

*The Lignin-Degrading Enzyme, Laccase
from Marine Fungi ;
Biochemical and Molecular Approaches*

Thesis submitted for the degree of

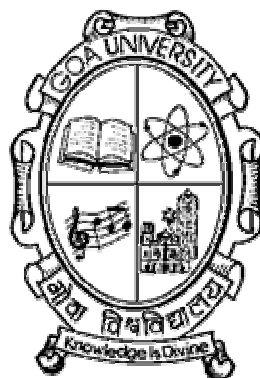
DOCTOR OF PHILOSOPHY

in

MARINE SCIENCES

to the

GOA UNIVERSITY



by

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STATEMENT

As per requirement, under the University Ordinance 0.19.8 (vi), I state that the present thesis titled “*The Lignin-Degrading Enzyme, Laccase from Marine Fungi; Biochemical and Molecular Approaches*” is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

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

Donna Trella D’Souza-Ticlo

CERTIFICATE

This is to certify that the thesis titled “*The Lignin-Degrading Enzyme, Laccase from Marine Fungi; Biochemical and Molecular Approaches*” submitted for the award of the degree of Doctor of Philosophy in the Department of Marine Sciences, Goa University, is the bona fide work of **Miss Donna T. D’Souza-Ticlo**. The work has been carried out under my supervision and the thesis or any part thereof has not been previously submitted for any degree or diploma in any university or institution.

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National Institute of Oceanography, Goa

Dedicated
to
Abba Father



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List of Abbreviations

AAs	amino acids
Abs	absorbance
ABTS	2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)
ACN	acetonitrile
ANOVA	analysis of variance
APS	ammonium per sulfate
AT	adenine thymine
B&K	Boyd and Kohlmeier
Bagasse	sugarcane bagasse
BLAST	basic local alignment search tool
BLASTP	protein-protein BLAST
BOD	biological oxygen demand
bps	base pairs
BSA	bovine serum albumin
CAS	chemical abstracts service
CBQase	cellobiose quinone oxidoreductase
CCD	central composite design
CcL	<i>Coprinus cinereus</i> laccase
CD	circular dichroism
CNS	carbon-nitrogen-sulfur
COD	chemical oxygen demand
conc	concentration
CTAB	cetyltrimethylammonium bromide/(1-hexadecyl)trimethylammonium bromide
D1	domain 1 (of 25-28s rDNA)
D2	domain 2 (of 25-28s rDNA)
Da	Dalton
DEAE	diethyl aminoethane
DHB	2,5-dihydroxybenzoic acid
DMHAP	3',5'-di methoxy-4'-hydroxy acetophenone
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DOC	dissolved organic carbon
DTT	dithiothreitol
E°	redox potential
E _{act}	energy of activation
EC	enzyme commission
EDTA	ethylene diamine tetra acetic acid
Endo H	endoglycosidase H
EPR	electron paramagnetic resonance
EPS	exopolymeric substance
EST	expressed sequence tag
ETS	external transcribed spacer
FPLC	fast protein liquid chromatography
g	g-force; Earths gravitational acceleration
g	gram (s)
GC	guanine cytosine

GPD	glyceraldehyde-3-phosphate dehydrogenase
h	hour (s)
ha	hectare = 10000 square meters
HBT	1-hydroxybenzotriazole
HCCA	α -cyano-4-hydroxycinnamic acid
HPLC	high performance / pressure liquid chromatography
HQ	hydroquinone / quinol,1,4-benzenediol
HSSP	homology derived secondary structure of proteins
HTMP	4-hydroxy-2,2,6,6 tetra-methyl piperidine-1-oxyl
ICBN	international code of botanical nomenclature
IEF	isoelectric focusing
IGS	inter generic spacer
IPG	immobilized pH gradients
ITS	internal transcribed spacer
IU	international units
kb	kilobase
kCal	kilo Calories
K_{cat}	catalytic constant
kDa	kilo Dalton
kJ	kilo Joules
K_m	Michaelis constant
LDE (s)	lignin-degrading enzyme (s)
LiP	lignin peroxidase
LN	low nitrogen
LNM	low nitrogen medium
LMS	laccase-mediator-system
LSU	large subunit
<i>m</i>	meta-position
M	molar
mA	milliampere
MaL	<i>Melanocarpus albomyces</i> laccase
MALDI	matrix-assisted laser desorption / ionization
MALDI-MS	matrix-assisted laser desorption / ionization mass spectroscopy
MALDI-TOF	matrix-assisted laser desorption / ionization time of flight
MANOVA	multivariate analysis of variance
MEA	malt extract agar
MEB	malt extract broth
MEGA	molecular evolutionary genetics analysis
milli Q water	ultra pure water
mM	millimolar
MnP	manganese dependant peroxidase
mol	moles
MSW	molasses spent wash
MTCC	Microbial Type Culture Collection
mtDNA	mitochondrial DNA
mU	milliunits
mV	millivolts
N	normal

NCBI	National Center for Biotechnology Information
nDNA	nuclear DNA
ng	nanogram
NIOCC	National Institute of Oceanography Culture Collection
NIOCC # 2a	MTCC 5159 / <i>Cerrena unicolor</i> MTCC 5159
NJ	neighbor-joining
nm	nanometre
nmol	nanomole
NS	nuclear small
NTS	non-transcribed spacer
<i>o</i>	ortho-position
O.D.	optical density
O ₂	molecular oxygen
<i>p</i>	para-position
PAGE	poly acrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	proof reading units
pI	isoelectric point
PITC	phenylisothiocyanate
POC	particulate organic carbon
ppm	parts per million
ppt	parts per thousand
PTH	phenylthiohydantoin
PVDF	polyvinylidene fluoride
PVP	poly vinyl pyrrolidine
Q ₁₀	temperature coefficient
R	gas constant (8.314472 Joules or 1.987 Calories)
RAPD	random amplified polymorphic DNA
RBBR	remazol brilliant blue R
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease enzyme
RO 16	reactive orange 16
RT	room temperature
S	sedimentation coefficient
S.E.E	standard error of estimate
SA	syringic acid / 4-hydroxy-3,5-dimethoxybenzoic acid
sDHB	super DHB
SDS	sodium dodecyl sulfate
sec	second (s)
sp.	species
SSU	small subunit
SW	seawater

T	temperature
T1	type 1
T2	type 2
T3	type 3
Taq	<i>Thermus aquaticus</i>
TBE	tris-borate-EDTA
TCA	trichloroacetic acid
TE	tris EDTA
TeA	textile effluent A
TeB	textile effluent B
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
THAP / DHAP	2,4,6-trihydroxyacetophenone
Tm	Melting point
TNT	2,4,6-trinitrotoluene
Tris	tris (hydroxymethyl) aminomethane
Tris-HCl	tris hydrochloride
TvL	<i>Trametes versicolor</i> laccase
U	unit
UV	ultra violet
v.	version
v/v	volume by volume
VA	veratryl alcohol / 3, 4-dimethoxy benzyl alcohol
Vgb	<i>Vitreoscilla</i> hemoglobin gene
Vhb	<i>Vitreoscilla</i> hemoglobin
V _{max}	maximum velocity
Vol (s)	volume (s)
VP	versatile peroxidase
w/v	weight by volume
X	times
λ	wavelength
μ	micron
μ mol	micromole
μg	microgram
μl	microlitre
μM	micromolar
ε	molar extinction coefficient / molar absorption coefficient
%	percent
Δ	delta / difference
°C	degree Celsius
15° 30' N	15 degrees and 30 minutes North
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
5' to 3'	5 prime to 3 prime direction
73° 55' E	73 degrees and 55 minutes East

Chapter 1

Review of Literature

Ph.D. Thesis



ABSTRACT

This chapter introduces marine and marine-derived fungi, their habitat and the factors that play a major influencing role in their survival and success in the marine environment. It also deals with the type of fungi found in the marine and marine-derived habitats such as the marine endophytes, marine animal-fungal associations and marine detritus-associated fungi. A brief write-up on *Cerrena*, the genus on which this study is based, has also been included.

It introduces the topic of lignin, the different kinds of associated wood-rots such as the soft rot, brown rot and the white-rot and the fungi responsible for these rots. The various lignin-degrading enzymes which include the peroxidase family and laccases have been described briefly. The molecular structure of laccase and how it affects the substrate range and activity of laccase has been described in detail. It also explains with illustrations, the mechanism of laccase action which consists of four, one-electron reductions of molecular oxygen to water. This mechanism of action can either lead to a polymerization or a depolymerization reaction depending on several conditions explained there-in. Finally, the various applications of laccase which are a consequence of either of the above two reactions have been briefly mentioned.

1. MARINE FUNGI form an ecological and not a taxonomic group (Hyde *et al.*, 2000) comprising the obligate and facultative marine fungi. Obligate marine fungi grow and sporulate exclusively in sea water and their spores are capable of germinating in sea water. On the other hand, facultative marine fungi are those fungi present in the marine environment which have a terrestrial origin but have undergone physiological adaptations that allow them to grow and possibly sporulate in the marine environment (Kohlmeyer and Kohlmeyer, 1979). About 800 species of obligate marine fungi have been reported so far (Hyde *et al.*, 2000). These belong mostly to ascomycetes, the anamorphs and a few basidiomycetes. Among the straminipilan fungi, the *Labyrinthulomycetes*, comprising of the thraustochytrids, aplanochytrids, and labyrinthulids are obligately marine (Raghukumar, 2002) and those belonging to the oomycetes are also fairly widespread in the marine environment.

Several factors and their combination thereof besides making the marine environment unique also greatly influence survival, growth and reproduction of fungi inhabiting this environment. Some of the physical factors that influence marine fungi are listed below:

- a) Salinity and pH
- b) Low water potential
- c) High concentrations of sodium ions
- d) Low temperature
- e) Oligotrophic nutrient conditions
- f) High hydrostatic pressure

A combination of the above parameters lends uniqueness to the marine environment.

- (a) Sea water on an average has a salinity of 33 - 35 ppt. Fresh water in comparison contains less than 0.05 % salts (0.5 ppt). Estuarine waters are the middle ground between the saline waters of the sea and the fresh waters of the rivers. The salinity in such habitats varies between the 0.5 ppt to almost full strength sea water, depending on the direction and strength of the tides as well as the seasons and the amount of

influx of fresh water from the contributing rivers and other connected fresh water sources. Hyper saline waters on the other hand such as those of the Dead Sea, contain far greater concentration of salts and have salinities ranging from 50 to 100 ppt.

Seawater pH is limited to the range 7.5 to 8.4 due to the buffering action of the carbonic acid-bicarbonate system. Although fungi are organisms that easily adapt to their environment, the alkaline pH of the sea water often limits the active proliferation of fungi. Terrestrial fungi generally grow best at pH 4.5 - 6, whereas facultative marine fungi were demonstrated to grow and produce various extracellular enzymes at pH 7 - 8 (Damare *et al.*, 2006; Raghukumar *et al.*, 1994; Raghukumar *et al.*, 1999).

- (b) Low water potential is one of the problems posed by seawater. Organisms living in it therefore, need to be hypertonic to enable water uptake. Marine fungi maintain this gradient by accumulating osmolytes such as glycerol, mannitol, polyol and trehalose (Blomberg and Adler, 1992).
- (c) The presence of high levels of sodium ions in seawater also confers some unique properties to the cells of organisms living in the sea. Sodium, even in small concentrations is generally toxic to most living cells in the terrestrial and freshwater environments. Many marine fungi are known to reduce the toxicity of sodium ions by sequestering them in vacuoles (Jennings, 1983) or have a very efficient sodium efflux ATPase (Benito *et al.*, 2002). The straminipilan fungi, the thraustochytrids and labyrinthulids on the other hand have an absolute requirement for sodium for their growth and sporulation (Jennings, 1986). Thus, these two groups are excellent tools for understanding the physiology of growth and enzyme production in the presence of sodium.
- (d) The temperature of sea water generally decreases with increasing depth, reaching about 2°C in the abyssal plain but the pattern varies with latitude and region (Mantyla and Reid, 1983). Such low temperatures place a great strain on the active growth of fungi and have often led to 'cold adaptation' of fungi wherein the fungi produce enzymes that are active at low temperatures (Damare *et al.*, 2006).

An interesting pattern termed '*Phoma*' pattern is seen in some marine fungi. This pattern is due to the interaction between salinity and temperature where the salinity optimum for growth in some marine fungi show upward shift with increasing incubation temperature. It was so named since it was first described in the marine species of *Phoma* (Lorenz and Molitoris, 1992).

- (e) Growth of fungi on microscope lenses, contact lenses and glass slides in terrestrial environments is indicative of oligotrophic existence of terrestrial fungi. In the water column, the organic nutrient levels are comparatively low and are mostly in steady state. Fungi have not been observed to grow freely in water columns. However, fungi probably form micro colonies in marine sediments.
- (f) There is an increase of 1 bar hydrostatic pressure with every 10 m depth in the sea. Thus deep-sea with elevated hydrostatic pressure, constant low temperatures of 2 - 4°C and darkness and "feast and famine" nutrient conditions is home to barotolerant and barophilic, cold-tolerant and psychrophilic microorganisms. Information about the presence of fungi in deep-sea is meagre as yet (Raghukumar, 2005).

Fungi in general require a substratum to attach on, hence marine fungi are found in special ecological niches in the marine environment. Their substrata range from decomposing wood and plant detritus in coastal waters (Kohlmeyer and Kohlmeyer, 1979; Kumaresan *et al.*, 2002) to calcareous animal shells (Raghukumar *et al.*, 1992), algae (Raghukumar, 2006) and corals (Golubic *et al.*, 2005). They have even been isolated from deep-sea sediments and detected in anoxic marine sediments (Stoeck *et al.*, 2003). A sound understanding of the ecology will help in revealing the novelty of an organism and its properties.

1.1.1 Marine endophytic fungi: Endophytic fungi live in the intercellular spaces of plant tissue causing no apparent damage to their host. Kobayashi and Ishibashi (1993) are of the opinion that marine endophytic fungi may have potential to produce different types of compounds from those of terrestrial sources. Several endophytic fungi have been isolated

and cultured from the mangrove plants *Rhizophora apiculata* and *Dendrophthoe falcate* (Kumaresan *et al.*, 2002). Endophytic fungi from marine algae and plants have been found to produce interesting secondary metabolites (Jensen and Fenical, 2002).

1.1.2 Animal-fungal associations: Association of fungi with marine animals ranges from saprotrophic to symbiotic to even parasitic. Saprotrophic fungi, some of them being common terrestrial fungi have been isolated from the surface, guts and coelomic fluids of holothurians or the sea cucumbers (Pivkin, 2000). Sponges are known for production of several bioactive molecules with antifungal, antibacterial, antimalarial and antiviral activities (Mayer and Hamann, 2004). Marine sponges, the natural bio-fermentors of microorganisms harbor a variety of microorganisms such as bacteria, fungi (Namikoshi *et al.*, 2002) and thraustochytrids (Rinkevich, 1999). Several bioactive compounds in sponges are assumed to be produced mostly by symbiotic bacteria or actinomycetes (Kobayashi and Ishibashi, 1993; Piel *et al.*, 2004). Jensen and Fenical (2000) have described 10 compounds from sponge-derived fungi. Several mycelial fungi isolated from sponges and other marine animals yielded not only new natural products but also compounds identical or related to those formerly attributed to their hosts (Proksch *et al.*, 2003). For any endosymbiotic association, it is necessary to reconfirm by direct visualization of the alleged symbionts inside their hosts. A recent report on the detection of an endosymbiotic yeast in a sponge by transmission electron microscopy and immunocytochemical labeling of β -1,4-N-acetyl-D-glucosamine residues of chitin walls, a fungal signature confirmed its presence (Maldonado *et al.*, 2005). Interestingly these yeasts are reported to be maternally transmitted from the soma through the oocytes to the fertilized eggs. Bioactive molecules from such sponge-fungal endosymbiosis are worth investigating. As many of these fungi are culturable, they can be easily tested for bioactive molecules and examined if similar molecules are also produced inside their hosts.

1.1.3 Detritus-associated fungi: Processes involved in detritus formation are crucial to remineralization processes in the marine ecosystem. Detritus, the dead organic matter with its associated microbes is an important link of marine food web to the detritivores

feeding on them (Mann, 1988). Detritus from coastal marine macrophytes, particularly mangroves, contribute an enormous amount of organic matter to the adjacent waters (Wafar *et al.*, 1997). The major role of microorganisms in detrital processes is the biochemical transformation of the detritus by production of extracellular degradative enzymes (Fig. 1). A great diversity of fungi were isolated from decaying mangrove and sea grass leaves (Coumo *et al.*, 1985; Fell and Newell, 1981).

An enormous amount of work is reported from lignin degrading enzymes from terrestrial white-rot fungi (Basidiomycetes). These fungi are well known for their lignin-degrading enzymes, the most common being laccase and the peroxidases such as lignin peroxidase (LiP) and manganese peroxidase (MnP) (Reddy, 1995).

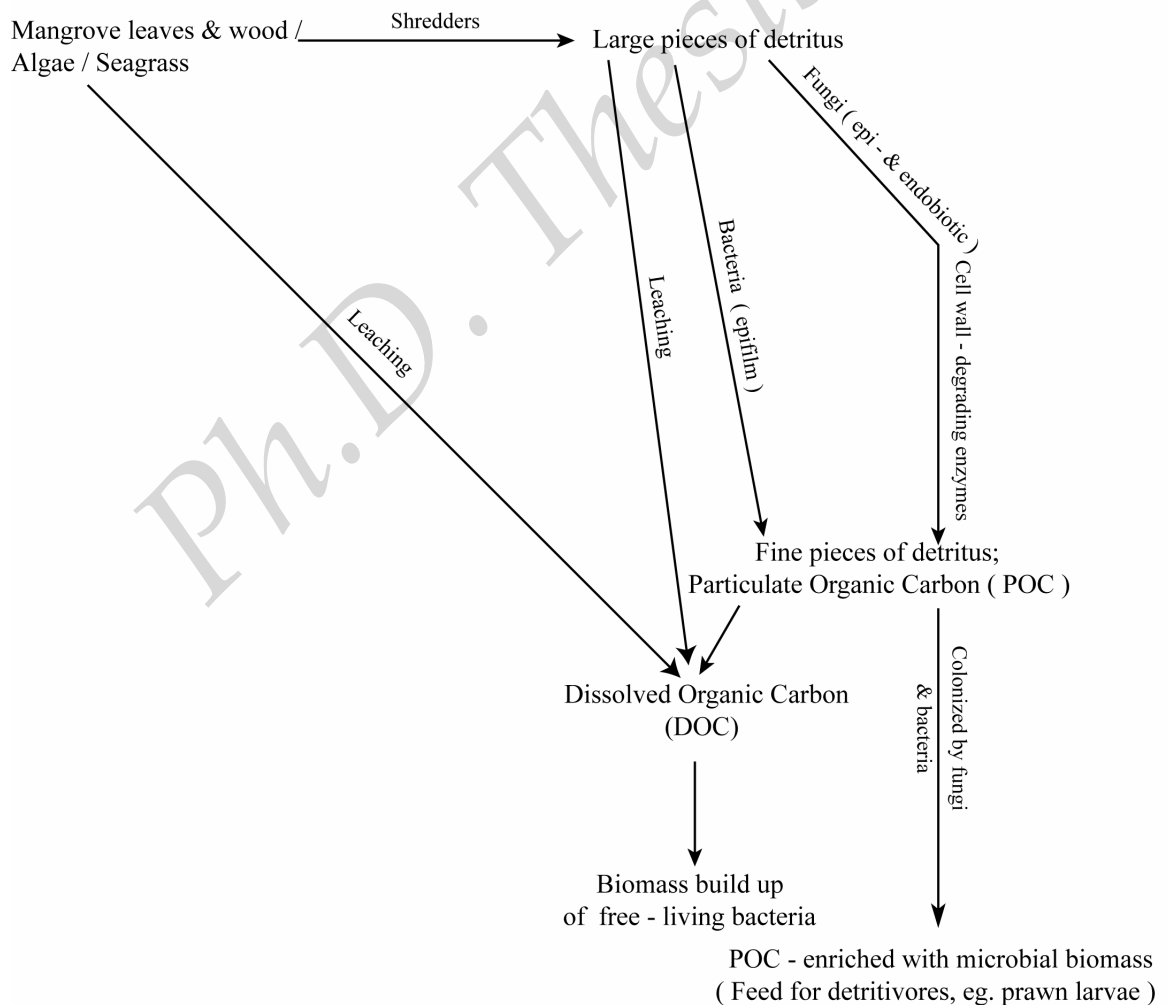


Fig. 1: Schematic diagram illustrating the process of degradation of plant material in the marine habitat. Modified from (Raghukumar *et al.*, 2008).

Several secondary metabolites are reported from fungi isolated from marine detritus. A polyketide metabolite, obionin-A was isolated from the liquid culture of the marine fungus *Leptosphaeria obiones*, a halotolerant ascomycete obtained from the salt marsh grass *Spartina alterniflora* (Poch and Gloer, 1989). These authors also reported two novel compounds helicacolides A and B from the Hawaiian mangrove ascomyceteous fungus *Helicascus kanaloanus*.

In ecology, r/k selection theory (MacArthur and Wilson, 1967) relates to the selection of traits which promote success in particular environments. **R-selection** occurs in unstable or unpredictable environments, as the ability to reproduce quickly is crucial and there is little advantage in adaptations that permit successful competition with other organisms since the environment is likely to change again. Whereas **k-selection** occurs in stable or predictable environments, as the ability to compete successfully for limited resources which is crucial and populations of K-strategists typically are very constant and close to the maximum carrying capacity of the environment.

The slow growing lignin-degrading fungi colonizing woody substrates in the marine environment are probably k-strategists and thus may be a good source of antibiotics (Strongman *et al.*, 1987). In fact, a number of antimicrobial compounds have been reported from fungi colonizing woody substrates in the marine environment (Bugni and Ireland, 2004). Such ecological information is important in drug discovery and novel metabolite-screening programs.

Cerrena is an interesting basidiomyceteous fungus, since it belongs to all of the above categories. It is known for its symbiotic relationship with both plants and animals (Pazoutova and Srutka, 2007) and is capable of maintaining a saprophytic lifestyle as well as thrive in the marine environment. Among the animals, *Cerrena* has a close symbiotic association with the horntail *Tremex fuscicornis* (wood wasp) both as larvae and adults. Adult female wood wasps oviposit in dying and dead standing or trees exposed to stress. As the eggs are laid, the wood is simultaneously inoculated with a mass of fungal oidia which are maintained in special pouches associated with the mycangia (egg-laying apparatus). The fungus permeates the surrounding wood and the larvae tunnel into this, ingesting both wood and fungal hyphae (Kukor and Martin, 1983). The fungal symbiont is indispensable for larval development especially beyond the first instar

(Pazoutova and Srutka, 2007). The reason for this symbiosis between fungus and animal is the large amount of lignin-degrading enzymes and proteases produced by this fungus, which helps it in breaking down wood components and making it palatable for the larvae and in return, fresh hosts for the fungus are found, via *Tremex*. The fungus can also choose to remain as an endophyte and upon death of the plant survive saprophytically. The major role for laccase-producing fungi in the marine environment is degradation of lignicolous plant material in mangrove and sea grass stands.

1.2 LIGNIN is a complex aromatic amorphous polymer, most commonly derived from wood and is an integral part of plant cell walls. The term was introduced in 1819 by de Candolle and is derived from the Latin word *lignum*, meaning wood and is the second most abundant biopolymer after cellulose, employing 30 % of non-fossil organic carbon and constituting from a quarter to a third of the dry mass of wood (Boerjan *et al.*, 2003; Lebo *et al.*, 2001; Sjöström, 1993).

It is relatively hydrophobic and aromatic in nature and is known for its heterogeneity in lacking a defined primary structure. The degree of polymerization in nature is difficult to measure since it is fragmented during extraction and the molecule consists of various types of substructures which appear to repeat in a random manner. However, it contains three basic monolignol monomeric units which are methoxylated to various degrees: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These monomers are incorporated into lignin in the form of the phenylpropanoids namely *p*-hydroxyphenyl, guaiacyl, and syringyl respectively. Gymnosperms have a lignin that consists almost entirely of guaiacyl with small quantities of *p*-hydroxyphenyl. Whereas dicotyledonic angiosperms have mostly a mixture of guaiacyl and syringyl with very little *p*-hydroxyphenyl, however monocotyledonic lignin is a mixture of all three. Many grasses have mostly guaiacyl, while some palms have mainly syringyl. All lignins contain small amounts of incomplete or modified monolignols, and other monomers are prominent in non-woody plants (Anonymous, 2001; Boerjan *et al.*, 2003; Ralph *et al.*, 2001).

Lignin fills the spaces in the cell wall between cellulose, hemicellulose and pectin components, especially in tracheids, sclereids and xylem. It is covalently linked to

hemicellulose and thereby cross-links different plant polysaccharides, conferring mechanical strength to the cell wall and to the plant as a whole (Chabannes *et al.*, 2001). It plays a crucial part in conducting water in plant stems. The polysaccharide components of plant cell walls are highly hydrophilic and thus permeable to water, whereas lignin is more hydrophobic. The cross-linking of polysaccharides by lignin is an obstacle for water absorption to the cell wall. Thus, lignin makes it possible for the plant's vascular tissue to conduct water efficiently (Sarkanen and Ludwig, 1971). Lignin is present in all vascular plants but not in bryophytes, supporting the idea that the original function of lignin was restricted to water transport.

Lignin is indigestible by mammalian and other animal enzymes due to its cross-linking with the other cell wall components, it also minimizes the accessibility of cellulose and hemicellulose to several microbial enzymes. Hence, lignin is generally associated with reduced digestibility of the overall plant biomass, which helps defend against pathogens and pests (Sarkanen and Ludwig, 1971). However, some fungi and bacteria are able to biodegrade the polymer. This ability is crucial for the organism as removal this barrier is required to reach the nutrient source which is the cellulose. The type of biodegradation of lignin that occurs depends on the type of wood-decaying fungi, either brown rot, soft rot or white rot. These categories are named based on visual characteristics of the advanced decay. Different types of fungi give rise to different types of wood rot. One can distinguish between the soft-rot, brown-rot and white-rot fungi (Eriksson, 1981).

1.3.1 SOFT ROT FUNGI attack wood causing a softening of the surface layer, hence the term 'soft-rot'. There are two distinct types of soft rot. Longitudinal cavities formed within the secondary wall of wood cells indicate *T₁ type* of soft rot whereas in the *T₂ type*, an erosion of the entire secondary wall occurs. In soft-rot, the middle lamella is not degraded (in contrast to cell wall erosion by white-rot fungi) but may be modified in advanced stages of decay. Large strength losses in wood can be associated with soft rot attack. Cavities formed in the wood as well as extensive cellulose degradation can result in extremely poor strength characteristics when soft-rot wood is visually evident. As decay progresses, extensive carbohydrate loss occurs and lignin concentrations increase

in the residual wood. Soft-rot is more common in hardwood than in softwood. It has been suggested that the reason for this, is the quality differences in the lignin of hard- and softwood. The methoxyl content of hardwood lignin is usually higher about 21 %, than in softwood lignin where the methoxyl content is about 14 %. Soft-rot is caused by fungi belonging to Ascomycetes and Fungi Imperfecti (Yoon and Singh, 2000).

1.3.2 BROWN ROT FUNGI mainly degrade cellulose and the hemicelluloses in wood. In an early stage of degradation they depolymerize cellulose faster than the degradation products are utilized (Wilcox, 1970). In brown rot decay, cellulose and hemicellulose are preferentially removed without affecting the lignin content. Here the wood retains its brown colouration but is left with a brittle matrix of modified lignin. This causes instability in the wood structure causing it to collapse. The residual wood is brown in colour, unstable and often cracks into cubical pieces when dry. *Serpula lacrymans* and *Meruliporia incrassata* are examples of fungi that cause brown rot (Coggins, 1977). Brown-rot and white-rot attack on wood is mainly caused by fungi belonging to Basidiomycetes. The hyphae of the fungi are normally localized in the cell lumen and these hyphae penetrate from one cell to another through openings or by producing bore holes in the cell walls (Wilcox, 1970).

1.3.3 WHITE ROT FUNGI is a rather heterogeneous group of organisms. They have in common the capacity to degrade lignin as well as the other wood components and the ability to produce extracellular enzymes which also oxidize phenolic compounds related to lignin. This is the reason why phenolic compounds have been utilized for the identification of white rot fungi. Their ability to metabolize large amounts of lignin in wood is unique among microorganisms. The relative amounts of lignin and polysaccharides degraded and utilized by these fungi vary and so does the order of preferential attack. Some species preferentially remove lignin from wood leaving pockets of white, degraded cells that consist entirely of cellulose, while others degrade lignin and cellulose simultaneously. They often cause a bleaching of normal wood colouration. The normal method of wood degradation by white-rot fungi is for the celluloses and the lignin to be attacked simultaneously. However, there are examples of a specific degradation of

the middle lamella lignin (Kaarik, 1974). It has been demonstrated that a totally specific attack on the lignin by white-rot fungi cannot be undertaken. This is most likely so because so much energy is required to degrade lignin that an additional, more easily accessible energy source is also necessary (Ander and Eriksson, 1978). A progressive erosion of the cell wall occurs when components are degraded simultaneously or a diffuse attack of lignin may occur by species that preferentially remove lignin.

1.4 LIGNIN-DEGRADING ENZYMES (LDEs) belong to two classes viz the heme-containing peroxidases and the copper-containing laccases. The peroxidases comprise of MnP, LiP and versatile peroxidase (VP). A series of redox reactions initiated by the LDEs degrade lignin or structures analogous to lignin subunits such as aromatic compounds (Fig. 2). The LDEs oxidize the aromatic compounds until the aromatic ring structure is cleaved, which is followed by further degradation with other enzymes

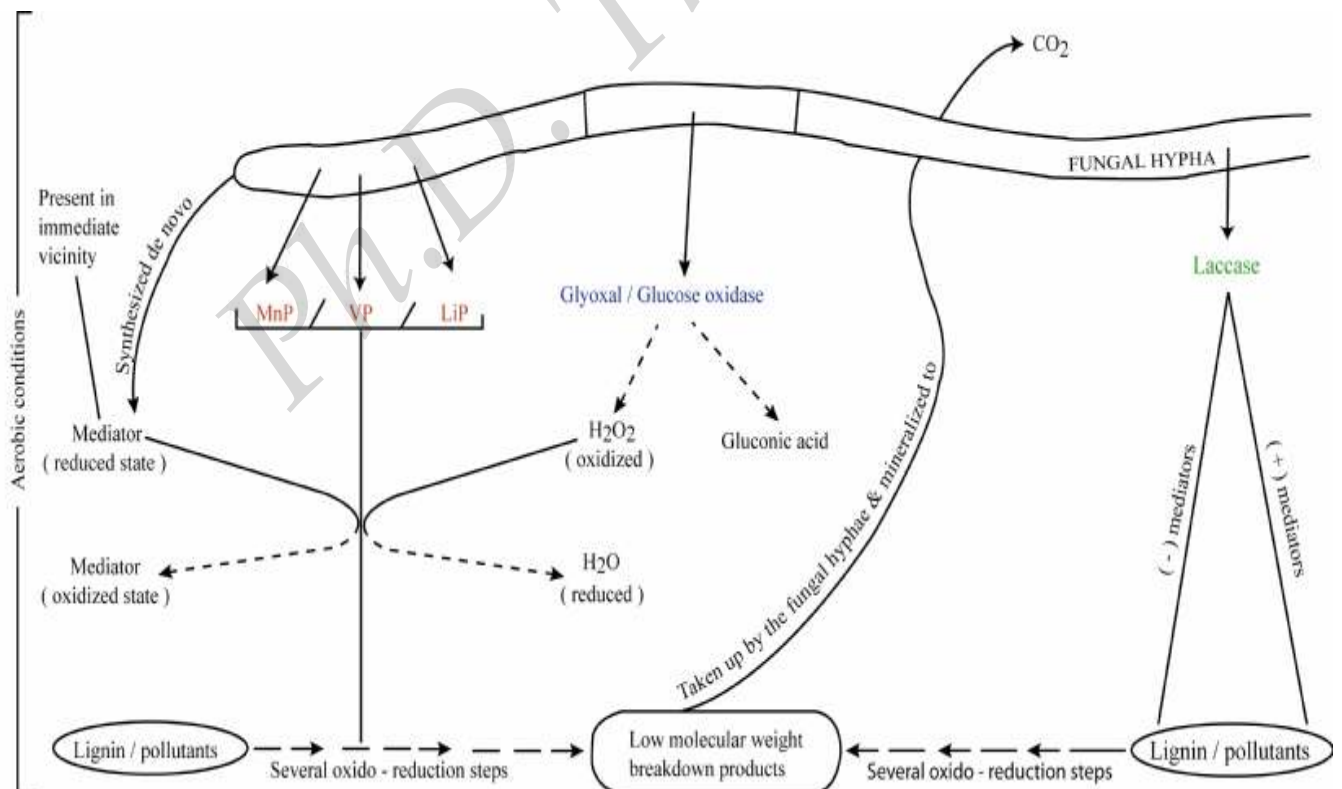


Fig. 2: Schematic diagram showing the role and activity of various extracellular lignin- degrading enzymes produced by fungi. Source: (Raghukumar *et al.*, 2008).

Based on the enzyme production patterns of white-rot fungi, Hatakka (1994) suggested three categories of fungi: (1) lignin peroxidase-manganese peroxidase group, (2) manganese peroxidase-laccase group and (3) lignin peroxidase-laccase group. The most efficient lignin degraders are able to mineralize lignin to carbon dioxide and these belong to the first category of fungi. Only moderate and very poor mineralization of lignin occurs in the second and third category of fungi respectively. The terrestrial white-rot fungus *Phanerochaete chrysosporium* which produces multiple isozymes of MnP and LiP and mostly no laccase, has been the laboratory model for physiological and molecular biological studies of LDEs (Fu and Viraraghavan, 2001).

1.4.1 Manganese-dependant peroxidase (EC 1.11.1.13) requires H_2O_2 as its co-substrate and the presence of Mn^{+2} (Mn^{+2} is naturally present in wood) which it oxidizes to Mn^{+3} producing an Mn^{+3} chelate oxalate, which in turn oxidizes the phenolic substrate. These Mn^{+3} -chelate-oxalates are small enough to diffuse into areas inaccessible even to the enzyme, as in the case of organopollutants buried deep within the soil, which may not necessarily be available to the enzyme (Tortella *et al.*, 2005).

1.4.2 Lignin peroxidase (EC 1.11.1.14) too, requires H_2O_2 , as its co-substrate and the presence of a mediator like veratryl alcohol to degrade lignin and other phenolic compounds. Here H_2O_2 gets reduced to H_2O by gaining an electron from LiP (which itself then gets oxidized). The oxidized LiP then returns to its native reduced state by gaining an electron from veratryl alcohol and converting it to veratryl aldehyde. Veratryl aldehyde gets reduced to veratryl alcohol by gaining an electron from lignin/pollutants. This results in the oxidation of lignin or the analogous aromatic compound (ten Have and Teunissen, 2001).

1.4.3 Versatile peroxidase is a novel enzyme which can utilize both veratryl alcohol and Mn^{+2} (EC 1.11.1.16) (Martínez, 2002; Martínez *et al.*, 2004). The most noteworthy aspect of VP is that it combines the substrate specificity characteristics of LiP, MnP as well as cytochrome c peroxidase (Du *et al.*, 1992). In this way, it is able to oxidize a variety of (high and low redox potential) substrates including Mn^{+2} , phenolic and non-phenolic lignin dimers, veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols and hydroquinones (Heinfling *et al.*, 1998). Versatile peroxidase has a Mn-

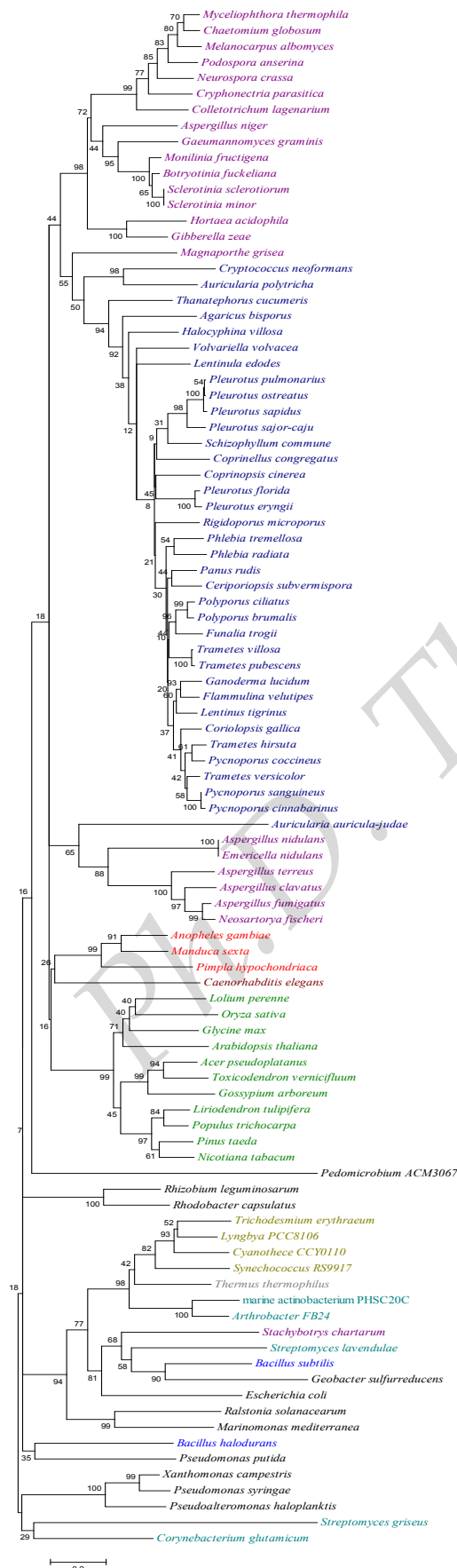
binding site similar to MnP and an exposed tryptophan residue homologous to that involved in veratryl alcohol oxidation by LiP. It is suggested that the catalytic properties of the new peroxidase is due to a hybrid molecular architecture combining different substrate binding and oxidation sites (Camarero *et al.*, 2000).

1.4.4 LACCASE (EC 1.10.3.2) is a benzenediol : oxygen oxidoreductase (a multi-copper enzyme), which has the ability to oxidize phenolic compounds. Unlike the peroxidases, it does not contain heme as the cofactor but copper, neither does it need H₂O₂ as the co-substrate but rather molecular oxygen (Baldrian, 2006). Laccase was first described by Yoshida (1883), and was characterized as a metal containing oxidase by Bertrand (1895). This makes it one of the oldest enzymes ever described.

Laccases can be roughly divided into two major groups depending on the source of its origin, i.e. those from higher plants and those from fungi (Mayer and Staples, 2002). The presence of laccase-like enzymes have been reported in bacteria (Claus and Filip, 1997) as well as in insects (Kramer *et al.*, 2001). The presence of laccase across kingdoms and their relationship with each other is presented in Fig. 3. Most laccases of fungal origin are of the secretory type, they are produced in the cells and then secreted and accumulate outside the hyphal filaments. Laccases occur as constitutive as well as inducible forms (Mougin *et al.*, 2003).

Laccase being an LDE has to have a non-specific type of cleavage, since lignin itself is an amorphous polymer, with no specific molecular structure. This non-specificity of laccase for the reducing substrate is its ultimate selling point, since this action of laccase can be extended to a variety of substrates. Often, more than one laccase isozyme is produced by the organism at any given time (Urzua *et al.*, 1995). Upon varying growth and / or nutrient conditions the kinds of isozymes produced also vary (D'Souza-Ticlo *et al.*, 2006). This consortium of laccases aids their action under varied conditions of pH, temperature as well as the type of substrate.

Laccase also often sports a high degree of glycosylation, which confers a degree of resistance to attack by proteases (Yoshitake *et al.*, 1993) which the enzyme often encounters in the wild. However, the redox potential although varying between different laccase isozymes, is not as high as those of the peroxidases, especially MnP.



In domain Eukaryota, laccases from kingdom Viridiplantae (Plants), phylum Streptophyta are indicated in green. Laccases from kingdom Metazoa (Animals), phylum Arthropoda and Nematoda are indicated in red and maroon respectively. Laccases from kingdom Eumycota (Fungi) are divided into two subphyla; Ascomycota and Basidiomycota, which are represented in purple and navy blue respectively.

In domain Prokaryota, kingdom Eubacteria, laccases from phyla Firmicutes, Actinobacteria, Deinococcus-Thermus, Proteobacteria and Cyanobacteria are represented in blue, teal, gray, black and olive green respectively.

1000 bootstrap repetitions were carried out and these values are indicated in the cladogram.

Fig. 3: Cladogram indicating the relationship between laccases across the kingdoms.

The presence of mediators increases laccases effective substrate range to include non-phenolics by reducing the effective redox potential. However, laccase does not have the exclusive need for their presence, unlike the other LDEs. Similarly, unlike other LDEs, laccase production need not be under strict carbon or nitrogen limitation (as often seen in the idiophase). This is vital since in nature, one cannot always expect to find such stringent conditions. Laccase production can be greatly increased in the presence of several inducers, the most common of which is copper ([Wesenberg *et al.*, 2003](#)).

1.4.4.1 Laccase structure: The laccase holoenzyme can either exist as a monomer, dimer or even as a tetramer. Each monomer contains 4 atoms of copper, which are distributed into three redox sites and are designated as Type 1 (T1), Type 2 (T2) and Type 3 (T3) copper.

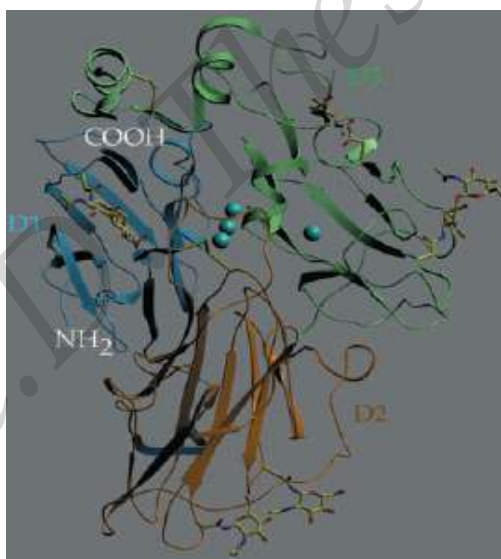


Fig. 4: Ribbon diagram of TvL. The arrangement of the domain structure is depicted in different colours (D1–D3). Copper ions are drawn as *blue spheres*. Carbohydrates and disulfide bonds are included as *stick models*. Source: [Pointek *et al.* \(2002\)](#).

Type 1 & T2 contains one copper atom each whereas T3 contains two copper atoms. The T2 and T3 copper together form the trinuclear cluster ([Farver and Pecht, 1984](#)). The apoprotein comprises of 3 domains of similar β -barrel type architecture. In the holoenzyme, T1 copper is contained in domain 3 whereas the trinuclear cluster is situated

between domains 1 and 3, with both domains providing residues for the co-ordination of the coppers. Fig. 4 illustrates the structure of laccase (Piontek *et al.*, 2002).

Type 1 copper is paramagnetic with a well-characterized Electron Paramagnetic Resonance (EPR) signal. Intense electronic absorption caused by the covalent copper-cysteine bond contributes to the blue colour attributed to laccases, observed at 610 nm (in the oxidized state). Due to its high redox potential of ~ 790 mV, T1 copper is the site where substrate oxidation takes place. Type 2 copper is also paramagnetic with a well-characterized EPR signal. However, this is the non-blue copper. This is strategically positioned close to the T3 copper pair. Type 2 copper is coordinated by two histidines. Type 3 is a diamagnetic spin-coupled copper-copper pair, which forms a bi-nuclear center with an absorbance maximum at 330 nm in the oxidized state. The anti-ferromagnetic coupling of the copper pair is the reason behind the absence of the EPR signal (Claus, 2004). However strong anion binding can activate an EPR signal (Gianfreda *et al.*, 1999). This strong coupling between the two copper atoms is maintained by a hydroxyl bridge. The T3 copper center is also the common feature of another protein super family including the tyrosinases and the haemocyanins. Type 3 copper is coordinated by 6 histidines (all conserved residues). The reduction of molecular oxygen and the release of molecular oxygen takes place at the trinuclear cluster.

Figure 5 illustrates the 11 conserved residues; 10 histidine and 1 cysteine which make a blue multi copper oxidase a laccase, along with the residues (AA's) which co-ordinate the 4 copper atoms. T1 copper has a trigonal coordination, with two histidines and a cysteine as conserved equatorial ligands and the axial ligand is usually variable. This axial ligand is methionine (M) in the bacterial (Cot A) and leucine (L) or phenylalanine (F) in fungal laccases. This critical residue is highlighted in bold forms a part of the crucial pentapeptide marked by a box, present across kingdoms as observed in Fig. 5. A mutation from phenylalanine to methionine significantly lowered the E° of a *Trametes villosa* laccase (Kumar *et al.*, 2003).

It has been widely argued that this axial position ligand strongly influences the E° of the enzyme, possibly providing the mechanism for regulating its activity (Claus, 2004).

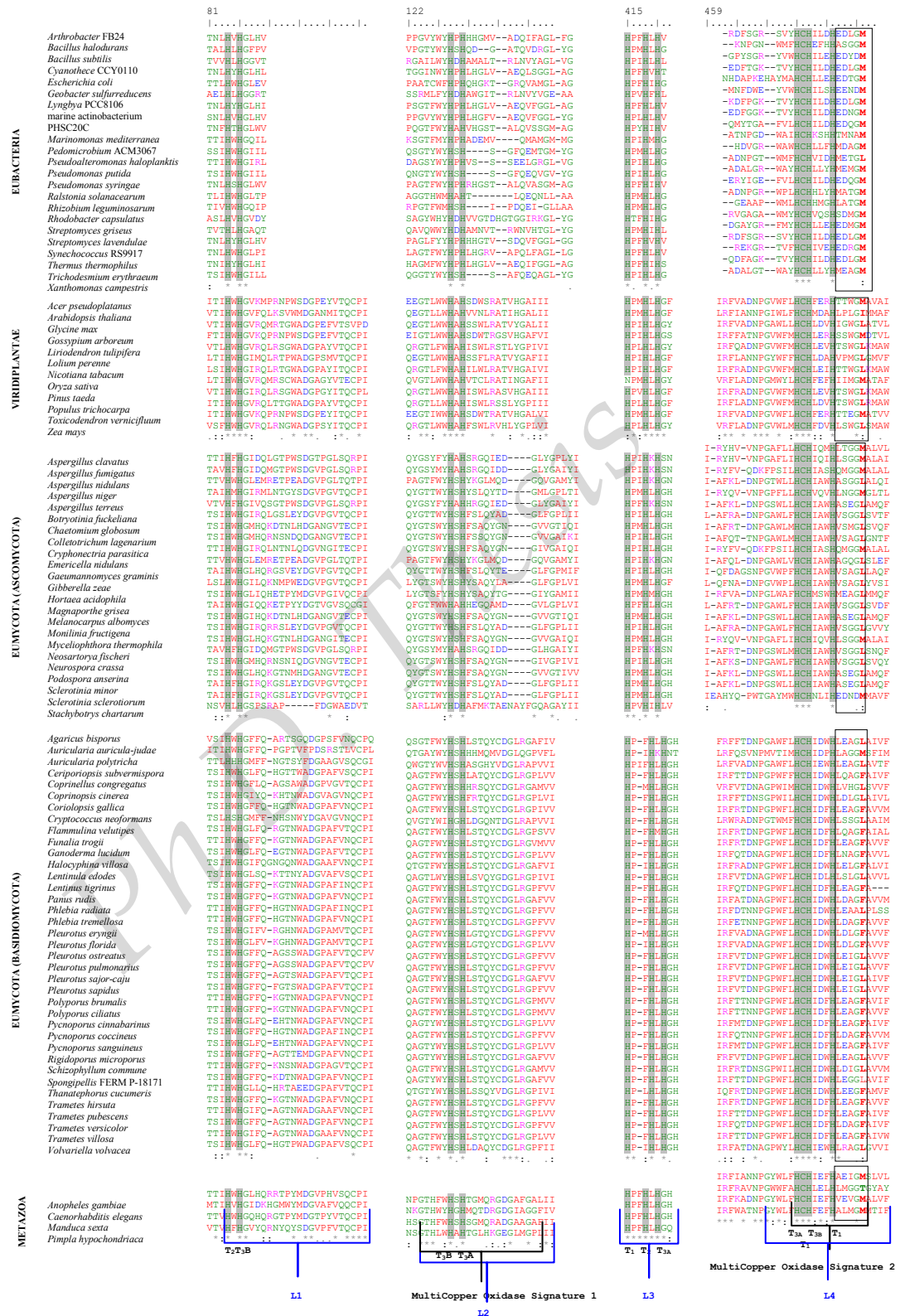


Fig. 5: Consensus sequence of laccases across the kingdoms. Conserved residues are in grey. L₁-L₄ are 4 laccase signatures; T₁, T₂, T_{3A} & T_{3B} are the 4 Cu atoms positioned adjacent to the AAs they bind to.

The crystal structure of *Trametes versicolor* laccase (TvL) has confirmed this hypothesis. The E° of laccase is a summation of a variety of factors, one of which is the trigonal coplanar coordination of the T1 copper. In most of the blue copper proteins, the copper of T1 is in a distorted tetrahedral (4-fold coordinated) configuration. Whereas in laccase, the T1 copper center is trigonal coplanar-coordinated (3-fold coordinated). This coordination of copper is brought about by the presence of ligands. The ligands are supplied by the S-atom (sulphur) of a cysteine and by the N δ 1 nitrogen of two histidines (Fig. 5).

In the other blue proteins, the T1 center has an additional axial ligand, which is contributed by the S-atom of a methionine. Whereas in laccase, methionine is replaced by either F or L at this position as seen in TvL and *Coprinus cinereus* laccase (CcL) respectively. Neither of these amino acids participate in the coordination, resulting in the absence of this additional ligand. As a consequence of this arrangement, the copper ion lies almost within the plane formed by the one S and two N (nitrogen) ligands, whereas in other copper proteins, due to the presence of an additional axial ligand, the copper lies above the plane towards the additional S ligand.

Thus, the coordination of the T1 copper in laccases is different from the ones found in blue copper proteins such as ascorbate oxidase, azurin, and plastocyanin, which supply an additional axial ligand. A modest elevation of the redox potential in laccase is attributed to the lack of this axial ligand, which is present in other blue copper proteins (Piontek *et al.*, 2002).

The E° varies within the laccases too, the presence of a longer Cu1-N δ 2 (His₄₅₈) bond distance has been shown to correspond with an increased E° of laccase (Piontek *et al.*, 2002). *Trametes versicolor* laccase (TvL) has been shown to have a high E° whereas the CcL has a low E° . A comparison between these two structures can account for the difference in redox potential. A small α -helix (residues 455 – 461), which carries the T1 copper-ligating His₄₅₈ in TvL, shows a greater displacement from the Type I copper atom, as compared to its corresponding position in CcL. Resulting in a greater Cu1-N δ 2 (His₄₅₈) distance in TvL than in CcL. An elongated Cu–N bond has an effect on the redox potential since the contribution of the free electron pair from the N to the copper is decreased, rendering the copper more electron-deficient. This would give rise to a destabilization of the higher oxidation states i.e. the redox potential of the T1 copper

should increase. The reason for such a displacement of the small α -helix carrying the T1 copper-ligating His₄₅₈ in TvL, is an H-bond which is formed between Glu₄₆₀ and Ser₁₁₃. The Ser₁₁₃ is situated in the opposite domain, domain 1 (D-1) and is one of the three residues that is responsible for the formation of this H-bond. This H-bond probably forces the α -helix into an unfavourable main chain conformation. As a consequence of this attractive H-bond formation, the whole helix, containing the His₄₅₈, is pulled towards D-1, thus increasing the Cu–N distance, thereby giving rise to an elongated Cu–N bond (Fig. 6).

In CcL, Glu₄₆₀ is replaced by a methionine and the position corresponding to Ser₁₁₃ by a glycine. Hence, an H-bond cannot be formed due to the lack of an appropriate H-bond donor and acceptor. [Pointek et al \(2002\)](#) go on to say that, the redox potential can be increased by more than 200 mV, by decreasing the electron density contribution at the metal cation (Cu) through a stretching of the bond between the metal and the ligating amino acid. This movement could be caused by an appropriate hydrogen bond that results in the displacement of the polypeptide segment, which carries the coordinating amino acid ([Piontek et al., 2002](#)).

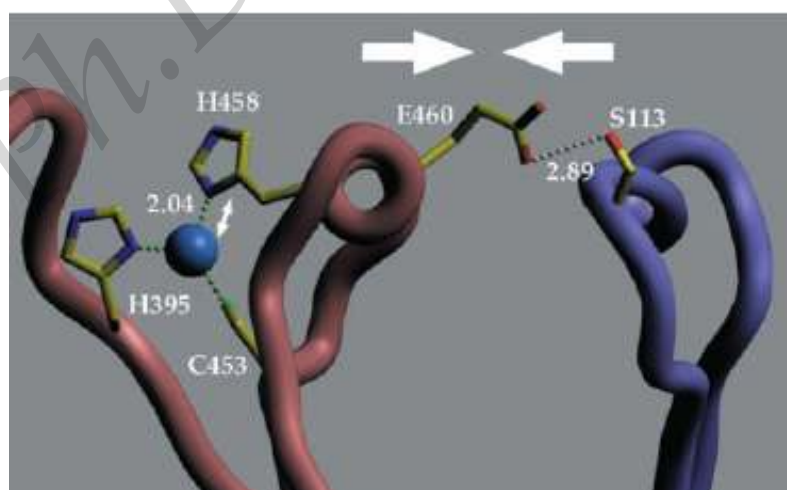


Fig. 6: Schematic drawing illustrating the movement of a helical segment in TvL.

The bond lengths are given in Angstroms. Source: [Pointek et al. \(2002\)](#).

1.4.4.2 Mechanism of catalysis (Fig. 7): The copper centers of laccase drive electrons from a reducing substrate to molecular oxygen without releasing toxic peroxide intermediates. Laccase catalysis is believed to comprise of three major steps (Gianfreda *et al.*, 1999):

1. Reduction of the mononuclear copper center: Here the reducing substrate (usually phenolic compounds) loses an electron to laccase (Gianfreda *et al.*, 1999). This electron reduces the T1 copper (at the mononuclear copper center), which is positioned just below the substrate-binding site (Piontek *et al.*, 2002). The oxidized substrate now becomes a radical, which can either donate the second electron to the T1 copper and become a quinone or directly take part in any non-enzymatic reactions leading to either polymerization or depolymerization. The reduced T1 copper oxidizes itself by transferring the electron to the trinuclear copper cluster. In this way, there are four such mono-electronic reductions of the T1 copper which occur sequentially (Gianfreda *et al.*, 1999).

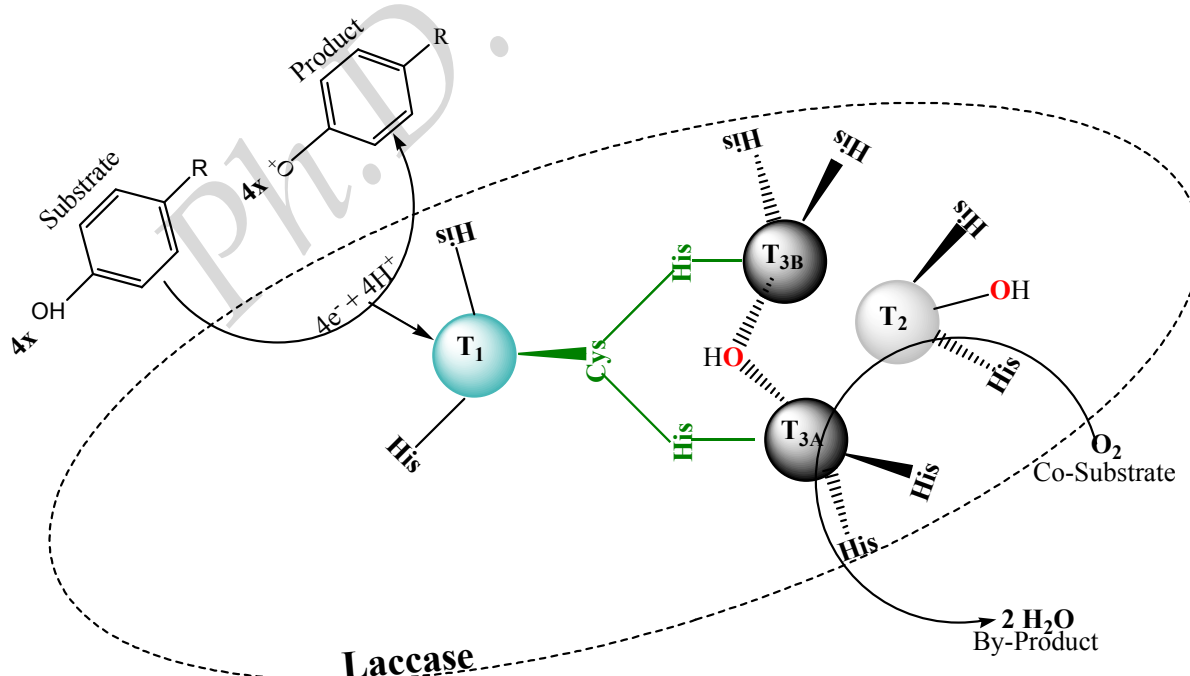


Fig. 7: Mechanism of laccase catalysis. Modified from Baldrian (2006).

2. Internal electron transfer from the mononuclear copper to the trinuclear copper center: A model has been proposed based on experimental evidence that the O₂ molecule first binds to the T2 and any one of the T3 copper atoms. This 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 appears to restrict the access of oxidizing agents besides molecular oxygen, which may account for the exclusivity of laccase for the oxidizing substrate, which is molecular oxygen (Gianfreda *et al.*, 1999) as opposed to its low affinity for the reducing substrate.

3. Reduction of molecular oxygen at the trinuclear copper center takes place at the trinuclear cluster with the concomitant release of water (Claus, 2004).

This basic reaction catalyzed by laccase has two very divergent fates due to the direction which the non-enzymatic reactions follow (Mayer and Staples, 2002). They can either lead to polymerization by the cross linking of monomers or depolymerization of the already existing polymers (Claus, 2004).

The fate of the reaction depends on the type of laccase catalyzing the reaction as well as the immediate microenvironment of the reaction. Ascomycete laccases such as *Melanocarpus albomyces* laccase (MaL) have structures different from basidiomycete laccases like TvL. Here oxygen binds with a novel geometry; the co-substrate oxygen enters the tri-nuclear cluster through a tunnel which is completely open in basidiomycete laccases whereas in MaL, the C-terminus forms a movable plug which can block this access. Thus after the oxygen has entered it may be trapped by this plug, resulting in stable dioxygen binding. This C-terminal blockage of ascomycete laccases drastically reduces the speed of the free inflow of O₂ and release of water molecules, thereby giving a chance for the oxidized free radicals in the surrounding environment to polymerize whereas in case of the basidiomycete laccases, the rapid exchange of O₂ and water does not allow for the build-up of free radicals in the microenvironment thus avoiding polymerization. This C-terminal blockage is probably characteristic of ascomycete laccases and may explain why ascomycete laccases are generally involved in

polymerization reactions whereas basidiomycete laccases, in depolymerization reactions (Hakulinen *et al.*, 2002).

Thus, laccase catalyzes two opposite types of reactions; depolymerization and polymerization reactions using a single mechanism of action, free radical generation. All the applications of laccase are a by-product of these two opposing reactions.

1.4.4.3 APPLICATIONS: The applications of laccase are very wide and are the subject of many reviews such as (Baldrian, 2006; Couto and Herrera, 2006; Gianfreda *et al.*, 1999; Mayer and Staples, 2002) to name few. Presented below are few of those applications:

1.4.4.3.1 APPLICATIONS IN NATURE

1.4.4.3.1.1 Lignification: Role-polymerization. The main role of laccases in plants is lignification, to increase rigidity of the stem (Claus, 2004).

1.4.4.3.1.2 Delignification: Role-depolymerization. The main role of laccases in fungi is delignification, Lignin is the structural component of wood and the white rots need to get past this barrier to reach the cellulose which is the energy source for the fungus. To do so lignin has to be degraded, one of the lignin degrading enzymes that these fungi have in their repertoire is laccase.

1.4.4.3.1.3 Fungal Pathogenesis: Role-polymerization. Laccase has been shown to be an important virulence factor in many diseases caused by fungi. Among other roles, laccase can protect the fungal phytopathogen from the toxic phytoalexins and tannins of the host (Pezet *et al.*, 1992). For example, in the root pathogen, the aggressiveness of *Heterobasidion annosum* is related to the presence of laccase, whereas laccase-negative strains have highly decreased virulence (Johansson *et al.*, 1999; Mayer and Staples, 2002). The effect of laccase in aiding fungal pathogenesis extends to humans as well.

Cryptococcus neoformans, an encapsulated fungus is known to infect immunocompromised patients. Laccase and its product melanin are important virulence factors of *C. neoformans*. Melanin may protect *C. neoformans* by acting as an anti-oxidant or by interacting with the cell wall surface, thereby offering protection against numerous effectors of cellular immunity. Liu *et al.* (1999) from their studies on infected mouse brain, which do not contain melanin, suggested that the iron oxidase activity of laccase may protect *C. neoformans* from alveolar macrophages by oxidation of phagosomal iron to Fe⁺³ with a resultant decrease in hydroxyl radical formation. The ability of a multinuclear copper enzyme to oxidize divalent iron has been reported previously (Hassett *et al.*, 1998). Since laccase is not produced in humans, it is a potential drug target (Mayer and Staples, 2002).

1.4.4.3.1.4 Plant Defense: Role-polymerization in wound healing but depolymerization when acting as a deterrent. The presence of laccases in resin ducts of Anacardiaceae suggests a rather obvious function, i.e. defense against predators, herbivores and invasion by bacteria and fungi. The excretion of an enzyme which will cause oxidative reactions at the wound surface certainly suggests a role as a deterrent or in wound healing (Mayer and Staples, 2002). However, this requires more research.

1.4.4.3.1.5 Sclerotization: Role-polymerization. In insects, laccase catalyzed oxidative coupling of catechols with proteins may be involved in the cuticle sclerotization (Kramer *et al.*, 2001).

1.4.4.3.1.6 Sporulation: Role-polymerization. In microorganisms, laccase-catalyzed cross-linking of protein residues e.g. tyrosine to dityrosine is required for the assembly of heat and UV-resistant *Bacillus* spores (Hullo *et al.*, 2001; Martins *et al.*, 2002).

1.4.4.3.1.7 Morphogenesis: Role-polymerization. An association between laccase synthesis in *Armillaria mellea* and formation of rhizomorphs (mycelial strands formed from large numbers of tightly adpressed hyphae) was hypothesized by Worrall *et al.* (1986). It was supported by an observed decrease of both laccase activity and rhizomorph production by the presence of laccase inhibitors.

1.4.4.3.2 EXTENDED APPLICATIONS

1.4.4.3.2.1 Bioremediation: Laccase-mediated processes may be regarded as much safer and less contaminating alternative methods than harsher chemical methods.

1.4.4.3.2.1.1 Soil Bioremediation: Role-polymerization, as a means of sequestering non-toxic polymers of toxic pollutants and depolymerization to degrade toxic pollutants to non-toxic breakdown products. Traditional techniques utilize oxygen- or chlorine-based agents that lead to the release of toxic contaminants in the environment. Laccase can immobilize soil pollutants by coupling to soil humic substances – a process analogous to humic acid synthesis in soils. Phenolic xenobiotics such as 3,4-dichloroaniline, 2,4,6-trinitrotoluene (TNT) or chlorinated phenols can be immobilized in this way. Immobilization lowers the biological availability of the xenobiotics and thus their toxicity (Baldrian, 2006).

Polycyclic aromatic hydrocarbons (PAHs) together with other xenobiotics are a major source of contamination in soil. Therefore, their degradation is of great importance for the environment. Laccases were able to mediate the coupling of reduced TNT metabolites to an organic soil matrix, which resulted in detoxification of the munition residue. Moreover, PAHs, which arise from natural oil deposits and utilization of fossil fuels, were also found to be degraded by laccases (Couto and Herrera, 2006).

Laccases from *Phanerochaete chrysosporium* and *T. versicolor*, are able to convert diketonitrile derivative, the active form of the herbicide isoxaflutole, which is attained in plants and soil, into the inactive benzoic acid analogue with the help of a redox mediator (Mougin *et al.*, 2000).

1.4.4.3.2.1.2 Decolourization of Dyes and Effluents: Role-depolymerization. The textile industry accounts for two-thirds of the total dyestuff market and consumes large volumes of water and chemicals. The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products. There are more than 100,000 commercially available dyes with over 7×10^5 tons of dyestuff produced annually. Due to their synthetic origin, most dyes are resistant to fading on exposure to light, water and various chemicals. Several dyes are made from known

carcinogens such as benzidine and other aromatic compounds and thus may have harmful repercussions. Most currently existing processes to treat such effluents are ineffective and not economical. Therefore, the development of bioprocesses such as the use of enzymes like laccases is an attractive solution due to their potential in degrading dyes of diverse chemical structure including synthetic dyes currently employed in the industry. The use of laccase in the textile industry is growing very fast, since besides decolourizing textile effluents mentioned above, laccase is also used to bleach textiles and even synthesize dyes. Related to textile bleaching, in 1996 Novozyme (Novo Nordisk, Denmark) launched a new industrial application of laccase enzyme in denim finishing, DeniLite®, the first industrial laccase and the first bleaching enzyme acting with the help of a mediator molecule. In addition, in 2001 the company Zytex (Zytex Pvt. Ltd., Mumbai, India) developed a formulation based on laccase-mediator-system (LMS) capable of degrading indigo in a very specific way. The trade name of the product is Zylite (Couto & Herrera, 2006).

1.4.4.3.2.2 Paper and Pulp Industry: Role-depolymerization in most cases and polymerization when used in adhesion. The industrial preparation of paper requires separation and degradation of lignin in wood pulp. Environmental concerns urge to replace conventional and polluting chlorine-based delignification or bleaching procedures. Oxygen delignification processes have been industrially introduced but pre-treatments of wood pulp with ligninolytic enzymes might provide milder and cleaner strategies of delignification that may not affect the integrity of cellulose. Although extensive studies have been performed to develop alternative bio-bleaching systems, few enzymatic treatments exhibit the delignification / brightening capabilities of modern chemical bleaching technologies.

One of the few exceptions to this generalization, is the development of LMS delignification technologies for kraft pulps. In addition, laccase is more readily available and easier to manipulate than both LiP and MnP and LMS has already found practical applications such as the Lignozym®-process. Several authors applied the LMS to pulp biobleaching. However, all these biobleaching studies were focused on wood pulps and little is known about the efficiency of the LMS on non-wood pulps including those used

for manufacturing specialty papers [Couto & Herrera, 2006](#)). In this sense, [Camarero *et al.* \(2004\)](#) explored the potential of LMS to remove lignin-derived products responsible for colour from a high quality flax pulp. They have shown the feasibility of LMS to substitute chlorine-containing reagents in manufacturing of these high-price paper pulps.

The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibers. For example, laccases can be used in the enzymatic adhesion of fibers in the manufacturing of lignocellulose-based composite materials such as fiberboards. Laccases have been proposed to activate the fiber bound lignin during manufacturing of the composites thus, resulting in boards with good mechanical properties without toxic synthetic adhesives. Another possibility is to functionalize lignocellulosic fibers by laccases in order to improve the chemical or physical properties of the fiber products. Preliminary results have shown that laccases are able to graft various phenolics acid derivatives onto kraft pulp fibers. This ability could be used in the future to attach chemically versatile compounds to the fiber surfaces, possibly resulting in fiber materials with completely novel properties such as hydrophobicity or charge [\(Couto & Herrera, 2006\)](#).

1.4.4.3.2.3 Food Industry: Laccases can be applied to certain processes that enhance or modify the colour and appearance of food or beverage.

1.4.4.3.2.3.1 Ethanol Production: Role-depolymerization. Laccase can improve the production of biofuel, ethanol from renewable raw materials. This has been experimentally proved by the expression of the *Trametes versicolor* laccase in *Saccharomyces cerevisiae*. Here, laccase increased resistance to phenolic inhibitors present in lignocellulose hydrolysates which inhibited their fermentation to ethanol [\(Larsson *et al.*, 2001\)](#).

1.4.4.3.2.3.2 Wine Clarification: Role-depolymerization. Laccase is also used to remove phenolic compounds from white grape must in wine. If the laccase is immobilized on a copper-chelate carrier that can be regenerated successfully, the process becomes more economical. Epicatechin, ferulic and *o*-coumaric acids are some of the phenolic

compounds which have been partially removed by the formation of complex compounds (Servili *et al.*, 2000).

1.4.4.3.2.3.3 Bread Making: Role-polymerization. Laccases are currently of interest in baking due to its ability to cross-link biopolymers. Thus, Selinheimo *et al.* (2006) have shown that a laccase from *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough.

1.4.4.3.2.4 Analytical Tools

1.4.4.3.2.4.1 Biosensors: Role-depolymerization. Morphine can be distinguished from codeine simultaneously in drug samples by injecting the sample into a flow detection system containing an enzyme sensor. This sensor contains laccase and glucose dehydrogenase immobilized at a Clark oxygen electrode. Morphine is oxidized by laccase with consumption of oxygen; laccase is then regenerated by glucose dehydrogenase. Laccase cannot oxidize codeine, so the sensor is selective for morphine. Morphine between 32 nM and 100 mM can be detected. The rapid and technically simple method allows discrimination between morphine and codeine in less than 1 min after injection at a sampling rate for 20 quantitative measurements per h (Bauer *et al.*, 1999).

The ability of laccase to catalyze the electro-reduction of oxygen via a direct mechanism, without the presence of an electrochemically active mediator, was used to design a potentiometric immunosensor (Ghindilis *et al.*, 1992). Similarly, a gas-phase oxygen biosensor was developed consisting of a laccase from *Rhus vernicifera*, using ascorbate as the reducing substrate enclosed in pouches of low-density polyethylene under nitrogen gas. In the presence of ascorbate, the blue-chromophore-prosthetic group of laccase was reduced and decolourized. When the enzyme was reoxidized by oxygen, there was a concomitant return of the blue colour. This could be recorded both visually and spectrophotometrically at 610 nm. This oxygen biosensor was very active and stable. It was proposed as an useful tool to measure oxygen levels in products packaged under low oxygen concentrations whose quality and safety is strictly dependent on these low oxygen levels (Gardiol *et al.*, 1996).

1.4.4.3.2.4.2 Enzyme Assay: Role-depolymerization. Murao *et al.* (1985) utilized a laccase from *Polyporus versicolor* to assay the levels of two enzymes, α -glucosidase and α -amylase, in different systems. The method was based on a colourimetric coupling reaction between a phenol and 4-aminoantipyrine mediated by laccase. The hydrolysis of phenyl- α -glucosides or phenyl-malto-pentaosides by glucosidase or amylase releases phenol that can be converted to a coloured substance by laccase.

A sensitive and quantitative assay for detecting two enzymes, cellobiose quinone oxidoreductase (CBQase) and glucose oxidase was set up by Roy and Archibald (1994). It was based on the capability of CBQase to reduce the cation radical formed by laccase oxidation of chlorpromazine. The attractive characteristics of this assay are its increased sensitivity, the high solubility of the substrate in water, and its ability to measure reductive activity in the presence of laccase and other oxidative enzymes.

1.4.4.3.2.5 Cosmetic Industry: Role-depolymerization. Laccase-based hair dyes are less irritating and easier to handle than current hair dyes, since laccases replace H_2O_2 as an oxidizing agent in the dye formulation. More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed (Couto and Herrera, 2006).

1.4.4.3.2.6 Synthetic Chemistry: In the future, laccases may also be of great interest in synthetic chemistry, where they have been proposed to be applicable for oxidative protection and production of complex polymers and medical agents. Recently, Mustafa *et al.* (2005) synthesized phenolic colourants by using an industrial laccase named Suberase® (Novo Nordisk A/S, Bagsvaerd, Denmark) (Couto and Herrera, 2006).

The most important obstacles to commercial application of laccases are the lack of sufficient enzyme stocks and the cost of redox mediators. Marked progress has been made over the last years to solve these problems. Thus, efforts have to be made in order to achieve economic overproduction in heterologous hosts and their modification by chemical means or protein engineering to obtain more robust and active enzymes. On the other hand, the development of an effective system for laccase immobilization also

deserves great attention. The general goal is to obtain stable economically viable catalysts with long shelf lives.

Laccase is an enzyme which has diverse commercial and environmental applications. Laccases with superior qualities such as thermo-stability, halo- and metal-tolerance as well as tolerance to either highly acidic or alkaline conditions, as per the specific requirement are commercially much sought after.

Marine fungi are a known source of novel metabolites and enzymes. In this thesis, marine-derived fungi have been screened for the production of laccases. A laccase-hyper producing fungal strain was selected, identified and the laccase characterized. The conditions for maximal production of laccase via medium modification have been determined. The bioremediation applications of the crude laccase preparations from this strain have also been investigated.