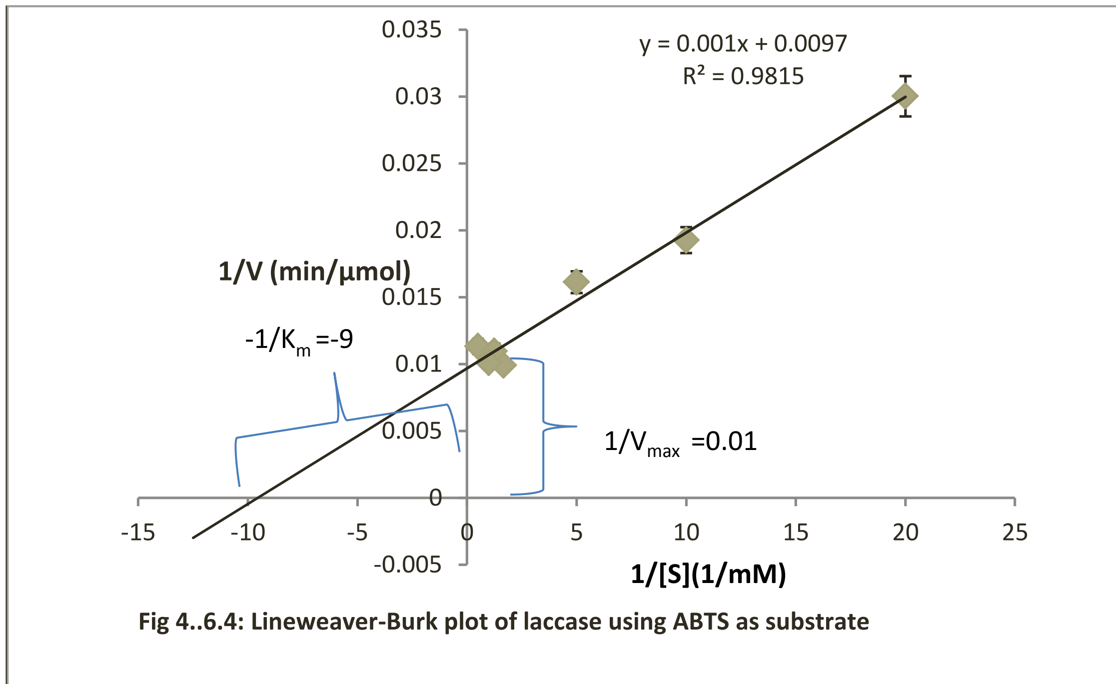


Fig 4..6.4: Lineweaver-Burk plot of laccase using ABTS as substrate

Table 6: Summary of purification of laccase from Isolate 'G1'

Enzyme	Total activity (U/ml)	Protein (µg/ml)	Specific activity (U/µg)	Fold purification
Crude filtrate	55.07	103.75	0.53	1.0
Ammonium sulfate fraction (after overnight dialysis)	882.0	33.65	26.21	49.45
Acetone precipitate	956.66	30.125	31.76	59.92

CHAPTER V: DISCUSSION

Lignocellulose is the major component of agricultural and farming waste which is composed of cellulose, hemicellulose and lignin (Sanchez, 2003). Lignin is the major barrier that encases cellulose and hemicellulose from hydrolysis. Some of fungi can attack the lignin to obtain energy from cellulose by secreting ligninolytic enzymes like laccases. Abundance of nutrients has been reported in agro industrial effluents which can be used for maintenance of microbial growth during enzyme fermentation process. Effluents of paper and pulp industry have been found rich in phenolic compounds (syringic acid, coumaric acid and ferulic acid) which stimulate laccase synthesis in white-rot fungi (Revankar and Lele, 2006). Different agricultural wastes (fruit peels, wheat bran, rice bran etc.) acting as lignocellulose substrates have been used by workers to stimulate the synthesis of laccase enzyme (Rolases *et al.,* 2007; Sathis kumar *et al.,* 2010).

Mostly *Pestalotiopsis* research is based on endophytic isolates (Liu et al. 2006; Wei *et al.,* 2007; Watanabe *et al.,* 2010; Alay *et al.,* 2010). Approximately all endophytic studies have used morphological characters and either gene sequence data(Hu *et al.,* 2007; Liu *et al.,* 2007; Wei *et al.,* 2007) or RFLP technique (Tejesvi et al 2007a) or a combination of gene sequence and RFLP technique (Tejesvi *et al.,* 2009) to distinguish species. I had used morphological characters and gene sequence for identification. The distribution of *Pestalotiopsis* is ubiquitous and is not largely influenced by geographical factors (Wei *et al.,* 2007; Tejesvi *et al.,* 2009). Tejesvi *et al.,* (2005) stated that the endophytic species of *Pestalotiopsis* dominant in the winter season and their colonization are comparatively low in monsoon season. The colonization frequency of species of *Pestalotiopsis* increases with increasing the age of host plant and colonization frequency was variable (Wei *et al.,* 2007). *Pestalotiopsis species* has been isolated from the human sinuses, fingernail, a bronchial biopsy, eyes, scalp and feet with corneal abrasions (Sutton 1999). One isolated from cotton was tested in a toxicity bioassay, which indicated that it caused reduction in weight, pathological abnormalities and even mortalities in rats (Diener *et al.,* 1976).

According to Index Fungorum (http://www.indexfungorumor genames/ names.asp; accession date, 2010,10,21) there are 235 pestalotiopsis names ; while in MycoBank(www.mycobank.org/ mycotaxo.aspx; accession date 2010,10,21) there are 232 names. More recently, some new species have been introduced based on host occurrence, plus morphological and molecular data. Wei and Xu (2004) isolated an endophytic species of *Pestalotiopsis* (*P. kunmingensis*) from *Podocarpus macrophyllus* (Thumb) species, status and host specificity within the genus *Pestalotiopsis* has been questioned previously and investigated (Zhu 1989; Jeewon *et al.,*2004; Wei *et al.,*2007; Hu *et al.,* 2007). These authors showed that different species isolated from the same host may not be phylogenetically closely related (Jeewon *et al.,* 2004; Wei *et al.,*2007). Wei *et al.,* (2007) investigated

endophytic species of *Pestalotiopsis* associated with plant species in the families' Podocarpaceae, Theaceae and Taxaceae.

The first gene and/or cDNA sequence were recorded for laccase from the Ascomycetes fungus, *Neurospora crassa* (German UA *et al.,* 1986), and sequence were published from 1990 onwards. Since then the number of laccase genes sequenced has increased considerably and searches from protein and gene sequence databases currently yield several hundreds of laccase gene sequence. However, a significant number of these are only partial stretches of putative laccase genes of which the corresponding protein products have been experimentally characterized is significantly lower.

Natural and synthetic lignins have been shown to enhance laccase production by many fungi. Culture conditions and medium composition can also play a major role in the level of laccase expression. In the presence of kraft lignin in medium, *P. cinnabarinus* showed 3 times and *Cyathus bulleri* showed 1.2 times increase in laccase activity than without lignin, (Gomez-Alarcon *et al.,* 1989; Vasdez and Kulad, 1994). Natural lignin extracted from cotton stalk, barley straw, potato extract, malt extract showed increased laccase production in several fungi, including *P. ostreatus* and *T. versicolor* (Gianfreda *et al.,* 1999). Increased laccase activity in culture media with lignin as compared to the culture media without lignin 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 radicals act as oxidant of lignin which depolymerizes lignin and give rise to phenolics (ferulic acid, vanillic acid, 3, 4 di-hydroxy benzoic acid) as well as non-phenolic structural polymers. The increase in laccase yield from G. sp. Rck 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 aromatic substances for enhancement of laccase production (D'souza *et al.,* 1991; Sharma *et al.,* 2013)

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 much biotechnological process. 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 (Patrick *et al.,* 2009).

Agitation is another factor which affects laccase production. Tavares *et al.,* (2006) observed that agitation did not play any role in the production of laccase by *T. versicolour*. Hess *et al.,* (2002) found that mycelia are damaged when fungus is grown in the stirred tank reactor and laccase production by *Trametes multicolour* is considerably decreased. Mohorcic *et al.,*

(2004) found that cultivation of white-rot fungus *Bjerkandera adusta* in a stirred tank reactor with very low activities was obtained.

The optimum temperature for laccase production was found to be 30°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°c (Ko *et al.,* 2001). But the enzyme was active over higher temperature range also. After 1 hour, laccase activity at 70°c decreased only by 28.42%.

Guaiacol did not showed any marked effect (Ikehata *et al.,* 2004). The promoter region encoding for laccase contains various recognition sites that are specific for xenobiotic. They bind to the recognition sites and induce laccase production (Faraco *et al.,* 2002).

In general, laccase respond similarly to several inhibitors of enzyme activity (Bollang and Leonowicz, 1984). In a study conducted by Bollang and Leonowicz (1984) it was found that azide, thioglycolic acid and diethyldithiocarbamic acid all inhibited laccase activity, whereas EDTA affected laccase activity to a lesser extent. In spite of inhibition, sodium azide showed inducing effect which might be due to increase in fungal biomass in the culture because azide ion mainly decreases the bacterial contamination in the culture. Small anions such as halides (excluding iodide), azide, cyanide and hydroxide bind to type 2 and type 3 copper, resulting in an interruption of internal electron transfer and activity inhibition.

Fungi require essential metal ions such as Mg^{++}, Ca^{++}, Mn^{++}, Zn^{++} or Cu^{2+} as cofactors/prosthetic groups of different metabolic enzymes but these metals are toxic when present in excess (Srinivasan and Murthy, 2000). Copper as micronutrients has a key role as a metal activator, induces both laccase transcription and plays an important role in laccase production (Palmeri *et al.,* 2000). Hence the effect of copper on laccase production was studied under optimized culture conditions but there was no marked effect of copper in laccase production.

V_{max} varies with the source of laccase (50–300 M/s). The kinetic constants differ in their dependence on pH but K_m 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.

This result is lower than that reported by Chakroun et al. (2010) who found that the Km value of *T. atroviride* laccase enzyme was 2.5 mM towards ABTS. Robles et al. (2002) reported that the Km of laccase from Chalara (syn. Thielaviopsis) paradoxa CH 32 by using ABTS as a substrate at pH 4.5 was 0.77 mM. While the Km 1.6 mM was found with *Ceriporiopsis subvermispora* at pH 3.5 using Guaiacol (Fukushima and Kirk, 1995). This observation of the different Km values confirms the suggestion of Banerjee and Vohra (1991) who attributed these differences to be due to alteration of culture conditions, pH and substrate used.

Other investigations said that *Ganoderma lucidum* laccase had K_m value of 3.7 mM for ABTS (Ko *et al.,* 2001) and 0.114 M (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 V_{max} was found to be 100 µM/min which is similar to that of *C. thermophilium* laccase (Benny chefetz, 1998) and K_m was 0.111 mM. 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 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)Wang *et al.,* 2010), and this variation could be attributed to the different ecological origins of each species or different culture conditions. In this study single band of 43 kDa was observed which is in agreement with Murugesan *et al.,* 2007). There may be isoforms of laccase but the isoforms were not visible in SDS PAGE which may be due to low concentration of protein. But some studies have also shown that 5 different isoforms of laccase with molecular weights from 40 to 66 kDa can be obtained from *Ganoderma* (Ko *et al.,* 2001). 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).

CHAPTER VI: SUMMARY

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is an extracellular enzyme that belongs to the blue multi-copper oxidases group. The broad specificity enzyme has the ability to oxidize wide range of aromatic compound especially phenolic compounds through radical-catalyzed mechanism involving four electrons reduction of oxygen molecule into water. Hence, laccase has been used in many biotechnological applications especially in pulp and paper industry, wastewater treatment, bioremediation, biosensor, etc. Laccase is mostly produced by white rot fungi which belong to the basidiomycetes. In this study, laccase was produced by a white rot fungus *Pestalotiopsis* sp. using submerged fermentation.

Different type of laccase producing microorganisms like actinomycetes, streptomycetes and fungi were isolated and their laccase producing activities were screened. Out of total fifty three isolates (30 were Actinomycetes, 20 were previously isolated Streptomycetes from our lab, 3 were fungal isolates), 12 isolates showed laccase positive test in different used media. Four and nine isolates showed laccase positive test in Guaicol and Tannic acid respectively. In 1-naphthol media none of the isolate were able to grow at used concentration. Genomic DNA was extracted from Isolate “G1” and PCR showed that the size was 658bp. The sequencing result was used to construct phylogenetic tree and the fungus was identified as *Pestalotiopsis sp*. was identified as the genus name. This study demonstrates that *Pestalotiopsis sp*. is an endophytic white-rot fungi which secretes extracellular laccase as the ligninolytic enzymes.

The laccase production was optimized in PDB containing 1% Kraft lignin which enhanced laccase production. pH and temperature optimization showed pH 5 and temperature 30°C were good to get highest laccase activity. Maximum laccase activity was observed at 200 RPM on 8th day. Maintaining agitation rate constant i.e 200 rpm, Dissolved oxygen was optimized which displayed maximum laccase activity at 40 DO (57.33± 2.33) on 6th day. During DO optimization, it was found that the days for maximum laccase activity was also going to be reduced. Among used different concentrations of different inducers, 1mM gallic acid induces maximum laccase activity (69.22± 10.84 U/ml) followed by vanillin (67.88± 14.92 U/ml) whereas guiaicol did not increases laccase activity. All concentrations of cysteine inhibited slightly but 0.5 and 2 mM EDTA had not inhibited. All concentrations of sodium azide and 1 mM EDTA gave opposite result. In spite of inhibition, sodium azide and 1 mM EDTA increases the laccase activity highly. Different concentrations of metal ions increases the laccase activity at different rates. 1 mM Magnesium sulphate gave highest laccase activity (50.52± 24.75U/ml) followed by 2 mM magnesium sulphate and then Zinc sulphate. This investigation said that Tween-80 was good surfactant than Tween-20. 0.015 gm/l Tween-80 is best surfactant than the rest concentrations.

The produced laccase from fermentation broth was partially purified by ammonium sulphate precipitation and acetone precipitation method. Specific activity of acetone precipitation was found to be 31.76 U/µg. *Pestalotiopsis sp.* laccase showed optimum temperature 60°C and 40°C is the most suitable tolerable temperature. The optimum pH of this laccase is 6 and this enzyme is most tolerable at pH 5. Acetone precipitation method gave positive SDS page result while Ammonium sulphate precipitation method didn't. Vmax and Km were found to be 100 µmol/min and 0.111 mM respectively and Kcat was found to be 33.195per minute.

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. During SDS- PAGE only single band was observed. 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 hyper-secretory 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.

CHAPTER VII: CONCLUSION AND RECOMMENDATION

Laccases are ubiquitous enzymes produced by many living species. Fungi produces laccases to oxidize lignin in order to get access to nutrients. This oxidative capacity has been used for various applications, including the removal/degradation of recalcitrant organic pollutants and for the synthesis of organic compounds. Laccase plays an important role in the carbon cycle and could help in degrading in wide range of xenoaromatics. Laccases have become important, industrially relevant enzymes that can be used for a number of diverse applications such as delignification of lignocellulosics, bioremediation applications such as waste detoxification and textile dye transformation, food technologic uses, personal and medical care applications, biosensor and analytical applications. Therefore it is not surprising that this enzyme has been studied intensively and yet remains a topic of intense research today.

The present work was done to study the molecular, biochemical and kinetic properties of laccase from *Pestalotiopsis sp.* collected from fungi of Nepal. Molecular properties helped to know the size of PCR product and genus of isolated fungus. Biochemical properties of enzyme activity which are crucial for understanding the mechanism of catalysis of enzyme. This thesis work gives a brief idea about the fungus and laccase enzyme produced by it. This enzyme was effective in broad pH range and at higher temperatures which suggests that the enzyme can be used over wide pH range and even at higher temperature without significant loss of enzyme activity. It had low Km value for ABTS (non-phenolic substrate).

Recommendations:

a. To study depolymerization of dye and waste water.

b. To clone laccase coding gene into suitable host.

c. To immobilize the laccase enzyme and study of its efficacy.

CHAPTER VIII: REFERENCES

Abari A, Emtiazi G, Roghanian R (2012) Production of none germinate spore ghost from a novel marine Bacillus with thermostable laccase activity. *African Journal of Microbiology Research*. **6**(2): 393-402.

Adler, E. (1977). Lignin chemistry past, present and future. Wood Science and Technology, 11(3), 169-218. doi:10.1007/bf00365615

Aggelis G, Iconomou D, Christou M, Bokas D, Kotzailias S, Christou G, Tsagou V and Papanikolaou S (2003) Phenolic removal in a model olive oil mill wastewater using< i> Pleurotus ostreatus</i> in bioreactor cultures and biological evaluation of the process. *Water Research*. **37**(16): 3897-3904.

Alcalde M. (2007) Laccase: biological functions, molecular structure and industrial applications. In industrial enzymes: structure, functions and applications. . J Polaina and AP MacCabe, eds (Dordrecht, The Netherlands: Springer) 459-74.

Aly, A. H., Debbab, A., Kjer, J., & Proksch, P. (2010). Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products.Fungal Diversity, 41(1), 1-16. doi:10.1007/s13225-010-0034-4

Annele Hatakka (1), & Kenneth E. Hammel (2). (1970, January 01). Fungal Biodegradation of Lignocelluloses. Retrieved July 05, 2017, from http://link.springer.com/chapter/10.1007/978-3-642-11458-8_15

Antorini M, Herpoël-Gimbert I, Choinowski T, Sigoillot J-C, Asther M, Winterhalter K and Piontek K (2002) Purification, crystallisation and X-ray diffraction study of fully functional laccases from two ligninolytic fungi. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*. **1594**(1): 109-114.

Aramayo R and Timberlake WE (1990) Sequence and molecular struture of the Aspergilus nidulans yA (laccase I) gene. *Nucleic acids research*. **18**(11): 3415-3415.

Aramayo R and Timberlake WE (1993) The Aspergillus nidulans yA gene is regulated by abaA. *The EMBO journal*. **12**(5): 2039.

Assavanig A, Amornikitticharoen B, Ekpaisal N, Meevootisom V and Flegel TW (1992) Isolation, characterization and function of laccase from Trichoderma. *Applied Microbiology and biotechnology*. **38**(2): 198-202.

Baldrian P (2003) Interactions of heavy metals with white-rot fungi. *Enzyme and Microbial Technology*. **32**(1): 78-91.

Baldrian P (2006) Fungal laccases–occurrence and properties. *FEMS microbiology reviews*. **30**(2): 215-242.

Baldrian, P. and Gabriel, J. (2002). Copper and Cadmium increase laccase activity in Pleurotus ostreatus. *FEMS Microbiol. Lett*. 206:69-74.

Banci L, Ciofi-Baffoni S and Tien M (1999) Lignin and Mn peroxidase-catalyzed oxidation of phenolic lignin oligomers. *Biochemistry*. **38**(10): 3205-3210.

Banerjee, U.C., Vohra, R.M., (1991). Production of laccase by Curvularia sp. Folia Microbial. 36, 343.

Baratto L, Candido A, Marzorati M, Sagui F, Riva S and Danieli B (2006) Laccase-mediated oxidation of natural glycosides. *Journal of Molecular Catalysis B: Enzymatic*. **39**(1): 3-8.

Barbosa A, Dekker R and Hardy GS (1996) Veratryl alcohol as an inducer of laccase by an ascomycete, Botryosphaeria sp., when screened on the polymeric dye Poly R-478. *Letters in Applied Microbiology*. **23**(2): 93-96.

Barilli A, Belinghieri F, Passarella D, Lesma G, Riva S, Silvani A and Danieli B (2004) Enzyme assisted enantioselective synthesis of the alkaloid (+)-aloperine. *Tetrahedron: asymmetry*. **15**(18): 2921-2925.

Barrière F, Ferry Y, Rochefort D and Leech D (2004) Targetting redox polymers as mediators for laccase oxygen reduction in a membrane-less biofuel cell. *Electrochemistry Communications*. **6**(3): 237-241.

Barton SC, Chen T, Heller A, Kim H-H, Mano N, Mao F and Zhang Y (2003). Miniature biological fuel cell that is operational under physiological conditions, and associated devices and methods, WO Patent App. PCT/US2003/013,806.

Bauer CG, Kühn A, Gajovic N, Skorobogatko O, Holt P-J, Bruce NC, Makower A, Lowe CR and Scheller FW (1999) New enzyme sensors for morphine and codeine based on morphine dehydrogenase and laccase. *Fresenius' journal of analytical chemistry*. **364**(1-2): 179-183.

Benny C. and Yitzhak H. (1998) Purification and Characterization of Laccase from Chaetomium thermophilium and Its Role in Humification. *American Society for Microbiology*. **64**(9): 3175–3179

Bermek H, Li K, Eriksson K-EL. (1998) Laccase-less mutants of the white rot fungus Pycnoporus cinnabarins cannot de-lignify kraft pulp. *J Biotechnol* 66:117-124.

Blanchette RA (1995) Degradation of the lignocellulose complex in wood. *Canadian Journal of Botany*. **73**(S1): 999-1010.

Boh B and Berovic M (2007) Grifola frondosa (Dicks.: Fr.) SF Gray (Maitake mushroom): Medicinal properties, active compounds, and biotechnological cultivation. *International Journal of Medicinal Mushrooms* 9:89-108

Böker R (1990) Untersuchungen zu Wirkstoffsynthesen und Fermentationen an Atropurpurascens und Cyathus striatus (Basidomycetes). Dissertation, Universität Kaiserslautern, Kaiserslautern, Germany

Bollag J-M and Leonowicz A (1984) Comparative studies of extracellular fungal laccases. *Applied and Environmental Microbiology*. **48**(4): 849-854.

Bourbonnais R and Paice MG (1990) Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. *FEBS letters*. **267**(1): 99-102.

Bourbonnais R, Leech D and Paice MG (1998) Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochimica et Biophysica Acta (BBA)-General Subjects*. **1379**(3): 381-390.

Bourbonnais R, Paice M, Reid I, Lanthier P and Yaguchi M (1995) Lignin oxidation by laccase isozymes from Trametes versicolor and role of the mediator 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. *Applied and Environmental Microbiology*. **61**(5): 1876-1880.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*. **72**(1): 248-254.

Braun S and Vecht-Lifshitz SE (1991) Mycelial morphology and metabolite production. *Trends in Biotechnology* 9:63-68

Brown NL, Barrett SR, Camakaris J, Lee BT and Rouch DA (1995) Molecular genetics and transport analysis of the copper-resistance determinant (pco) from Escherichia coli plasmid pRJ1004. *Molecular microbiology*. **17**(6): 1153-1166.

Bulter T, Alcalde M, Sieber V, Meinhold P, Schlachtbauer C and Arnold FH (2003) Functional expression of a fungal laccase in Saccharomyces cerevisiae by directed evolution. *Applied and Environmental Microbiology*. **69**(2): 987-995.

Buswell JA, Cai Y and Chang St (1995) Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by Lentinula (Lentinus) edodes. *FEMS microbiology letters*. **128**(1): 81-87.

Call H and Mücke I (1997) History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). *Journal of Biotechnology*. **53**(2): 163-202.

Camarero J, Sort J, Hoffmann A, García-Martín JM, Dieny B, Miranda R and Nogués J (2005) Origin of the asymmetric magnetization reversal behavior in exchange-biased systems: Competing anisotropies. *Physical review letters*. **95**(5): 057204.

Carmichael RD and Pickard MA (1989) Continuous and batch-production of chloroperoxidase by mycelial pellets of Caldariomyces fumago in an airlift fermenter. Applied and Environmental Microbiology 55:17-20

Carpita, N. C. (1996). Structure And Biogenesis Of The Cell Walls Of Grasses.Annual Review of Plant Physiology and Plant Molecular Biology, 47(1), 445-476. doi:10.1146/annurev.arplant.47.1.445

Carter DN, McKenzie D, Johnson A and Idner K (1997) Performance parameters of oxygen delignification. *Tappi journal*. **80**(10): 111-117.

Cassland P and Jönsson L (1999) Characterization of a gene encoding Trametes versicolor laccase A and improved heterologous expression in Saccharomyces cerevisiae by decreased cultivation temperature. *Applied Microbiology and biotechnology*. **52**(3): 393-400.

Chai CC (2008). Enhanced Production Of Lignin Peroxidase And Manganese Peroxidase By Phanerochaete Chrysosporium In A Submerged Culture Fermentation And Their Application In Decolourisation Of Dyes TD897.[C434 2008 f rb], Universiti Sains Malaysia.

Chakroun, H., Mechichi, T., Martinez, M.J., Dhouib, A., Sayadi, S., (2010). Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: application on bioremediation of phenolic compounds. *Process Biochem*. 45, 507–513.

Chefetz B, Chen Y and Hadar Y (1998) Purification and characterization of laccase fromChaetomium thermophilium and its role in humification. *Applied and Environmental Microbiology*. **64**(9): 3175-3179.

Chen C-Y, Yeh K-L, Aisyah R, Lee D-J and Chang J-S (2011) Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. *Bioresource Technology*. **102**(1): 71-81.

Chivukula M and Renganathan V (1995) Phenolic azo dye oxidation by laccase from Pyricularia oryzae. *Applied and Environmental Microbiology*. **61**(12): 4374-4377.

Choi SH (2003). Implantable biofuel cell system based on nanostructures, Google Patents.

Chunxia Lu HW, Yuanming Luo, Lin Guo Abdel-Banat, B. M. A. (2010) An efficient system for pre-delignification of gramineous biofuel feedstock. *Elsevier* **45**: 1141–1147.

Claus H (2004) Laccases: structure, reactions, distribution. *Micron*. **35**(1): 93-96.

Claus H and Strong PJ (2009) Laccase. *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology*.

Colberg P and Young L (1985) Anaerobic degradation of soluble fractions of [14C-lignin] lignocellulose. *Applied and Environmental Microbiology*. **49**(2): 345-349.

Collins, P.J. and. Dobson, A.D.W. (1997). Regulation of laccase gene transcription in Trametes versicolor. *Appl. Environ. Microbiol*. 63:3444-3450

Côté, W. A. (1977, January 01). Wood Ultrastructure in Relation to Chemical Composition. Retrieved July 05, 2017, from http://link.springer.com/chapter/10.1007/978-1-4615-8873-3_1

Couto, S. R., & Herrera, J. L. T. (2006). Industrial and biotechnological applications of laccases: *A review. Biotechnology Advances*, 24(5), 500-513.

Cui YQ, Okkerse WJ, van der Lans RGJM and Luyben KCAM (1998) Modelling and measurements of fungal growth and morphology in submerged fermentations. *Biotechnology and Bioengineering* 60:216-229

Cui YQ, van der Lans RGJM and Luyben KCAM (1997) Effect of agitation intensities on fungal morphology of submerged fermentation. *Biotechnology and Bioengineering* 55:715-726

D'Acunzo F, Galli C, Gentili P and Sergi F (2006) Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. *New Journal of Chemistry*. **30**(4): 583-591.

Daâssi, D., Frikha, F., Zouari-Mechichi, H., Belbahri, L., Woodward, S., & Mechichi, T. (2012). Application of response surface methodology to optimize decolourization of dyes by the laccase-mediator system. *Journal of Environmental Management*, 108(0), 84-91.

Di Fusco, M., Tortolini, C., Deriu, D., & Mazzei, F. (2010). Laccase-based biosensor for the determination of polyphenol index in wine. *Talanta,* 81(1–2), 235-240.

Diener, U. L., Morgan-Jones, G., Wagener, R. E., & Davis, N. D. (1981). Toxigenicity of fungi from grain sorghum. Mycopathologia, 75(1), 23-26. doi:10.1007/bf00439062

Dombrovskaya, E. N. and Kostyshin, S. S. (1996). Effects of surfactants of different ionic nature on the ligninolytic complexes of the white rot fungi Pleurotus floridae and Phellinus igniarius. *Biochem. (Moscow).* 61:215-219

Dosoretz CG, Chen AHC and Grethlein HE (1990) Effect of oxygenation conditions on submerged cultures of Phanerochaete chrysosporium. *Applied Microbiology and Biotechnology* 34:131-137

D'souza S (2001) Microbial biosensors. *Biosensors and Bioelectronics*. **16**(6): 337-353.

D'Souza TM, Merritt CS and Reddy CA (1999) Lignin-modifying enzymes of the white rot basidiomycete Ganoderma lucidum. *Appl Environ Microbiol*. **65**(12): 5307-5313.

Ducros V, Brzozowski AM, Wilson KS, Brown SH, Østergaard P, Schneider P, Yaver DS, Pedersen AH and Davies GJ (1998) Crystal structure of the type-2 Cu depleted laccase from Coprinus cinereus at 2.2 Å resolution. *Nature Structural & Molecular Biology*. **5**(4): 310-316.

Durán N, Rosa MA, D'Annibale A and Gianfreda L (2002) Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme and Microbial Technology*. **31**(7): 907-931.

Durbeej, B., Wang, Y., & Eriksson, L. A. (2003). Lignin Biosynthesis and Degradation — a Major Challenge for Computational Chemistry. Lecture Notes in Computer Science High Performance Computing for Computational Science — VECPAR 2002, 137-165. doi:10.1007/3-540-36569-9_10

Dwivedi UN, Singh P, Pandey VP and Kumar A (2011) Structure–function relationship among bacterial, fungal and plant laccases. *Journal of Molecular Catalysis B: Enzymatic*. **68**(2): 117-128.

Edenhofer OR. (2011) Summary for policy makers. An intergovernmental panel for climate, change special report on renewable energy sources and climate change mitigation. *Cambridge university Press, Cambridge UK.*

Eggert C, Temp U and Eriksson K-E (1996) The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase. *Applied and Environmental Microbiology*. **62**(4): 1151-1158.

Eggert, C., Temp, U., & Eriksson, K. L. (1997). Laccase is essential for lignin degradation by the white-rot fungusPycnoporus cinnabarinus. FEBS Letters,407(1), 89-92. doi:10.1016/s0014-5793(97)00301-3

Eggert, C., Temp, U., Dean, J. F., & Eriksson, K. L. (1996). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Letters, 391(1-2), 144-148. doi:10.1016/0014-5793(96)00719-3

Endo K, Hayashi Y, Hibi T, Hosono K, Beppu T and Ueda K (2003) Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by Streptomyces griseus. *Journal of biochemistry*. **133**(5): 671-677.

Enguita FJ, Marçal D, Martins LO, Grenha R, Henriques AO, Lindley PF and Carrondo MA (2004) Substrate and dioxygen binding to the endospore coat laccase from Bacillus subtilis. *Journal of Biological Chemistry*. **279**(22): 23472-23476.

Eriksson K-EL, Blanchette RA and Ander P (1990)*Microbial and enzymatic degradation of wood and wood components*, Springer-Verlag

Fåhraeus G and Reinhammar B (1967) Large scale production and purification of laccase from cultures of fungus *Polyporus versicolor* and some properties of laccase A. *Acta Chemica Scandinavica* 21:2367-2378

Fang QH and Zhong JJ (2002) Two-stage culture process for improved production of ganoderic acid

Faraco V, Giardina P, Palmieri G and Sannia G (2002) Metal-activated laccase promoters. *Progress in Biotechnology*. **21**: 105-111.

Farnet A, Criquet S, Tagger S, Gil G and Petit JL (2000) Purification, partial characterization, and reactivity with aromatic compounds of two laccases from Marasmius quercophilus strain 17. *Canadian journal of microbiology*. **46**(3): 189-194.

Farnet A-M, Tagger S and Petit JL (1999) Effects of copper and aromatic inducers on the laccases of the white-rot fungus< i> Marasmius quercophilus</i>. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie*. **322**(6): 499-503.

Farneth WE, Hasty NM, Damore MB and Chisholm DA (2004). Oxygen scavenging compositions and methods of use, Google Patents.

Fazenda ML, Seviour R, McNeil B and Harvey LM (2008) Submerged culture fermentation of "higher fungi": the macrofungi. *Advances in Applied Microbiology* 63:33-103

Fenegel D, Wegener G. (1984) Wood: chemistry, ultrastructure, reactions. de Gruyter Inc., Berlin.

Fenice M, Giovannozzi SG, Federici F and D'Annibale A (2003) Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. *Journal of Biotechnology* 100:77-85

Fernandes SC, de Oliveira IRW, Fatibello-Filho O, Spinelli A and Vieira IC (2008) Biosensor based on laccase immobilized on microspheres of chitosan crosslinked with tripolyphosphate. *Sensors and Actuators B: Chemical*. **133**(1): 202-207.

Ferraroni M, Myasoedova NM, Schmatchenko V, Leontievsky AA, Golovleva LA, Scozzafava A and Briganti F (2007) Crystal structure of a blue laccase from Lentinus tigrinus: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. *BMC Structural Biology*. **7**(1): 60.

Fukushima Y and Kirk TK (1995) Laccase component of the Ceriporiopsis subvermispora lignin-degrading system. *Applied and Environmental Microbiology*. **61**(3): 872-876.

Galhaup C, Wagner H, Hinterstoisser B and Haltrich D (2002) Increased production of laccase by the wood-degrading basidiomycete< i> Trametes pubescens</i>. *Enzyme and Microbial Technology*. **30**(4): 529-536.

Garavaglia S, Teresa Cambria M, Miglio M, Ragusa S, Iacobazzi V, Palmieri F, D'Ambrosio C, Scaloni A and Rizzi M (2004) The Structure of *Rigidoporus lignosus* Laccase Containing a Full Complement of Copper Ions, Reveals an Asymmetrical Arrangement for the T3 Copper Pair. *Journal of molecular biology*. **342**(5): 1519-1531.

Gardner, K., & Blackwell, J. (1974). The hydrogen bonding in native cellulose.Biochimica et Biophysica Acta (BBA) - General Subjects, 343(1), 232-237. doi:10.1016/0304-4165(74)90256-6

Gehrig I, Bart HJ, Anke T and Germerdonk R (1998) Influence of morphology and rheology on the production characteristics of the basidiomycete *Cyathus striatus*. *Biotechnology and*

Germann, U. A., & Lerch, K. (1986). Isolation and partial nucleotide sequence of the laccase gene from Neurospora crassa: amino acid sequence homology of the protein to human ceruloplasmin. Proceedings of the National Academy of Sciences, 83(23), 8854-8858. doi:10.1073/pnas.83.23.8854

Ghindilis A (2000) Direct electron transfer catalysed by enzymes: application for biosensor development. *Biochemical Society Transactions*. **28**(2): 84.

Gianfreda, L., Xu, F., & Bollag, J. (1999). Laccases: A Useful Group of Oxidoreductive Enzymes. Bioremediation Journal, 3(1), 1-26. doi:10.1080/10889869991219163

Giardina P, Cannio R, Martirani L, Marzullo L, Palmieri G and Sannia G (1995) Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete Pleurotus ostreatus. *Applied and Environmental Microbiology*. **61**(6): 2408-2413.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and G. Sannia. 2009. Laccases: a never-ending story. *Cellular and Molecular Life Sciences*. doi 10.1007/s00018-009-0169-1

Gil, D. A., & Rebelo, M. F. (2010). Evaluating the antioxidant capacity of wines: a laccase-based biosensor approach. *European Food Research and Technology*, 231(2), 303-308.

Giorno, L., and E. Drioli. 2000. Biocatalytic membrane reactors: applications and perspectives. TIBTECH. 18:339-349.

Givaudan A, Effosse A, Faure D, Potier P, Bouillant M-L and Bally R (1993) Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: Evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. *FEMS microbiology letters*. **108**(2): 205-210.

Gomez-Alarcon, T., Lohoz, G., and Molina, D. 1989. Production of extracellular enzymes during growth and autolysis of Pycnoporus cinnabanhus. J. *Basic Microbiol*. 29: 23-29.

Graves C, Ebbesen SD, Mogensen M and Lackner KS (2011) Sustainable hydrocarbon fuels by recycling CO_2 and H_2O with renewable or nuclear energy. *Renewable and Sustainable Energy Reviews*. **15**(1): 1-23.

Grimm LH, Kelly S, Krull R and Hempel DC (2005) Morphology and productivity of filamentous fungi. *Applied Microbiology and Biotechnology* 69:375-384

Gross GG. (1980) The biochemistry of lignifications. In: Advances in botanical research. pp.25-63. Academic Press Inc., London.

Hadibarata, T., Yusoff, A., Aris, A., Salmiati, Hidayat, T., & Kristanti, R. (2012). Decolorization of Azo, Triphenylmethane and Anthraquinone Dyes by Laccase of a Newly Isolated Armillaria sp. F022. Water, Air, & Soil Pollution, 223(3), 1045-1054.

Han M, Choi H and Song H (2005) Purification and characterization of laccase from the white rot fungus Trametes versicolor. *Journal of Microbiology-seoul-*. **43**(6): 555.

Harkin JM, Larsen MT, Obst JR. (1974) Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. *Mycolog Soc Amer* 66(3):469-476

Harkin JM, Obst JR. (1973) Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi.Experienta 29(4):381-508

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS microbiology reviews*. **13**(2): 125-135.

Hatakka A, Steffen K, Tuomela M and Hofrichter M (2001) Fungal processes for bioremediation. *Proceedings of the First European Bioremediation Conference* 2-5

Heinzkill M, Bech L, Halkier T, Schneider P and Anke T (1998) Characterization of laccases and peroxidases from wood-rotting fungi (family Coprinaceae). *Applied and Environmental Microbiology*. **64**(5): 1601-1606.

Ho Wong J, Bun Ng T, Jiang Y, Liu F, Cho Wing Sze S and Yanbo Zhang K (2010) Purification and characterization of a laccase with inhibitory activity toward HIV-1 reverse transcriptase and

tumor cells from an edible mushroom (Pleurotus cornucopiae). *Protein and Peptide Letters*. **17**(8): 1040-1047.

Hu HL, Jeewon R, Zhou DQ, Zhou TX, Hyde KD (2007) Phylogenetic diversity of endophytic Pestalotiopsis species in Pinus armandii and Ribes spp.: evidence from rDNA and β- tubulin gene phylogenies. Fungal Divers 24:1–22

Jenkins, P., Tuurala, S., Vaari, A., Valkiainen, M., Smolander, M., & Leech, D. (2012). A mediated glucose/oxygen enzymatic fuel cell based on printed carbon inks containing aldose dehydrogenase and laccase as anode and cathode. *Enzyme and Microbial Technology*, 50(3), 181-187.

Jeon J-R, Murugesan K, Kim Y-M, Kim E-J and Chang Y-S (2008) Synergistic effect of laccase mediators on pentachlorophenol removal by Ganoderma lucidum laccase. *Applied Microbiology and biotechnology*. **81**(4): 783-790.

Jiménez-Tobon G, Kurzatkowski W, Rozbicka B, Solecka J, Pocsi I and Penninckx MJ (2003) In situ localization of manganese peroxidase production in mycelial pellets of Phanerochaete chrysosporium. *Microbiology-SGM* 149:3121-3127

Jönsson LJ, Saloheimo M and Penttilä M (1997) Laccase from the white-rot fungus Trametes versicolor: cDNA cloning of lcc1 and expression in Pichia pastoris. *Current genetics*. **32**(6): 425-430.

Joo SS, Lee DI and Hwang KW (2010) Inhibition of Cytokine-Induced β Cell Apoptosis via Laccase and Its Therapeutic Advantages for Insulin-Dependent Diabetes Mellitus, Type 1 Diabetes. *Biological and Pharmaceutical Bulletin*. **33**(11): 1854-1860.

Joo SS, Ryu IW, Park J, Yoo YM, Lee D, Hwang KW, Choi H, Lim C, Lee DI and Kim K (2008) Molecular Cloning and Expression of a Laccase from Ganoderma Iucidum, and Its Antioxidative Properties. *Molecules and cells*. **25**(1): 112.

Jurado M, Prieto A, Martinez-Alcalá A, Martinez AT, Martinez MJ. (2009) Laccase detoxification of steamexplodedwheat straw for second generation. *Biores Technol,* 100:6378-6384.

Karamyshev AV, Shleev SV, Koroleva OV, Yaropolov AI and Sakharov IY (2003) Laccase-catalyzed synthesis of conducting polyaniline. *Enzyme and Microbial Technology*. **33**(5): 556-564.

Karos M, Subkowski T, Lemaire H-G, Bollschweiler C, Nüßlein B and Fischer R (2006). Verwendung von Laccasen als Reportergene, DE Patent 102,004,047,409.

Kelly S, Grimm LH, Jonas R, Hempel DC and Krull R (2006) Investigations of the morphogenesis of filamentous microorganisms. *Engineering in Life Sciences* 6:475-480

Kersten, P., & Cullen, D. (2007). Extracellular oxidative systems of the lignin-degrading Basidiomycete Phanerochaete chrysosporium. Fungal Genetics and Biology, 44(2), 77-87. doi:10.1016/j.fgb.2006.07.007

Kiiskinen L-L, Kruus K, Bailey M, Ylösmäki E, Siika-aho M and Saloheimo M (2004) Expression of Melanocarpus albomyces laccase in Trichoderma reesei and characterization of the purified enzyme. *Microbiology*. **150**(9): 3065-3074.

Kiiskinen LL, Rättö M and Kruus K (2004) Screening for novel laccase-producing microbes. *Journal of applied microbiology*. **97**(3): 640-646.

Kim C, Lorenz WW, Hoopes JT and Dean JF (2001) Oxidation of phenolate siderophores by the multicopper oxidase encoded by the Escherichia coli yacK gene. *Journal of Bacteriology*. **183**(16): 4866-4875.

Kim YS and Singh AP (2000) Micromorphological characteristics of wood biodegradation in wet environments: a review. *IAWA journal*. **21**(2): 135-155.

Kirk TK and Cullen D (1998) Enzymology and molecular genetics of wood degradation by white-rot fungi. *Young R, Akhtar M, editors. Environmentally friendly technologies for the pulp and paper industry. New York: John Wiley and Sons, Inc*.

Kirk TK and Farrell RL (1987) Enzymatic" combustion": the microbial degradation of lignin. *Annual Reviews in Microbiology*. **41**(1): 465-501.

Kirk TK, Schultz E, Connors WJ, Lorenz LF and Zeikus JG (1978) Influence of culture parameters on lignin metabolism by Phanerochaete chrysosporium. *Archives of Microbiology* 117:277-285

Klonowska A, Gaudin C, Asso M, Fournel A, Réglier M and Tron T (2005) LAC3, a new low redox potential laccase from *Trametes sp.* strain C30 obtained as a recombinant protein in yeast. *Enzyme and Microbial Technology*. **36**(1): 34-41.

Ko EM, Leem YE and Choi HT (2001) Purification and characterization of laccase isozymes from the white-rot basidiomycete Ganoderma lucidum. *Appl Microbiol Biotechnol*. **57**(1-2): 98-102.

Kojima Y, Tsukuda Y, Kawai Y, Tsukamoto A, Sugiura J, Sakaino M and Kita Y (1990) Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete Coriolus hirsutus. *The Journal of biological chemistry*. **265**(25): 15224-15230.

Koo, B., Min, B., Gwak, K., Lee, S., Choi, J., Yeo, H., & Choi, I. (2012). Structural changes in lignin during organosolv pretreatment of Liriodendron tulipifera and the effect on enzymatic hydrolysis. Biomass and Bioenergy, 42, 24-32. doi:10.1016/j.biombioe.2012.03.012

Krogh KBRM. (2008) Biomass degrading enzymes from Penicillium – cloning and characterization. Dissertation, University of Denmark.

Kuhad RC, Singh A and Eriksson K-EL (1997)*Microorganisms and enzymes involved in the degradation of plant fiber cell walls*, Springer

Kumarasamy Murugesan I-HN, Young-Mo Kim, Yoon-Seok Chang (2006) Decolorization of reactive dyes by a thermostable laccase produced by Ganoderma lucidum in solid state culture. *Elsevier*. 790-784.

Kunamneni A, Ballesteros A, Plou FJ and Alcalde M (2007) Fungal laccase—a versatile enzyme for biotechnological applications. *Communicating current research and educational topics and trends in applied microbiology*. **1**: 233-245.

Kunamneni A, Camarero S, García-Burgos C, Plou FJ, Ballesteros A and Alcalde M (2008) Engineering and applications of fungal laccases for organic synthesis. *Microbial Cell Factories*. **7**(1): 32.

Kunamneni, A., Plou, F., Ballesteros, A., & Alcalde, M. (2008). Laccases and Their Applications: A Patent Review. Recent Patents on Biotechnology, 2(1), 10-24. doi:10.2174/187220808783330965

Laccase Enzymology in Relation to Lignocellulose Processing. (2017) Laccase-enzymology-in-relation-to-lignocellulose-processing.htm http://docplayer.net/46094845-

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**(5259): 680-685.

LaFayette PR, Eriksson K-EL and Dean JF (1999) Characterization and heterologous expression of laccase cDNAs from xylem tissues of yellow-poplar (Liriodendron tulipifera). *Plant molecular biology*. **40**(1): 23-35.

Larrondo LF, Avila M, Salas L, Cullen D and Vicuna R (2003) Heterologous expression of laccase cDNA from Ceriporiopsis subvermispora yields copper-activated apoprotein and complex isoform patterns. *Microbiology*. **149**(5): 1177-1182.

Larsson S, Cassland P and Jönsson LJ (2001) Development of a Saccharomyces cerevisiae strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. *Applied and Environmental Microbiology*. **67**(3): 1163-1170.

Leatham GF, Stahmann, Mark A (1981) Studies on the laccase of Lentinus edodes: specificity, localization and association with the development of fruiting bodies. *Journal of General Microbiology*. **125**(1): 147-157.

Lecault V, Patel N and Thibault J (2007) Morphological characterization and viability assessment of *Trichoderma reesei* by image analysis. *Biotechnology Progress* 23:734-740

Lee I-Y, Jung K-H, Lee C-H and Park Y-H (1999) Enhanced production of laccase in Trametes vesicolor by the addition of ethanol. *Biotechnology letters*. **21**(11): 965-968.

Leisola MSA, Thaneiwyss U and Fiechter A (1985) Strategies for production of high ligninase activities by Phanerochaete chrysosporium. *Journal of Biotechnology* 3:97-107

Leontievsky AA, Myasoedova NM, Baskunov BP, Evans C and Golovleva L (2000) Transformation of 2, 4, 6-trichlorophenol by the white rot fungi Panus tigrinus and Coriolus versicolor. *Biodegradation*. **11**(5): 331-340.

Leontievsky, A. A., Vares, T., Lankinen, P., Shergill, J. K., Pozdnyakova, N. N., Myasoedova, N. M., Kalkkinen, N., Golovleva, L. A., Cammack, R., Thurston, C. F., and A. Hatakka. (1997). Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiology Letters*.156:9-14.

Leštan, D., Leštan, M. and Perdih, A. (1994). Physiological aspects of Biosynthesis of Lignin Peroxidases by Phanerochaete chrysosporium. *Appl. Environ. Microbiol*. 60:606-612.

Levine WG (1966) Laccase, a review. *The biochemistry of copper. Academic Press Inc., New York*. 371-387.

Lin Z-B and Zhang H-N (2004) Anti-tumor and immunoregulatory activities of Ganoderma lucidum and its possible mechanisms. *Acta Pharmacologica Sinica*. **25**: 1387-1395.

Liu W, Chao Y, Liu S, Bao H and Qian S (2003) Molecular cloning and characterization of a laccase gene from the basidiomycete Fome lignosus and expression in Pichia pastoris. *Applied Microbiology and biotechnology*. **63**(2): 174-181.

Lloret L, Eibes G, Lú-Chau T, Moreira M, Feijoo G and Lema J (2010) Laccase-catalyzed degradation of anti-inflammatories and estrogens. *Biochemical Engineering Journal*. **51**(3): 124-131.

Lonergan G, Baker WL. (1995) Comparative study of substrates of fungal laccases. *Lett Appl Microbiol* 21:31-33.

Lynd, L. R., Weimer, P. J., Zyl, W. H., & Pretorius, I. S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiology and Molecular Biology Reviews, 66(3), 506-577. doi:10.1128/mmbr.66.3.506-577.2002

M EL-Fakharany E, M Haroun B, Ng T and M Redwan E-R (2010) Oyster mushroom laccase inhibits hepatitis C virus entry into peripheral blood cells and hepatoma cells. *Protein and Peptide Letters*. **17**(8): 1031-1039 .

Machczynski, M. C., Vijgenboom, E., Samyn, B., and G. W. Santers. 2004. Characterisation of SLAC: a small laccase from Streptomyces coelicolor with unprecedented activity. *Protein Science.* 13(9):2388-2397.

Madhavi V and Lele S (2009) Laccase: properties and applications. *BioResources*. **4**(4): 1694-1717.

Madzak C, Otterbein L, Chamkha M, Moukha S, Asther M, Gaillardin C and Beckerich JM (2005) Heterologous production of a laccase from the basidiomycete Pycnoporus cinnabarinus in the dimorphic yeast Yarrowia lipolytica. *FEMS yeast research*. **5**(6-7): 635-646.

Majcherczyk A, Johannes C and Hüttermann A (1998) Oxidation of Polycyclic Aromatic Hydrocarbons (PAH) by Laccase of *Trametes Versicolor*. *Enzyme and Microbial Technology*. **22**(5): 335-341.

Mansur M, Suarez T, Fernández-Larrea JB, Brizuela MA and Gonzalez AE (1997) Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. *Applied and Environmental Microbiology*. **63**(7): 2637-2646.

Márquez-Rocha FJ, Guillén GK, Sánchez JE and Vázquez-Duhalt R (1999) Growth characteristics of Pleurotus ostreatus in bioreactors. *Biotechnology Techniques* 13:29-32

Martinez AT, Speranza M, Ruiz-Dueñas FJ, Ferreira P, Camarero S, Guillén F, Martinez MJ, Gittiérez A, del Rio JC. (2005) Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attach of lignin. *Int Microbiol* 8:195-204.

Maruyama T, Komatsu C, Michizoe J, Ichinose H and Goto M (2006) Laccase-Mediated Oxidative Degradation of the Herbicide Dymron. *Biotechnology progress*. **22**(2): 426-430.

Marzorati M, Danieli B, Haltrich D and Riva S (2005) Selective laccase-mediated oxidation of sugars derivatives. *Green Chemistry*. **7**(5): 310-315.

Mayer, A. M., and R. C. Staples. 2002. Laccase: new functions for an old enzyme. Phytochemistry. 60:551-565.

McIntyre M, Dynesen J and Nielsen J (2001) Morphological characterization of *Aspergillus nidulans*: growth, septation and fragmentation. *Microbiology-UK* 147:239-246

Mellano MA and Cooksey DA (1988) Nucleotide sequence and organization of copper resistance genes from Pseudomonas syringae pv. tomato. *Journal of Bacteriology*. **170**(6): 2879-2883.

Messerschmidt A and huber R (1990) The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin Modelling and structural relationships. *European journal of Biochemistry*. **187**(2): 341-352.

Michel FC, Grulke EA and Reddy CA (1990) Development of a stirred tank reactor system for the production of lignin peroxidases (ligninases) by Phanerochaete chrysosporium Bkm-F-1767. *Journal of Industrial Microbiology* 5:103-112

Michel FC, Grulke EA and Reddy CA (1992) Determination of the respiration kinetics for mycelial pellets of Phanerochaete chrysosporium. *Applied and Environmental Microbiology* 58:1740-1745

Miele A (2009). New oxidative enzimes for industrial application, Università degli studi di Napoli Federico II.

Mikolasch A, Hammer E, Jonas U, Popowski K, Stielow A and Schauer F (2002) Synthesis of 3-(3, 4-dihydroxyphenyl)-propionic acid derivatives by N-coupling of amines using laccase. *Tetrahedron*. **58**(38): 7589-7593.

Min K-L, Kim Y-H, Kim YW, Jung HS and Hah YC (2001) Characterization of a Novel Laccase Produced by the Wood-Rotting Fungus *Phellinus ribis*. *Archives of Biochemistry and Biophysics*. **392**(2): 279-286.

Minussi RC, Pastore GM and Duran N (2002) Potential applications of laccase in the food industry. *Trends in Food Science & Technology*. **13**(6): 205-216.

Moilanen U, Kellock M, Galkin S, Viikari L. (2011) The laccase-catalyzed modification of lignin for enzymatic hydrolysis. *Enz Microb Technol* 49:492-498.

Molina-Guijarro, J. M., Pérez, J., Muñoz-Dorado, J., Guillén, F., Moya, R., Hernández, M., and M. E. Arias. 2009. Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from Streptomyces ipomoea. International Microbiology. 12:13-21.

Molino B, Haydar S, Yang Z, Michels P, Hemenway M, Rich J and Khmelnitsky Y (2004) Preparation of novel cyclosporins. *Patent WO2004082629 A2*.

More SS, PS R and Malini S (2011) Isolation, Purification, and Characterization of Fungal Laccase from Pleurotus sp. *Enzyme research*. **2011**.

Mougin C, Kollmann A and Jolivalt C (2002) Enhanced production of laccase in the fungus Trametes versicolor by the addition of xenobiotics. *Biotechnology letters*. **24**(2): 139-142.

Munoz C, Guillén F, Martinez A and Martínez M (1997) Laccase isoenzymes of Pleurotus eryngii: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn2+ oxidation. *Applied and Environmental Microbiology*. **63**(6): 2166-2174.

Murugesan, K., Nam, I., Kim, Y., & Chang, Y. (2007). Decolorization of reactive dyes by a thermostable laccase produced by Ganoderma lucidum in solid state culture. Enzyme and Microbial Technology, 40(7), 1662-1672. doi:10.1016/j.enzmictec.2006.08.028

Nyanhongo G, Gomes J, Gübitz G, Zvauya R, Read J and Steiner W (2002) Production of laccase by a newly isolated strain of< i> Trametes modesta</i>. *Bioresource Technology*. **84**(3): 259-263.

O. V. Morozova GPS, M. A. Gorbacheva, S. V. Shleev, and A. I. Yaropolov (2007) “Blue” Laccases. *Biochemistry* **72**(10): 1396-1412.

Osma JF, Toca-Herrera JL and Rodríguez-Couto S (2010) Uses of laccases in the food industry. *Enzyme research*. **2010**.

Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. (2017). Retrieved July 05, 2017, from http://www.sciencedirect.com/science/article/pii/001457939080298W

Palmeiri G, Giardina P, Marzullo L, Desiderio B, Nittii G, Cannio R and Sannia G (1993) Stability and activity of a phenol oxidase from the ligninolytic fungus Pleurotus ostreatus. *Applied Microbiology and biotechnology*. **39**(4-5): 632-636.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A., and G. Sannia. 1997. A novel white laccase from Pleurotus ostreatus. *Journal of Lipid Research*. 272(50):31301-31307.

Palmore GTR (2004) Bioelectric power generation. *TRENDS in Biotechnology*. **22**(3): 99-100.

Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances* 22:189-259

Patel I, Ludwig R, Haltrich D, Rosenau T and Potthast A (2011) Studies of the chemoenzymatic modification of cellulosic pulps by the laccase-TEMPO system. *Holzforschung*. **65**(4): 475-481.

Patrick F, Mtui G, Mshandete AM, Johansson G and Kivaisi A (2009) Purification and characterization of a laccase from the basidiomycete Funalia trogii (Berk.) isolated in Tanzania. *African Journal of Biochemistry Research*. **3**(5): 250-258.

Paul GC and Thomas CR (1998) Characterisation of mycelial morphology using image analysis. *Advances in Biochemical Engineering/Biotechnology* 60:1-59

Paul GC, Kent CA and Thomas CR (1993) Viability testing and characterization of germination of fungal spores by automatic image analysis. *Biotechnology and Bioengineering* 42:11-23

Pedroza, A. M., Mosqueda, R., Alonso-Vante, N., & Rodríguez-Vázquez, R. (2007). Sequential treatment via Trametes versicolor and UV/TiO2/RuxSey to reduce contaminants in waste water resulting from the bleaching process during paper production. *Chemosphere*, 67(4), 793-801.

Peláez, F., Martínez, M., & Martínez, A. (1995). Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. Mycological Research, 99(1), 37-42. doi:10.1016/s0953-7562(09)80313-4

Pinto, P. C., Evtuguin, D. V., & Neto, C. P. (2005). Chemical Composition and Structural Features of the Macromolecular Components of PlantationAcacia mangiumWood. Journal of Agricultural and Food Chemistry, 53(20), 7856-7862. doi:10.1021/jf058081b

Piontek K, Antorini M and Choinowski T (2002) Crystal Structure of a Laccase from the FungusTrametes versicolor at 1.90-Å Resolution Containing a Full Complement of Coppers. *Journal of Biological Chemistry*. **277**(40): 37663-37669.

Pita, M., Gutierrez-Sanchez, C., Olea, D., Velez, M., Garcia-Diego, C., Shleev, S., et al. (2011). High redox potential cathode based on laccase covalently attached to gold electrode. *The Journal of Physical Chemistry C*, 115(27), 13420-13428.

Pointing S and Vrijmoed L (2000) Decolorization of azo and triphenylmethane dyes by Pycnoporus sanguineus producing laccase as the sole phenoloxidase. *World Journal of Microbiology and Biotechnology*. **16**(3): 317-318.

Ponzoni C, Beneventi E, Cramarossa MR, Raimondi S, Trevisi G, Pagnoni UM, Riva S and Forti L (2007) Laccase-Catalyzed Dimerization of Hydroxystilbenes. *Advanced Synthesis & Catalysis*. **349**(8-9): 1497-1506.

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J and van den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. *Trends in Biotechnology* 20:200-206

Quaratino D, Fenice M, Federici F and D'Annibale A (2006) Mn-peroxidase production by Panus tigrinus CBS 577.79: response surface optimisation and bioreactor comparison. *Journal of Chemical Technology and Biotechnology* 81:832-840

Radzio R and Kück U (1997) Synthesis of biotechnologically relevant heterologous proteins in filamentous fungi. *Process Biochemistry* 32:529-539

Rajesh K, Dhanasekaran D, and Panneerselvam A (2014), Isolation and taxonomic characterization of medicinal mushroom *Ganoderma* spp. *Academia Journal of Microbiology Research* 2(2): 061-070 DOI: http://dx.doi.org/10.15413/ajmr.2014.0109

Ranocha P, Chabannes M, Chamayou S, Danoun Sd, Jauneau A, Boudet A-M and Goffner D (2002) Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. *Plant Physiology*. **129**(1): 145-155.

Record E, Punt PJ, Chamkha M, Labat M, van den Hondel CA and Asther M (2002) Expression of the Pycnoporus cinnabarinus laccase gene in Aspergillus niger and characterization of the recombinant enzyme. *European journal of Biochemistry*. **269**(2): 602-609.

Robles, A., Lucas, R., Martı´nez-Can˜ amero, M., Omar, N.B., Pe´rez, R., Ga´ lvez, A., (2002). Characterisation of laccase activity produced by the hyphomycete *Chalara (syn. Thielaviopsis) paradoxa* CH32. *Enzyme Microb. Technol*. 31, 516–522.

Rochefort D, Burbonnais R, Leech D, Paice MG. (2002) Oxidation of lignin model compounds by organic and transition metal-based electron transfer mediators. *Chem Commun* 11:1182-1183.

Rodri´guez Couto, S., Sanromán, M. A., Hofer, D., & Gübitz, G. M. (2004). Stainless steel sponge: a novel carrier for the immobilisation of the white-rot fungus Trametes hirsuta for decolourization of textile dyes. *Bioresource Technology*, 95(1), 67-72.

Rodríguez Couto S and Toca Herrera JL (2006) Industrial and biotechnological applications of laccases: a review. *Biotechnology Advances*. **24**(5): 500-513.

Rosgaard L, Pedersen S, Meyer AS. (2005) Fra holm til alkohol. Aktuel Naturvidenskab 3:21-25.

Rothschild, N., Hadar, Y. and Dosoretz, C. (1995). Ligninolytic System Formation by Phanerochaete chrysosporium in Air. Appl. Environ. Microbiol. 61: 1833-1838.

Ryan, D. R., Leukes, W. D., & Burton, S. G. (2005). Fungal bioremediation of phenolic wastewaters in an airlift reactor. *Biotechnology Progress*, 21(4), 1068-1074.

Saloheimo M and Niku-Paavola M-L (1991) Heterologous production of a ligninolytic enzyme: expression of the Phlebia radiata laccase gene in Trichoderma reesei. *Nature Biotechnology*. **9**(10): 987-990.

Scott SL, Chen WJ, Bakac A and Espenson JH (1993) Spectroscopic parameters, electrode potentials, acid ionization constants, and electron exchange rates of the 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) radicals and ions. *The Journal of Physical Chemistry*. **97**(25): 6710-6714.

Selig, M., Viamajala, S., Decker, S., Tucker, M., Himmel, M., & Vinzant, T. (2007). Deposition of Lignin Droplets Produced During Dilute Acid Pretreatment of Maize Stems Retards Enzymatic Hydrolysis of Cellulose. Biotechnology Progress, 23(6), 1333-1339. doi:10.1021/bp0702018

Selinheimo E, Kruus K, Buchert J, Hopia A and Autio K (2006) Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. *Journal of Cereal Science*. **43**(2): 152-159.

Servili M, De Stefano G, Piacquadio P and Sciancalepore V (2000) A novel method for removing phenols from grape must. *American journal of enology and viticulture*. **51**(4): 357-361.

Sharma KK, Shrivastava B, Sastry VR, Sehgal N and Kuhad RC (2013) Middle-redox potential laccase from Ganoderma sp.: its application in improvement of feed for monogastric animals. *Sci Rep*. **3**: 1299.

Sharma P, Goel R and Capalash N (2007) Bacterial laccases. *World Journal of Microbiology and Biotechnology*. **23**(6): 823-832.

Shleev S, Christenson A, Serezhenkov V, Burbaev D, Yaropolov A, Gorton L and Ruzgas T (2005) Electrochemical redox transformations of T1 and T2 copper sites in native Trametes hirsuta laccase at gold electrode. *Biochem. J*. **385**: 745-754.

Shleev S, Persson P, Shumakovich G, Mazhugo Y, Yaropolov A, Ruzgas T, Gorton L. (2006) Interaction of fungal laccases and laccase-mediator system with lignin. *Enz Microb Technol* 39:841-847.

Shrestha P, Joshi B, Joshi J, Malla R, Sreerama L (2016) Isolation and Physicochemical Characterization of Laccase from *Ganoderma lucidum*-CDBT1 Isolated from Its Native Habitat in Nepal. *Hindawi Publishing Corporation,* Volume 2016, Article ID 3238909, 10 pages, http://dx.doi.org/10.1155/2016/3238909

Simkus R, Laurinavicius V, Boguslavsky L, Skotheim T, Tanenbaum S, Nakas J and Slomczynski D (1996) Laccase containing sol-gel based optical biosensors. *Analytical letters*. **29**(11): 1907-1919.

Sjöström, E. (1993). Wood Polysaccharides. Wood Chemistry, 51-70. doi:10.1016/b978-0-08-092589-9.50007-3

Skálová T, Dohnálek J, Østergaard LH, Østergaard PR, Kolenko P, Dušková J, Štěpánková A and Hašek J (2009) The Structure of the Small Laccase from< i> Streptomyces coelicolor</i> Reveals a Link between Laccases and Nitrite Reductases. *Journal of molecular biology*. **385**(4): 1165-1178.

Soares, G. M. B., de Amorim, M. T. P., & Costa-Ferreira, M. (2001). Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. *Journal of Biotechnology*, 89(2–3), 123-129.

Soden D, O'callaghan J and Dobson A (2002) Molecular cloning of a laccase isozyme gene from Pleurotus sajor-caju and expression in the heterologous Pichia pastoris host. *Microbiology*. **148**(12): 4003-4014.

Soden DM and Dobson ADW (2001) Differential regulation of laccase gene expression in Pleurotus sajor-caju. *Microbiology*. **147**(7): 1755-1763.

Solano F, Garcia E, Perez D and Sanchez-Amat A (1997) Isolation and Characterization of Strain MMB-1 (CECT 4803), a Novel Melanogenic Marine Bacterium. *Applied and Environmental Microbiology*. **63**(9): 3499-3506.

Solomon, E. I., Sundaram, U. M., & Machonkin, T. E. (1996). Multicopper Oxidases and Oxygenases. Chemical Reviews, 96(7), 2563-2606. doi:10.1021/cr950046o

Srebotnik E and Hammel KE (2000) Degradation of nonphenolic lignin by the laccase/1-hydroxybenzotriazole system. *Journal of Biotechnology*. **81**(2): 179-188.

Stahl P, Kissau L, Mazitschek R, Giannis A and Waldmann H (2002) Natural Product Derived Receptor Tyrosine Kinase Inhibitors: Identification of IGF1R, Tie-2, and VEGFR-3 Inhibitors. *Angewandte Chemie International Edition*. **41**(7): 1174-1178.

Steyaert RL (1949) Contributions à l'étude monographique de Pestalotia de Not. et Monochaetia Sacc. (Truncatella gen. nov.et Pestalotiopsis gen. nov.). Bull. Jard. Bot. Bruxelles 19:285–354

Sun J, Wang H and Ng TB (2011) Isolation of a laccase with HIV-1 reverse transcriptase inhibitory activity from fresh fruiting bodies of the Lentinus edodes (Shiitake mushroom).

Susmel P and Stefanon B (1993) Aspects of lignin degradation by rumen microorganisms. *Journal of Biotechnology*. **30**(1): 141-148.

Tavares APM, Coelho MAZ, Coutinho JAP and Xavier AMRB (2005) Laccase improvement in submerged cultivation: induced production and kinetic modelling. *Journal of Chemical Technology and Biotechnology* 80:669-676

Tejesvi, M. V., Mahesh, B., Nalini, M. S., Prakash, H. S., Kini, K. R., Subbiah, V., & Shetty, H. S. (2005). Endophytic Fungal Assemblages from Inner Bark and Twig of Terminalia arjuna W. & A. (Combretaceae). World Journal of Microbiology and Biotechnology, 21(8-9), 1535-1540. doi:10.1007/s11274-005-7579-5

Telke AA, Ghodake GS, Kalyani DC, Dhanve RS and Govindwar SP (2011) Biochemical characteristics of a textile dye degrading extracellular laccase from a< i> Bacillus</i> sp. ADR. *Bioresource Technology*. **102**(2): 1752-1756.

Telke AA, Joshi SM, Jadhav SU, Tamboli DP and Govindwar SP (2010) Decolorization and detoxification of Congo red and textile industry effluent by an isolated bacterium Pseudomonas sp. SU-EBT. *Biodegradation*. **21**(2): 283-296.

Thiruchelvam AT and Ramsay JA (2007) Growth and laccase production kinetics of Trametes versicolor in a stirred tank reactor. *Applied Microbiology and Biotechnology* 74:547-554

Thurston CF (1994) The structure and function of fungal laccases. *Microbiology*. **140**: 19-26.

Tišma, M., Tnidaršic-Plazl, P., Vasic-Racki, Đ., & Zelic, B. (2012). Optimization of laccase production by Trametes versicolor cultivated on industrial waste. *Applied Biochemistry and Biotechnology*, 166(1), 36-46.

Torrecilla, J. S., Mena, M. L., Yáñez-Sedeño, P., & García, J. (2007). Application of artificial neural network to the determination of phenolic compounds in olive oil mill wastewater. *Journal of Food Engineering,* 81(3), 544-552.

Tsuchiya R and Petersen BR (2000). Reduction of malodour, Google Patents.

Tucker KG and Thomas CR (1992) Mycelial morphology - the effect of spore inoculum level.

Tuomela M, Vikman M, Hatakka A and Itävaara M (2000) Biodegradation of lignin in a compost environment: a review. *Bioresource Technology*. **72**(2): 169-183.

Tuor, U., Winterhalter, K., & Fiechter, A. (1995). Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay.Journal of Biotechnology, 41(1), 1-17. doi:10.1016/0168-1656(95)00042-o

Vanhoutte B, Pons MN, Thomas CR, Louvel L and Vivier H (1995) Characterization of *Penicillium chrysogenum* physiology in submerged cultures by color and monochrome image analysis. *Biotechnology and Bioengineering* 48:1-11

Vasconcelos AFD, Barbosa AM, Dekker RF, Scarminio IS and Rezende MI (2000) Optimization of laccase production by< i> Botryosphaeria</i> sp. in the presence of veratryl alcohol by the response-surface method. *Process Biochemistry*. **35**(10): 1131-1138.

Vasdez, K., and Kulad, R. 1994. Induction of laccase productions in Cyathus bulleri under shaking and static condition. Folia Microbiol. 39: 326-330.

Vecht-Lifshitz SE, Magdassi S and Braun S (1990) Pellet formation and cellular aggregation in Streptomyces tendae. *Biotechnology and Bioengineering* 35:890-896

Vite-Vallejo, O., Palomares, L. A., Dantán-González, E., Ayala-Castro, H. G., Martínez-Anaya, C., Valderrama, B., & Folch-Mallol, J. (2009). The role of N-glycosylation on the enzymatic activity of a Pycnoporus sanguineus laccase.Enzyme and Microbial Technology, 45(3), 233-239. doi:10.1016/j.enzmictec.2009.05.007

Voelker SL, Lachenbruch B, Meinzer FC, Jourdes M, Ki C, Patten AM, Davin LB, Lewis NG, Tuskan GA and Gunter L (2010) Antisense down-regulation of 4CL expression alters lignification, tree growth, and saccharification potential of field-grown poplar. *Plant Physiology*. **154**(2): 874-886.

Wahleithner JA, Xu F, Brown KM, Brown SH, Golightly EJ, Halkier T, Kauppinen S, Pederson A and Schneider P (1996) The identification and characterization of four laccases from the plant pathogenic fungusRhizoctonia solani. *Current genetics*. **29**(4): 395-403.

Wang DWS. (2009) Structure and action mechanism of ligninolytic enzymes. *Appl Biochem Biotechnol* 157:174-209.

Wang, Z., Cai, Y., Liao, X., Tao, G., Li, Y., Zhang, F., & Zhang, D. (2010). Purification and characterization of two thermostable laccases with high cold adapted characteristics from Pycnoporus sp. SYBC-L1. Process Biochemistry, 45(10), 1720-1729. doi:10.1016/j.procbio.2010.07.011

Watanabe, K., Motohashi, K., & Ono, Y. (2010). Description of Pestalotiopsis pallidotheae: a new species from Japan. Mycoscience, 51(3), 182-188. doi:10.1007/s10267-009-0025-z

Wei JG, Xu T, Guo LD, Liu AR, Zhang Y, Pan XH (2007) Endophytic Pestalotiopsis species associated with plants of Podocarpaceae, Theaceae and Taxaceae in southern China. Fungal Divers 24:55–74

Wei, D., Houtman, C. J., Kapich, A. N., Hunt, C. G., Cullen, D., & Hammel, K. E. (2010). Laccase and Its Role in Production of Extracellular Reactive Oxygen Species during Wood Decay by the Brown Rot Basidiomycete Postia placenta. Applied and Environmental Microbiology, 76(7), 2091-2097. doi:10.1128/aem.02929-09

Wesenberg D, Kyriakides I and Agathos SN (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances*. **22**(1): 161-187.

Wolfanden BS, Willson RL. (1982) Radical-cations as reference chromogens in kinetic studies of one-electron transfer reactions: Pulse radiolysis studies of 2.2’-azinobis-(3-ethylbenzthiazoline-6-sulphonate). *J Chem Soc Perkin Trans II*:805-812.

Wong, D. W. (2008). Structure and Action Mechanism of Ligninolytic Enzymes.Applied Biochemistry and Biotechnology, 157(2), 174-209. doi:10.1007/s12010-008-8279-z

Xavier A, Evtuguin D, Ferreira R and Amado F (2001) Laccase production for lignin oxidative activity. *Proceeding of the 8th International Conference on Biotechnology in the Pulp and Paper Industry* 4-8

Xu F (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry*. **35**(23): 7608-7614.

Xu F (1997) Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *Journal of Biological Chemistry*. **272**(2): 924-928.

Xu F, Kulys JJ, Duke K, Li K, Krikstopaitis K, Deussen H-JW, Abbate E, Galinyte V and Schneider P (2000) Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compounds. *Applied and Environmental Microbiology*. **66**(5): 2052-2056.

Yao PX, Matsumoto N, Tsujimura H, Takaya M and Toda K (1995) Enhanced production of peroxidase in continuous culture of Arthromyces ramosus by selective bleeding of mycelium. *Journal of Fermentation and Bioengineering* 79:307-312

Yaropolov A, Skorobogat'Ko O, Vartanov S and Varfolomeyev S (1994) Laccase. *Applied Biochemistry and Biotechnology*. **49**(3): 257-280.

Yaver DS and Golightly EJ (1996) Cloning and characterization of three laccase genes from the white-rot basidiomycete< i> Trametes villosa</i>: genomic organization of the laccase gene family. *Gene*. **181**(1): 95-102.

Yaver DS, Overjero MDC, Xu F, Nelson BA, Brown KM, Halkier T, Bernauer S, Brown SH and Kauppinen S (1999) Molecular characterization of laccase genes from the basidiomycete Coprinus cinereus and heterologous expression of the laccase Lcc1. *Applied and Environmental Microbiology*. **65**(11): 4943-4948.

Yazhong X, Shuxiang Z, Qiaoyan H, Wei J, Chunlei P and Yunyu S (2003) Immobilization of fungal laccase on chitosan and its use in phenolic effluents treatment. *ACTA MICROBIOLOGICA SINICA-CHINESE EDITION-*. **43**(2): 245-250.

Yoshitake A, Katayamay Y, Nakamuram, Iimura Y,Kawai S and Morohoshi N(1993) N-linked carbohydrate chains protect laccase III from proteolysis in Coriolus versicolor. *Journal of General Microbiology*. **139**(1): 179-185.

Youn, H., Hah, Y. C., & Kang, S. (1995). Role of laccase in lignin degradation by white-rot fungi. FEMS Microbiology Letters, 132(3), 183-188. doi:10.1111/j.1574-6968.1995.tb07831.x

Zaidel DNA, Meyer AS. (2012) Biocatalytic cross-linking of pectic polysaccharides for designed food functionality: Structures, mechanisms, and reactions. *Biocatal Agric Biotechnol* 1(3):207-219.

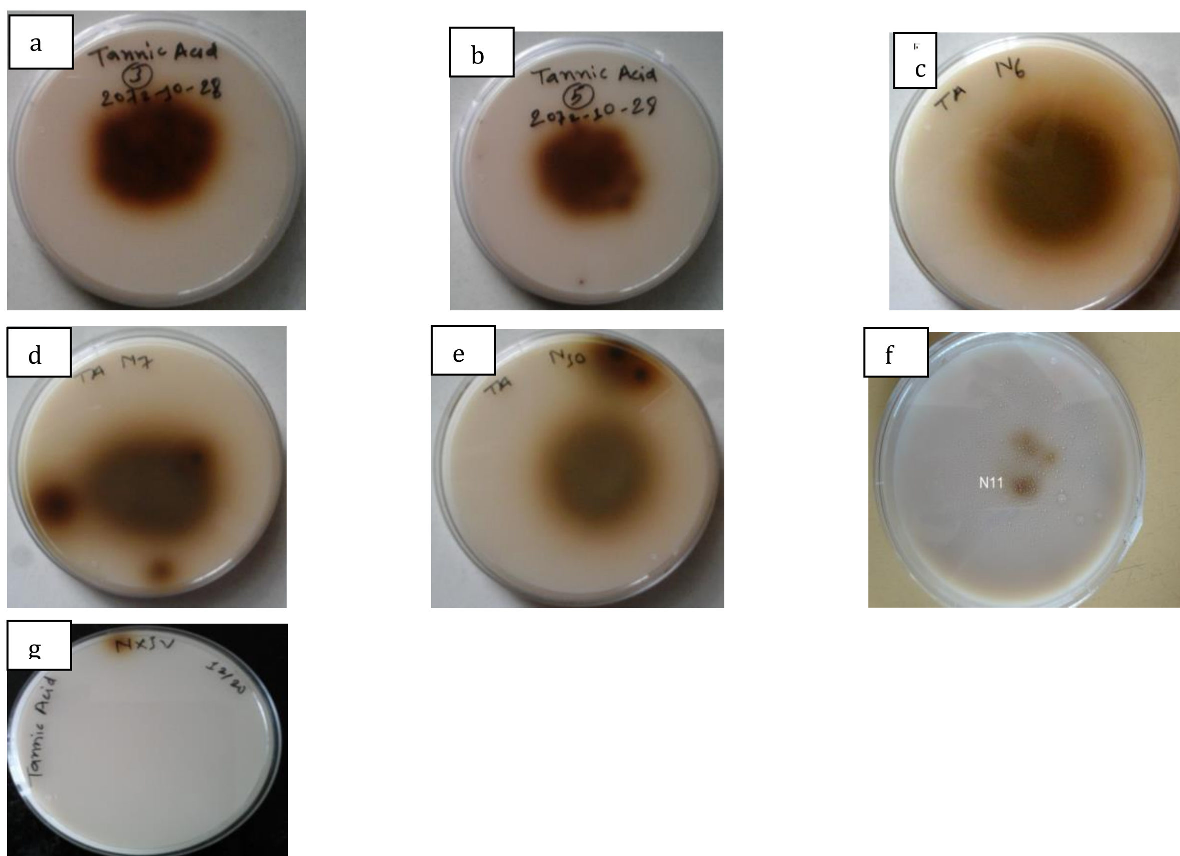
Zhang A-P, Liu C-F and Sun R-C (2010) Fractional isolation and characterization of lignin and hemicelluloses from Triploid of *Populus tomentosa* Carr. *Industrial Crops and Products*. **31**(2): 357-362.

Zhongyang Ding LP, Youzhi Chen, Liang Zhang, ZhenghuaGu, Guiyang Shi and Kechang Zhang (2011) Production and characterization of thermostable laccase from the mushroom, Ganoderma lucidum, using submerged fermentation. *African Journal of Microbiology Research*. **6**(6): 1147-1157.

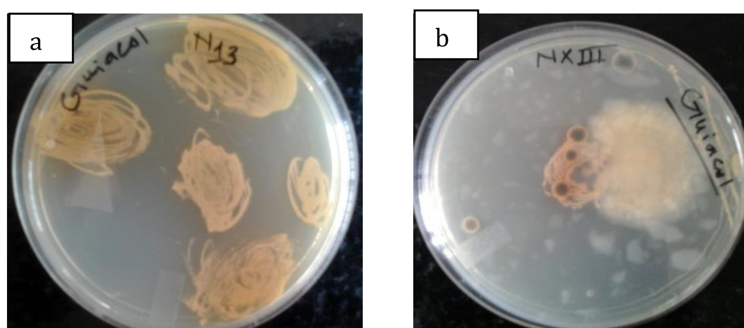
Žmak PM, Podgornik A, Podgornik H and Koloini T (2006) Impact of pellet size on growth and lignin peroxidase activity of Phanerochaete chrysosporium. *World Journal of Microbiology & Biotechnology* 22:1243-1249

Zouari, N., Romette, J.-L., & Thomas, D. (1994). Laccase electrode for the continuous-flow determination of phenolic compounds. *Biotechnology Techniques*, 8(7), 503-508.

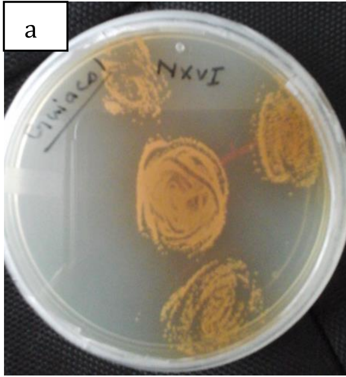
Appendix

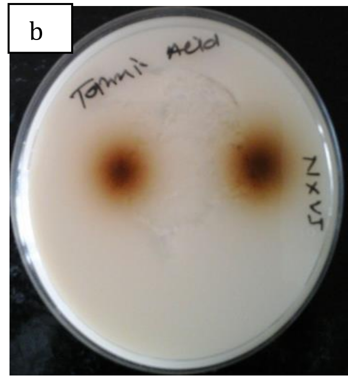


A) Screening of laccase activities of different isolates in tannic acid media in day 5, a) 3, b) 5, c) N6, d) N7, e) N10, f) N11, g) NXIV

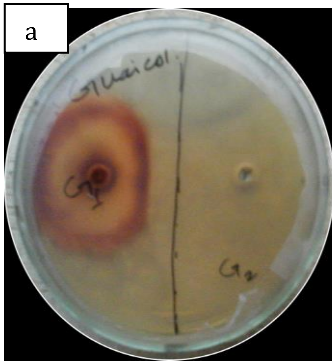


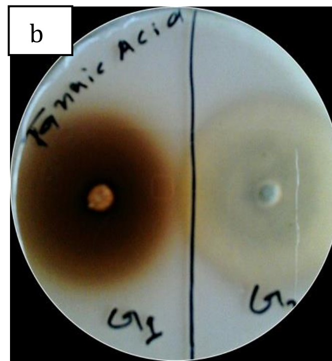
B) Screening of laccase activities of different isolates in Guaiacol media a) N13, b)NXII



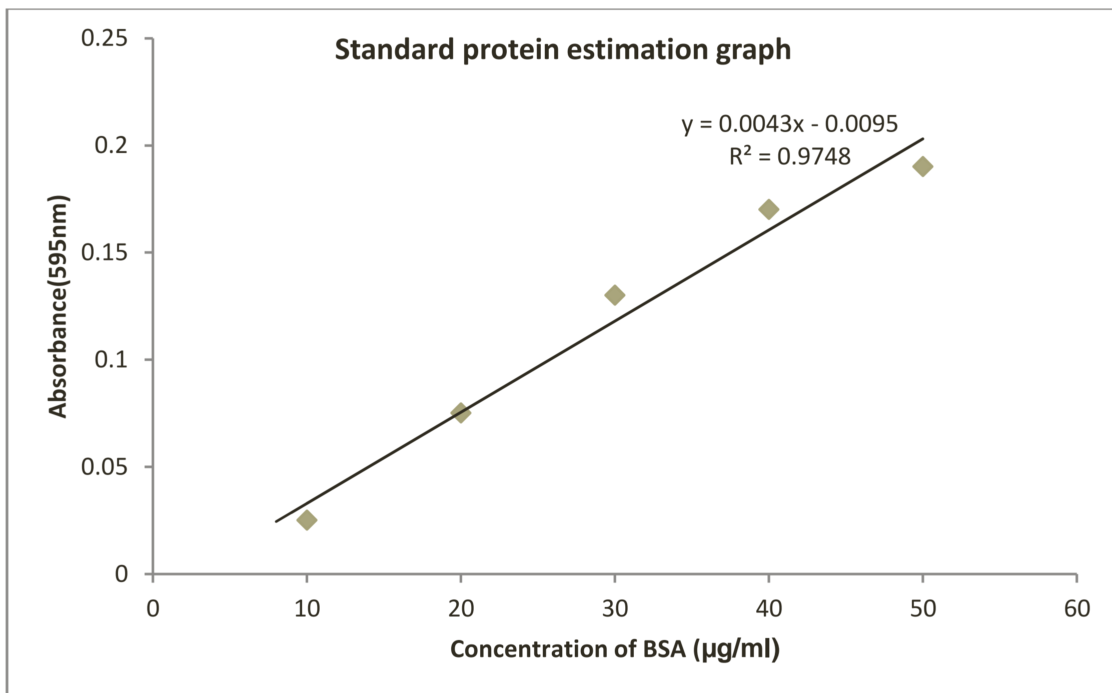


C) Screening of laccase activities of isolate NXVI in j) Guaiacol in day 6, k) Tannic acid in day 5





D) Screening of laccase activities of isolate G1 and G2 in a) Guaiacol in day 6, b) Tannic acid in day 5



E) Components of SDS-PAGE

30% acrylamide solution preparation (for 100 ml)

1gm bisacrylamide (neurotoxic) mixed with 29 gm acrylamide in 50 ml TDW and final volume Maintained to 100 ml.

Lower tris pH 8.8 (for 100 ml)

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 tris	1.25 ml	
10% SDS	3 ml	
Glycerol	4.75 ml	
2-mercaptoethanol/B-mercaptoethanol	0.5 ml	
0.1% bromophenol blue	0.5ml	-store at 4°c

Resolving gel

10% (For 10 ml)

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

Stacking gel (for 5% - 4 ml)

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

Running buffer for 1000ml

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

F) Fixative solution:

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

G) ABTS Solution (Molecular of ABTS= 548.68 g)

27.4g ABTS dissolved in 50ml and 137 g ABTS dissolved in 50 ml of sodium acetate buffer of pH 5 to make 1mM ABTS and 5mM solution and kept at 4°C for further use.

H) 1 % Guaiacol solution

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

I) Sodium acetate buffer (0.1 M, pH 5)

1.05ml 0f 100% acetic acid (sp. Gravity 1.04) and 4.32g of 100% sodium acetate were mixed in to make final volume 500ml and pH 5.

J) Sequence of 18S rRNA of Isolate ;G1’

>Isolate G1
CTGCGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATT
AAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGCCTGGCTGGCCGGTCCGCCT
CACCGCGTGCACTGGTTCGGCCGGGCCTTTCCCTCTGGGGAACCTCATGGTCTTCACTGA
TCGTGATGGGGAACCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTAT
GCTCGAATACATTAGCATGGAATAATAAAATAGGACGTGTGGTTCTATTTTGTTGGTTTC
TAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGGCATCAGTATTCAATTGTCAGAGG
TGAAATTCTTGGATTTATTGAAGACTAACTACTGCGAAAGCATTTGCCAAGGATGTTTTC
ATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACC
ATAAACTATGCCGACTAGGGATCGGACGGTGTTACTTATTGACCCGTTCGGCACCTTACG
AGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGGCGGCTAAAAGGTGAACTCATTGA
ATTG

K) Electrolab Fermenter of Central Department of Biotechnology, TU