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

Thesis submitted for the degree of

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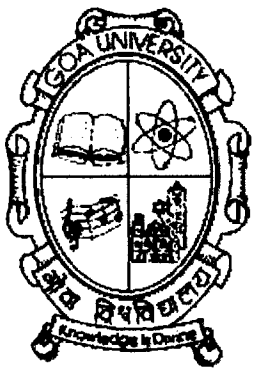

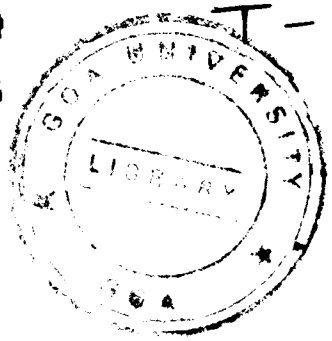

by

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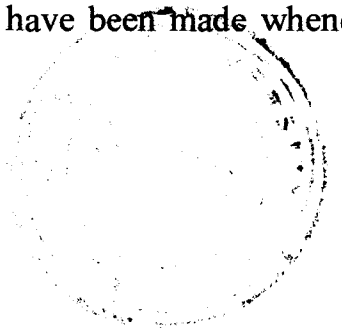
October 2008

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.



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Understanding the seas

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.

Place: Dona Paula

Date: 20 Oct, 2008

22/10/09

All the corrections suggested by the examiners have been incorporated.

22-10-2009

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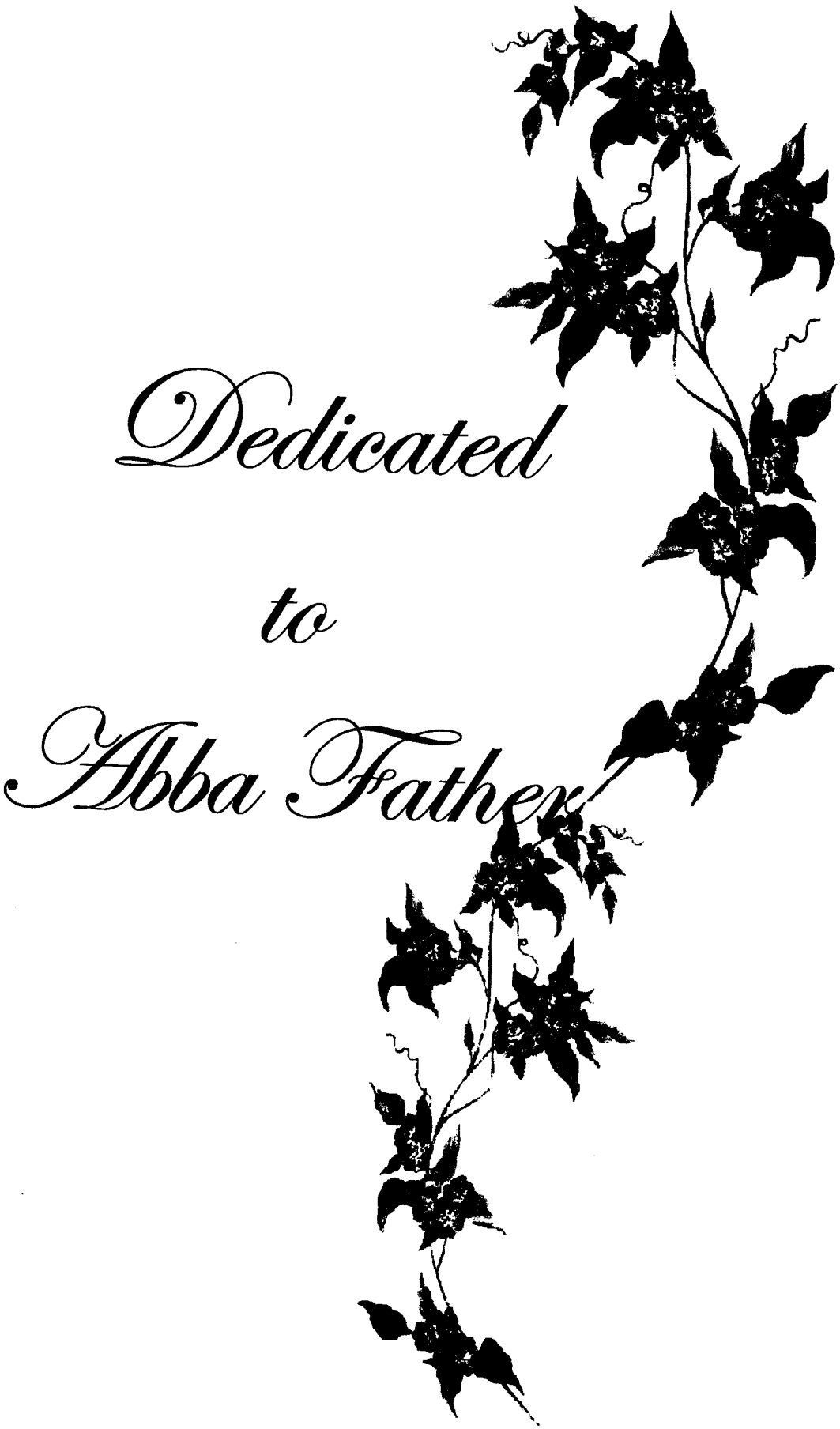
Dedicated
to
Abba Father

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D'Souza Ticlo

Donna Trella D'Souza Ticlo

Table of Contents

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 Kohlmeyer
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	*Coprinus cinereus* 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
m	meta-position
M	molar
mA	milliampere
MaL	*Melanocarpus albomyces* 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 / *Cerrena unicolor* MTCC 5159
NJ	neighbor-joining
nm	nanometre
nmol	nanomole
NS	nuclear small
NTS	non-transcribed spacer
o	ortho-position
O.D.	optical density
O_2	molecular oxygen
p	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 pyrolidine
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	*Thermus aquaticus*
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	*Trametes versicolor* laccase
U	unit
UV	ultra violet
v.	version
v/v	volume by volume
VA	veratryl alcohol / 3, 4-dimethoxy benzyl alcohol
Vgb	*Vitreoscilla* hemoglobin gene
Vhb	*Vitreoscilla* 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
€	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

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, aplanochtrids, 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 environemnt, 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).

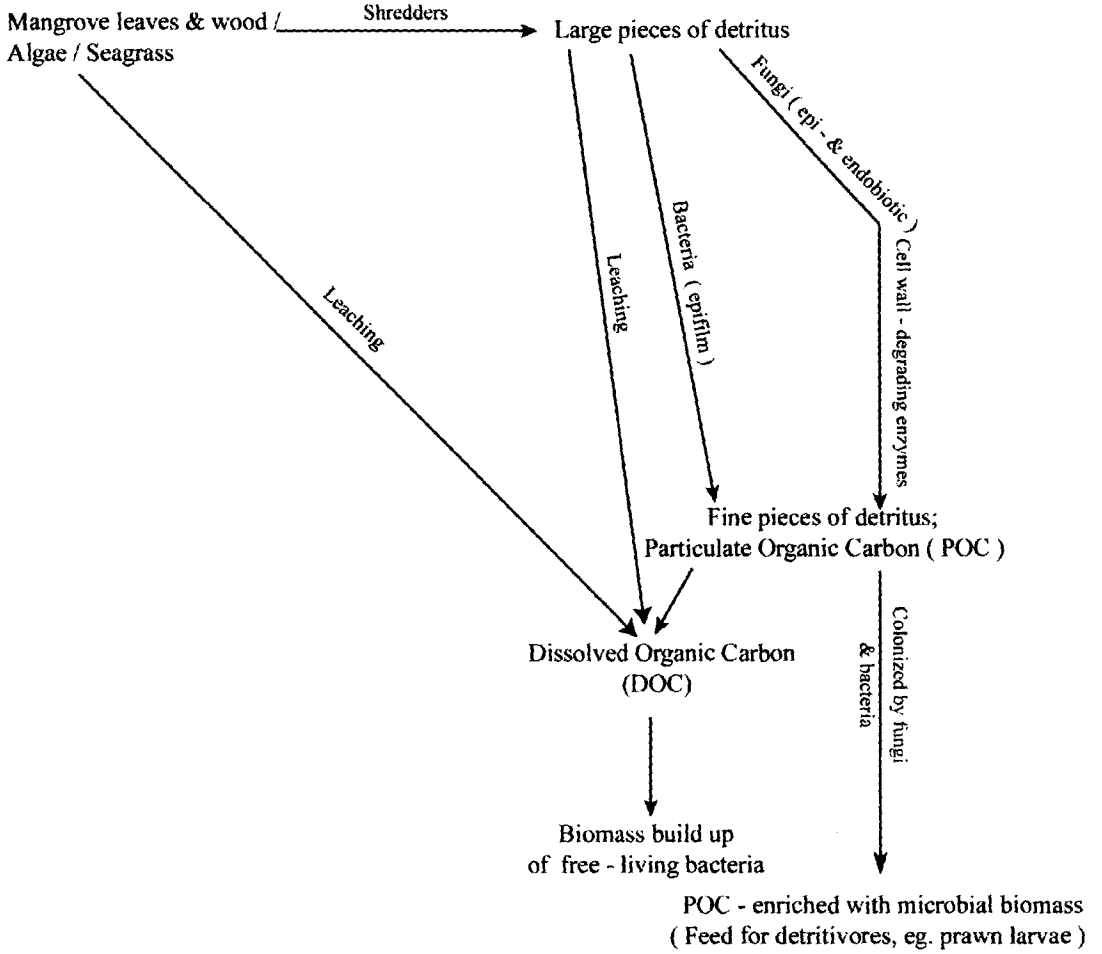


Fig. 1: Schematic diagram illustrating the process of degradation of plant material in the marine habitat. Modified from (Raghukumar *et al.*, 2008).

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 helicascolides 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-stratergists 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 ***T_1 type*** of soft rot whereas in the ***T_2 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

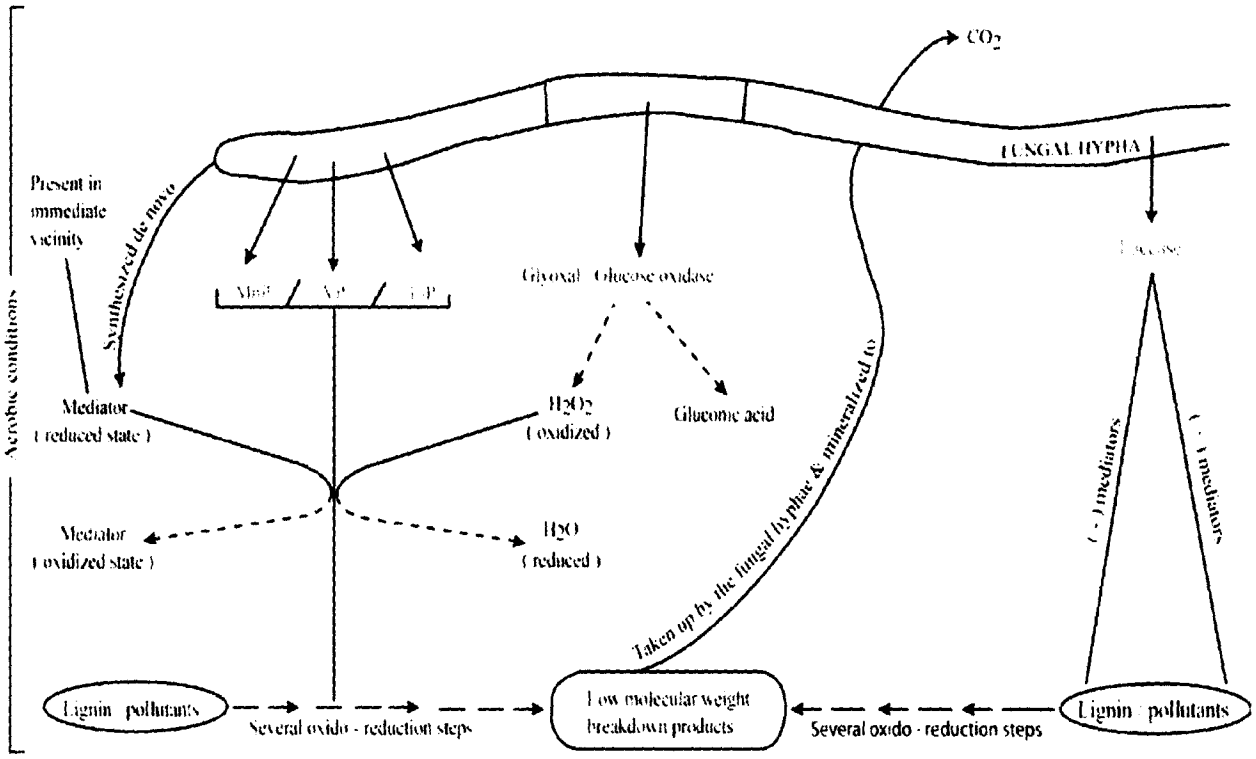


Fig. 2: Schematic diagram showing the role and activity of various extracellular lignin- degrading enzymes produced by fungi. Source: (Raghukumar *et al.*, 2008).

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-

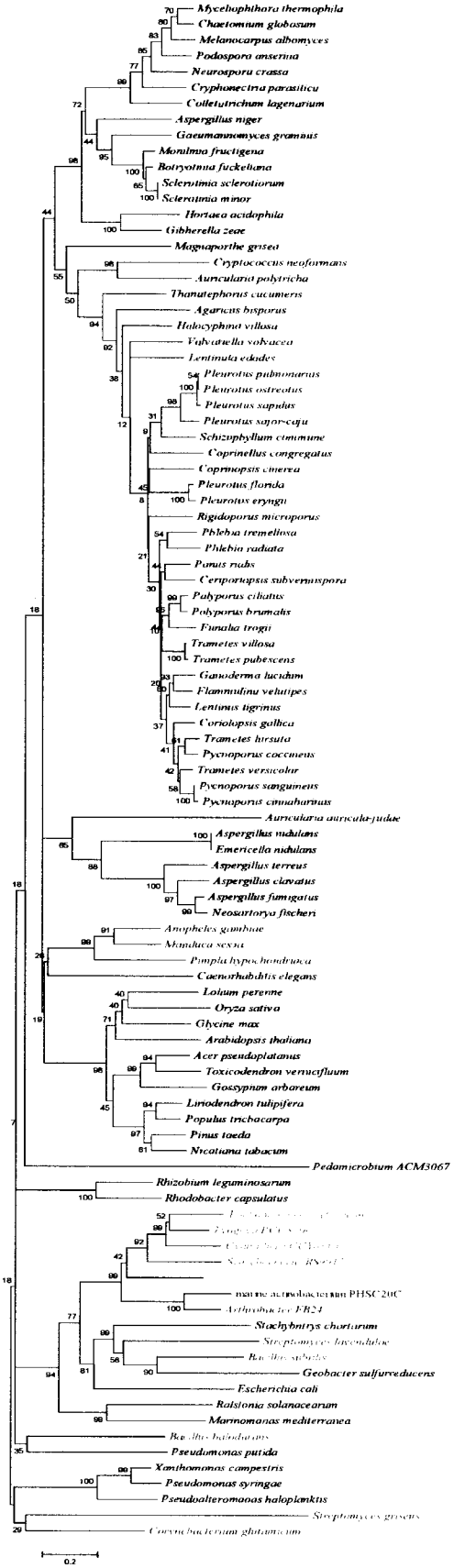
binding site similar to MnP and an exposed tryptophan residue homologous to that involved in veratryl alcohol oxidation by LiP. It is suggested that the catalytic properties of the new peroxidase is due to a hybrid molecular architecture combining different substrate binding and oxidation sites (Camarero *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 H_2O_2 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 seceretory 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, , 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.

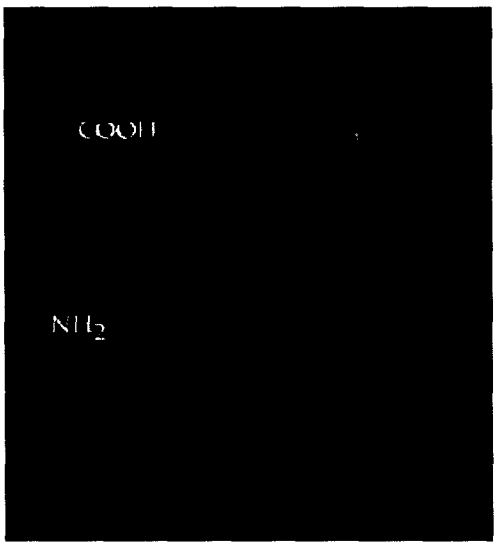


Fig. 4: Ribbon diagram of TvL. The arrangement of the domain structure is depicted in different colours (D1–D3). Copper ions are drawn as *blue spheres*. Carbohydrates and disulfide bonds are included as *stick models*. Source: Pointek *et al.* (2002).

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).

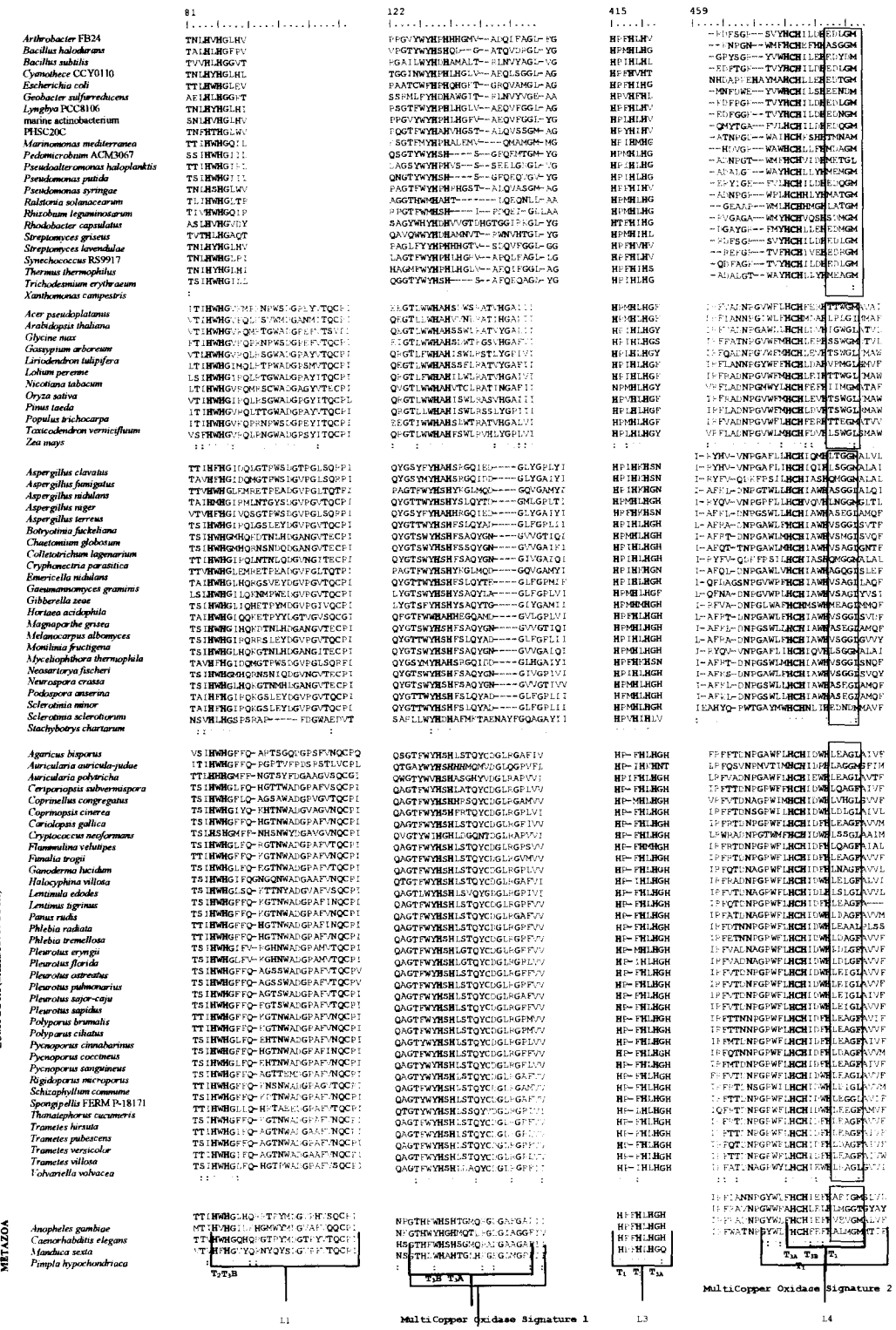


Fig. 5: Consensus sequence of laccases across the kingdoms. Conserved residues are in grey. L_1-L_4 are 4 laccase signatures; T_1, T_2 ,T_{3A} &T_{3B} are the 4 Cu atoms positioned adjacent to the AAs they bind to.

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 Nδ1 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-Nδ2 (His_{458}) 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 theses two structures can account for the difference in redox potential. A small α-helix (residues 455 – 461), which carries the T1 copper-ligating His_{458} 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-Nδ2 (His_{458}) 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 His_{458} in TvL, is an H-bond which is formed between Glu_{460} and Ser_{113}. The Ser_{113} 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 His_{458}, is pulled towards D-1, thus increasing the Cu–N distance, thereby giving rise to an elongated Cu-N bond (Fig. 6).

In CcL, Glu_{460} is replaced by a methionine and the position corresponding to Ser_{113} 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).

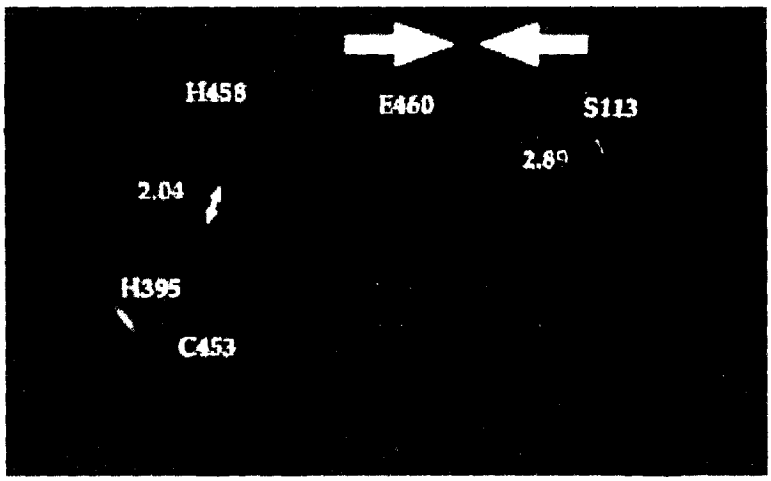


Fig. 6: Schematic drawing illustrating the movement of a helical segment in TvL.
The bond lengths are given in Angstroms. Source: Pointek *et al.* (2002).

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).

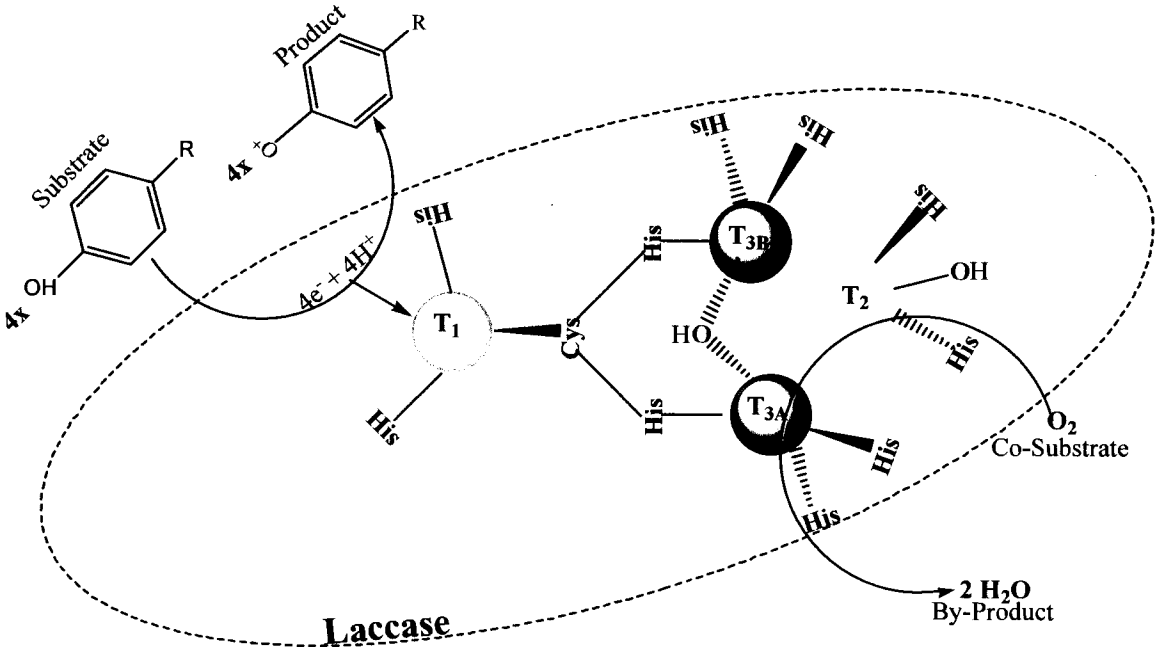


Fig. 7: Mechanism of laccase catalysis. Modified from Baldrian (2006).

2. Internal electron transfer from the mononuclear copper to the trinuclear copper center: A model has been proposed based on experimental evidence that the O_2 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 O_2 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 O_2 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 immuno-compromised 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 Anarcardiaceae 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, Bagsvaerdt, 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.

Chapter 2

Isolation, Screening & Identification of Laccase-producing Marine Fungi

ABSTRACT

Fungi were isolated from decaying wood pieces, leaves and other plant detritus from the mangrove swamps of Chorao Island in Goa, India. Seventy-five fungi were isolated using techniques such as particle-plating, hyphal and single spore isolation methods out of which, 15 isolates were laccase-producers. From the laccase-producing fungi, the anamorphic fungi designated NIOCC #2a, NIOCC #7a and NIOCC #Xa were the most efficient dye decolourizers and laccase producers as well. NIOCC #2a was the laccase hyper-producing strain selected for this study. It was deposited at the Microbial Type Culture Collection (MTCC) under accession no. 5159. Since it was an anamorph, it was identified using rDNA homology (18S rDNA) to be a *Cerrena unicolor* strain. The fungus NIOCC #2a is therefore referred to as *Cerrena unicolor* MTCC 5159. These results were confirmed by ITS1-5.8S-ITS2 and D1/D2 of the 25-28S rDNA. Its identity as a marine-derived fungus was confirmed by obtaining maximum biomass production in full strength seawater of 34 ppt and maximum laccase production in seawater of 25 ppt.

2.1 INTRODUCTION

Marine fungi are an ecological rather than taxonomic group and comprise about roughly 1500 species, excluding those that form lichens. According to their biogeographical distribution, marine fungi can be grouped into temperate, subtropical, tropical and cosmopolitan species (Abdel-Wahab & El-Sharouny, 2002).

Fungi which grow and sporulate exclusively under marine conditions, have been defined as ***obligate marine fungi***. In order to accomodate the possibility that terrestiral species might also be active in the sea, these authors offered a defination for fungi which originate from freshwater or terrestrial environment and are capable of growth and sporualtion in the sea as ***facultative marine fungi***. Marine obligate and facultative filamentous fungi are known to occur in algae, corals and detritus of marine macrophytes (Kohlmeyer & Kohlmeyer, 1979).

Marine-derived fungi mostly include facultative marine fungi, that are capable of growth in the marine environment but similar strains also occur on land. If the marine-derived strains have been actively growing and present for sufficient time in the marine environment, chances that specific adaptations occur, increase (Coumo *et al.*, 1995). These authors also suggest that five times as many new compounds from marine-derived strains could be isolated as compared to their terrestrial counterparts, suggesting that the rate of successful isolation of new compounds may be habitat dependant. Genetic comparison of the terrestrial and the marine-derived strains can determine whether these adaptations have occurred at the genetic level (Jensen & Fenical, 2002).

The vast majority of compounds reported from marine fungi are in fact from marine-derived strains, which based on morphological characteristics have been shown to be identical or at least closely related to terrestrial species. Given the inherent salt tolerance of many fungal species, transitioning the land / sea barrier may in fact be commonplace and most marine-derived strains may have been growing in the environments from which they were isolated. Since these strains are chemically prolific and compared to obligate marine fungi are fast growing as well as exhibit a high degree of salt tolerance, they have been the focus of a vast majority of research. Marine-derived

strains, which have been metabolically active in the marine environment for sufficient amount of time to allow secondary metabolite production to be influenced by specific marine parameters, are a good source for the isolation of novel marine metabolites (Jensen & Fenical, 2002).

The term 'mangrove', apart from indicating a tropical intertidal community, also refers to the constituent vegetation of this community (Tomlinson, 1986) that exists at the boundary between the terrestrial and marine environments. This covers approximately one-fourth of the entire tropical coastline and extend over 15.5 million ha worldwide (Bandaranayake, 1998). The mangroves have specialized features like pneumatophores (aerial roots), viviparous form of reproduction, prop roots and high salt tolerance all of which make them unique. The mangroves are home to mangrove fungi, which include some obligate marine fungi, marine-derived fungi and special ***mangicolous fungi***, fungi specific for mangroves. The age of the mangrove stand, diversity of the mangrove and terrestrial tree flora as well as various microhabitats (e.g. salinity, temperature, humidity, pH) inside mangroves are the most important factors controlling the diversity of mangrove fungi (Kohlmeyer & Volkmann-Kohlmeyer, 1993).

These fungi play a mostly saprophytic role, in the microbial processes occurring in the mangroves especially the mangrove food web and the surrounding coastal areas, to release nutrients which can again be used by plants and animals as organic sources for metabolism (Raghukumar, 2004). This role involves the degradation and mineralization of lignocellulosic substrates and fungi that do so are termed as ***lignicolous*** or ***lignin-degrading fungi*** (this term applies for all fungi that have the capacity to degrade lignin, irrespective of their source). Mangrove plants, found in estuaries of the tropical and subtropical belts contain about 50 % lignocellulosic structural polymers and about 50 % soluble organics which include tanins and phenolics (Benner & Hodson, 1985).

Next to cellulose, lignin is the most abundant and widely distributed renewable aromatic polymer (Boominathan & Reddy, 1992) and is one of the major structural components of woody plants. About 70 % of the dry weight of woody plants consists of lignocellulose of which 20 - 30 % is contributed by lignin. The lignin-degrading fungi initiate the process of lignin degradation using a specialized set of

enzymes, the lignin-degrading enzymes (LDEs). These enzymes include the heme-containing peroxidases and the copper-containing glycoproteins, laccases. The peroxidases include the manganese dependant peroxidase (MnP), lignin peroxidase (LiP) and versitaile peroxidase (VP), which is a hybrid of MnP and LiP.

Besides existing as saprophytes, mangrove fungi also exist as '***endophytes***'. The term endophyte refers to all organisms inhabiting plant tissues, which at some time of their life cycle, colonize internal plant tissues without causing apparent harm to the host (Petrini, 1991). Successful colonization of the host tissues by endophytic fungi can be achieved only when they are able to breach the protective layers of the host. An endophyte occupies essentially the same ecological niche as most fungal pathogens and hence it is expected to adopt the same strategy as the pathogens to enter into the host tissues (Petrini *et al.*, 1992). Mangrove plants provide a hostile environment for endophytes, by the presence of phenolics like tannins, which are known to inhibit the growth of litter and soil fungi (Kumaresan *et al.*, 2002). Ability to grow in the presence of such phenolic compounds entails some adaptations on the part of the fungal endophytes. These include the production of extracellular cell wall degrading enzymes, ability to grow in the presence of phenolic compounds and halotolerance. Kumaresan *et al* (2002) have shown that most of endophytic mangrove fungi examined produced lipolytic and pectinolytic activities which degrade the cuticular waxes on the leaf surface and the middle lamella of the leaf cells. Most of them produced the non-specific enzyme, laccase which also degraded other phenolic compounds in addition to the lignin polymer, indicating their involvement in litter degradation as well.

Many mangrove plants either accumulate or exclude salt from their leaves; the salt concentration in their leaves was similar to that of seawater. In addition, salt excreting mangrove plants such as *Avicennia* and *Aegiceras*, mangrove plants often have crystals of salt on their leaf surface (Tomlinson, 1986). Thus, the foliar endophytes of mangrove plants have to encounter a saline milieu during and after leaf penetration. Halotolerance in endophytes, would be essential (Kumaresan *et al.*, 2002). Some latent pathogens, survive as endophytes on their host tissues and become active when the host is stressed (Carrol, 1988).

The ability of endophytic basidiomyceteous fungi to produce extracellular enzymes including laccase that lead to breakdown of the host cell walls, contributes to their overall success in the colonization of the host via entry through host cell walls. Endophytes can grow and produce fruiting bodies on dead and fallen mangrove leaves thus building up the endophyte inoculum (Kumaresan & Suryanarayanan, 2002). This suggests the ability of these endophytes to lead a saprobic lifestyle upon the senescence of the plant (Pointing, 2001).

Even with the comparatively lesser abundance of oligate and facultative marine fungi, their taxonomy is of importance and the presence and type of LDEs produced have also been used a critrerion in fungal taxonomy, among the lignicolous fungi (Guarro *et al.*, 1999). Fungal taxonomy is a dynamic, progressive discipline that consequently requires changes in nomenclature. The baseline of traditional fungal taxonomy and nomenclature is morphological criteria or their phenotypes. Numerous alternative approaches have been developed including nutritional and physiological studies, serologic tests, secondary metabolites, ubiquinone system, and fatty acids. Although some of these are very useful for identifying poorly differentiated fungi such as yeasts and black yeasts, in most cases they are only complementary tools of morphological data. The fungus, as a whole, comprises a teleomorph (sexual state) and one or more anamorphs (asexual states). Traditional fungal classification is possible when the fungus is at its teleomorphic stage (asexual spore morphology also help); however the snag occurs in cases of fungi where only the anamorphic stage is available.

The dual modality of fungal propagation, i.e., sexual and asexual, has led to (Saccardo, 1880) a dual nomenclature. The anamorph and teleomorph generally develop at different times and on different substrates, (although in zygomycetes they often occur together). Each phase has been described in total ignorance of the existence of the other in many cases, although the International Code of Botanical Nomenclature (ICBN) maintains that it is legal to give them separate binomials, many mycologists regard it as unsatisfactory to have several names for a single fungus (Guarro *et al.*, 1999). For a long time, the anamorphs that occurred alone have been grouped into a separate major high-level taxon called Deuteromycotina or Deuteromycetes. The phenotypic approach has been largely criticized for its lack of standardized and stable terminology and for its high

subjectivity. Moreover, some phenotypic characteristics have been considered unstable and dependent on environmental conditions, as with growth in artificial culture. A clear limitation of phenotypic approaches is that they cannot be applied to fungi that do not grow or sporulate in culture. Consequently, many fungi will remain unclassified as long as taxonomists rely solely on phenotypic characteristics.

Another notable problem of classification based on morphological criteria is the above-mentioned dual system of classification, with no consistent correlation between the taxonomies of the Ascomycetes and Deuteromycetes (Hennebert & Sutton, 1994). This is an important difficulty in establishing the taxonomic concept of the fungus as a whole. In addition, traditional fungal systematics does not significantly contribute to the understanding of the evolutionary relationships among fungi. The simple morphology, the lack of a useful fossil record and fungal diversity have been major impediments to phylogenetic studies (Berbee & Taylor, 1992). Classically, studies on fungal evolution have been based on comparative morphology, cell wall composition, cytologic testing, ultrastructure, cellular metabolism and the fossil record, yet wide disparities remain (Guarro *et al.*, 1999). It is here where modern molecular techniques not only come to the rescue but also allow for the anamorphic and teleomorphic stages of a particular fungus to be connected and integrated into a more natural taxonomic scheme, which was previously possible only to a limited extent in traditional fungal taxonomy.

With the advent of universally applicable molecular approaches in fungal taxonomy, some mycologists have advocated abandoning the dual system of naming because unified classification of all fungi may be possible on the basis of the rDNA sequences of the anamorphs (Blackwell, 1993). Population studies and molecular data have shown that many widely used morphospecies actually comprise several biological or phylogenetic species. One of the problems for morphologists involves deciding how many base differences are required for strains to be considered different species. This has been partly solved by the phylogenetic-species concept, especially when based on cladistic analysis of molecular characteristics, which offers consistency in the delineation of species. Cladogram topology indicates the existence of monophyletic groups, which may represent species or supra- or subspecific taxa. Peterson and Kurtzman (1991) correlated

the biological-species concept with the phylogenetic-species concept by comparing the fertility of genetic crosses among heterothallic yeasts.

Two important technical advances have stimulated the use of molecular techniques. Firstly, the advent of PCR has allowed the analysis of small numbers of fungal cells or even single spores, dried herbarium material, or extinct organisms. Second, the selection of universal oligonucleotide primers specific to fungi, have provided easy access to nucleotide sequences (Guarro *et al.*, 1999).

One group of genes that is most frequently targeted for phylogenetic studies is the one that codes for ribosomal RNA (rRNA). Introns of several protein-encoding genes, such as the β-tubulin and actin, chitin synthase, acetyl coenzyme A synthase, glyceraldehyde-3-phosphate dehydrogenase, lignin peroxidase or orotidine 5'-monophosphate decarboxylase genes can also be applied and can provide important information (Guarro *et al.*, 1999). The main reasons for the popularity of rDNA are that it is a multiple-copy, non-protein-coding gene, whose repeated copies in tandem are homogenized by concerted evolution, and it is therefore almost always treated as a single-locus gene. Furthermore, ribosomes are present in all organisms, with a common evolutionary origin. Parts of the molecule are highly conserved (van de Peer *et al.*, 1997) and serve as reference points for evolutionary divergence studies. The conserved regions alternate with variable regions or divergent domains (Hassouna *et al.*, 1984). The 5.8S, 18S, and 25-28S rDNAs are transcribed as a 35S to 40S precursor, along with internal and external transcribed spacers (ITS and ETS). All spacers are spliced out of the transcript. Between each cluster is a non-transcribed spacer (NTS) or intergenic spacer (IGS) that serves to separate the repeats from one another on the chromosome. The 5S gene takes a variable position and is transcribed in the opposite direction. The total length of one DNA repeat is between 7.7 and 24 kb (Hibbett, 1992). Comparisons of the 18S (also called the small-subunit [SSU]) rRNA sequences have been performed to assess the relationships of the major groups of living organisms (Woese *et al.*, 1990). For phylogeny of filamentous fungi, the 18S sequence is mostly used completely or in subunits of over 600 bps (Wilmotte *et al.*, 1993). In the yeasts, the D1 and D2 variable regions of 25-28S rDNA regions are almost exclusively used (Demain *et al.*, 1998). This technique is currently being extended to Heterobasidiomycetes (Begerow *et al.*, 1997) and sometimes

also to filamentous Ascomycetes (Masclaux *et al.*, 1995). Peterson and Kurtzman (1991) demonstrated that the D2 region, a variable region of the 25-28S rDNA gene, is sufficiently variable to recognize biological species of yeasts and that con-specific species generally show less than 1 % nucleotide substitution. A schematic diagram representing various rDNA regions is illustrated in Fig. 1.

In only a limited number of fungi have both regions been sequenced. Due to this different choice of target regions, comparison of fungi to all possible relatives is hampered. The 25-28S variable domains are very informative and allow comparisons from high taxonomic levels down to the species level, although only a limited number of variable positions remain (Guého *et al.*, 1993). In the 18S gene, the variable domains mostly provide insufficient information for diagnostic purposes (de Hoog *et al.*, 1997), and thus large parts of the molecule must be sequenced to obtain the resolution required (Haase *et al.*, 1995). The ITS regions are much more variable but sequences can be aligned with confidence only between closely related taxa. These regions are generally used for species differentiation but may also demonstrate patterns of microevolution (Gräser *et al.*, 1999). In contrast, 5.8S rDNA is too small and has the least variability compared to the ITS regions. The 5S rDNA has been used mainly to infer relationships, based on the secondary structure of the ribosomal molecule (Blanz & Unseld, 1987) and not merely the DNA sequence.

The evolutionary distance is generally displayed in the form of trees, and a wide diversity of algorithms is available to construct them. Two basic methods are available: distance matrix methods, resulting in phenograms, and maximum-parsimony methods, resulting in cladograms. The statistical significance of the tree found is tested by using resampling algorithms such as the bootstrap method (Hillis *et al.*, 1994). The tree is usually rooted with an organism at a moderate distance (outgroup) that still can be aligned with confidence. For groups which have unexpected heterogeneity such as the fission yeasts and the black yeasts, the correct choice of out-group is quite important (Stasz *et al.*, 1989).

Among the classical DNA-based methods is the determination of the nuclear DNA (nDNA) guanine-plus-cytosine (G+C) content. The G+C content of nDNA has been established for many fungi, primarily yeasts.

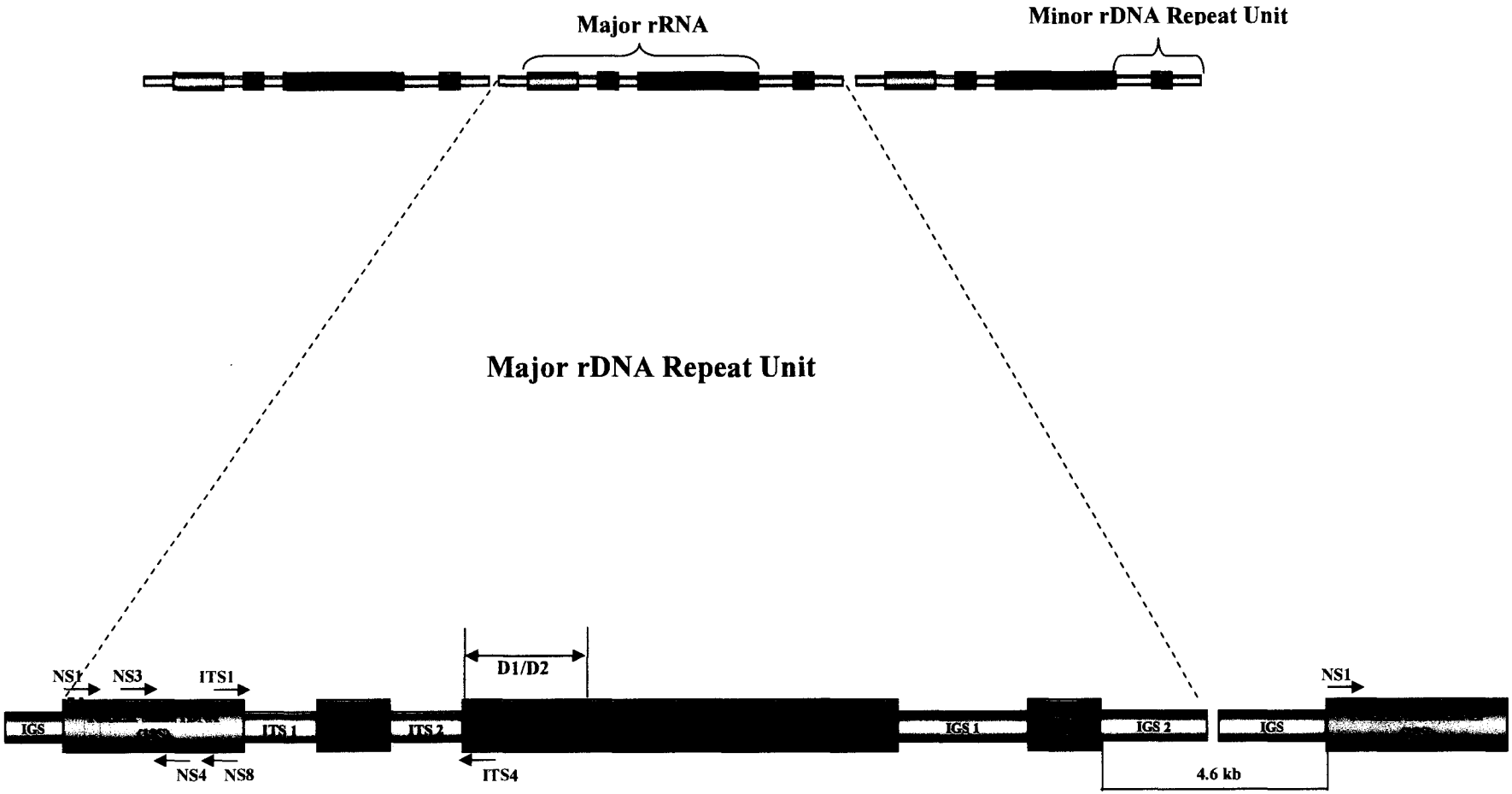


Fig. 1: Schematic representation of the fungal rRNA gene.

The primers used to amplify the various rDNA regions used in this study are represented as one-direction (5' to 3') arrows. The D1/D2 region of the 25-28 S rDNA is represented by a double sided arrow (http://plantbio.berkeley.edu/~bruns/picts/results/map.pdf, ; http://www.biology.duke.edu/fungi/mycolab/primers.htm, ; Sugita & Nishikawa, 2003).

A difference of 2 % in the G+C content has been considered to indicate that two strains should be assigned to different species (Kurtzman, 1994). In some insufficiently resolved fungal groups, a difference of 8 % has been allowed within species (Boekhout, 1991). In more precise recent studies with ecologically defined taxa, this difference was reduced to 1 % (Guého *et al.*, 1992). The G+C content is determined by using the Tm from the S-shaped melting curve of the DNA. Occasionally the shape of the curve is deviant; by determination of the first derivative, this could be traced back to the presence of DNAs with different melting velocities. Guého *et al.* (1997) found that these differences may be characteristic of sub-specific entities.

The identity or nonidentity of closely related strains can be determined by DNA-DNA hybridization, estimating the velocity of heteroduplex formation compared to the standard kinetics of the individual strains. A relative hybridization value of over 80 % is generally regarded as indicating membership in the same species, whereas values of less than 20 % are proof of nonidentity (Vilgalys, 1988). Intermediate values have increasingly been found recently, and these probably indicate subspecific entities (Smith *et al.*, 1995).

In recent years, the methods most widely used for taxonomy at the species level have been sequencing and electrophoretic methods. Many authors have sequenced closely related species to investigate the relationship of the taxa. Such studies have been carried out with larger genera such as *Penicillium*, *Fusarium* and *Trichoderma*. Teleomorph and anamorph variation is not always congruent. The speed of evolution seems heterogeneous and characterized by different rates of variation between groups. In general, about 2 % intraspecific variability is maintained within species, when ITS homology is used as the basis for distinction (Guarro *et al.*, 1999).

Among the electrophoretic methods, restriction fragment length polymorphism (RFLP) is particularly significant for taxonomy. This technique involves digesting DNA samples with a panel of restriction enzymes. The patterns can be tabulated and compared or phenetic trees can be constructed. The RFLP technique is widely used in taxonomy to compare patterns of mitochondrial DNA (mtDNA). This DNA is generally indicative of differences somewhat below the species level but in groups where microspecies are currently distinguished, such as in the dermatophytes, the differences

seem to correspond to teleomorph species (Mochizuki *et al.*, 1990). RFLP-based typing methods have been used to reveal anamorph-teleomorph connections (Gené *et al.*, 1996). Most commonly, the RFLP of PCR-amplified rDNA is used. This technique is also known as amplified rDNA restriction analysis (Vaneechoutte *et al.*, 1992) and provides a quick insight into relationships between moderately distant fungi (de Hoog *et al.*, 1997). Therefore, homogeneity of ITS profiles corresponds well to final ITS sequencing diversity (Uijthof *et al.*, 1998). The method is primarily confirmatory; i.e. new strains are quickly assigned to sequenced strains with the use of restriction maps (Vilgalys & Hester, 1990). This strategy was used, for example, by Yan *et al* (1995) to study *Phialophora.* Amplified rDNA restriction analysis is particularly useful as an inexpensive and simple alternative to SSU rDNA sequencing when broad relationships have already been determined. However, the frequent occurrence of introns in SSU rDNA (Gargas & DePriest, 1996) may hamper quick comparison of strains.

Random primed methods (Hadrys *et al.*, 1992) are particularly useful to determine relationships below the level of species but depending on the length of the primers and the recognized taxonomic diversity of the group under study, the method may help to discriminate species (Uijthof *et al.*, 1994). A popular technique is random amplified polymorphic DNA (RAPD) with 10-mer primers. However, this method is gradually being abandoned due of poor reproducibility. Microsatellites are a special class of tandem repeats, which have a base motif of up to 10 bps that is frequently repeated (up to about 100 times); they are found in many genomic loci with an almost ubiquitous distribution (Tautz, 1993). A general profile comparison of microsatellites enables species recognition (Niesters *et al.*, 1993). In addition, due to the high level of polymorphism, individual bands can be informative for the characterization of strains (Longato & Bonfante, 1997).

Karyotyping, the migration of chromosomes under the influence of an electric field, has been commonly used for a long time in plant, mammal and bacterial taxonomy. However, some problems have complicated the use of this technique in fungal taxonomy (e.g. the small size of the fungal chromosomes and the difficulty of observing condensed chromosomes during meiosis) (Kohn, 1992). Techniques such as electron microscopy, pulsed-field gel electrophoresis (Mills & McCluskey, 1990) and its recent

modification, contour-clamped homogeneous electric field electrophoresis (Tateishi *et al.*, 1996) contribute to the precise determination of karyotypes. The high intraspecific variability of the electrophoretic karyotypes of fungi makes this technique especially applicable for studies of populations in pathogenic yeasts (Doebbeling *et al.*, 1993) and filamentous fungi (Tateishi *et al.*, 1996).

Before DNA-sequencing methods became available, it was practically impossible to infer a reliable evolutionary tree containing all forms of life. Molecular biology techniques especially the analysis of rRNA sequences are currently used for reliable phylogenetic studies. These enable a more natural classification system to be established. However, the reliability of the new system depends on the skill of the investigators and the quality of data submitted to the database, which cannot be always verified. This is the Achilles heel of classification based solely on molecular techniques.

Traditionally, marine-derived lignicolous fungi have been classified into Ascomycetes and Basidiomycetes based on their distict spore structures that set them apart from their terrestrial counterparts. The anamorphic fungi are placed under 'Deuteromycetes' and it is the taxonomy of this group of fungi that benefit the most from the use of molecular techniques. The integration of traditional fungal taxonomy and molecular taxonomy would lead to a more natural taxonomic scheme.

2.2 OBJECTIVES

The objectives were to isolate lignin-degrading marine fungi, make a comparison between their respective lignin-degrading capabilities in order to select the fungal strain with the maximum lignin-degrading capacity and identification of this strain for further study.

2.3 MATERIALS AND METHODS

2.3.1 SAMPLING

The sampling site for the isolation of lignin-degrading fungi was the mangrove swamps of Chorao island in Goa, India (73° 55' E and 15° 30' N). The physico-chemical conditions prevalent at the site during the time of sampling were recorded. These included dissolved oxygen content, chlorinity, (Appendix 8.2.1.1 & 8.2.1.2) pH, temperature and salinity of the immediate surrounding surface water. Salinity was determined by measurement of the conductivity of the water sample with Autosal, Laboratory Salinometer (Guildline instruments Ltd., Canada). For pH determination, a pH meter (LabIndia, India) was used. Temperature was measured with a Celsius thermometer.

The decaying wood and leaf matter were rinsed with the surrounding estuarine water to remove attached soil particles and other extraneous matter. They were then placed in sterile (pre-rinsed with 70 % ethanol) transparent plastic 'zip-lock' bags to seal the moisture in. They were examined within 3 h after collection under a stereo-microscope to detect fruiting bodies (ascocarps, basidiocarps, pycnidia) and spores of higher marine fungi. Fungal structures are usually confined to the outer 1 cm unless the wood is at an advanced stage of decay, although such wood usually supports fewer fungal species, most of them are exclusively involved in lignin degradation (Vrijmoed, 2000).

Whether the fruiting bodies were present or not, the samples were incubated at ambient temperature in sterile bags lined with moist filter paper for a fortnight or until, fungal mycelia were observed to colonize the detritus pieces and/or produce fruiting bodies.

2.3.2 FUNGAL ISOLATION METHODS

2.3.2.1 Particle plating method: The leaves were cut into pieces of 1 cm^2 under sterile conditions and surface sterilized for 5 min with a mixture of 0.5 % sodium hypochlorite and 0.1 % detergent prepared in sterile distilled water (Zak & Bryan, 1963). The solution

was then drained and the leaf pieces washed using sterile seawater. These pieces were homogenized using sterile mortar and pestle and passed through sterile stainless steel sieves of 200 μm and 100 μm in succession, followed by washing with sterile seawater. The particles retained on the 100 μm sieve were then plated on malt extract agar prepared with 50 % seawater and fortified with 10 % antibiotic solution, to prevent bacterial growth. The antibiotic solution contained 400,000 U of procaine penicillin and 1 g of streptomycin sulphate in 100 mL of sterile distilled water. In addition, 1 % (w/v) of benomyl, a benzimidazole fungicide, was added in order to select for the slower growing wood decay fungi (Maloy, 1974). The plates were then incubated at room temperature.

2.3.2.2 Single-spore isolation method: Decaying wood pieces, which were incubated in a moist chamber for over a fortnight, were thoroughly examined for the presence of fruiting bodies of sporulating fungi using a stereo-microscope. A sterile glass needle was used to transfer a single fruiting body onto the slide, containing a drop of sterile seawater. This was then placed under the low power of a stereo microscope and crushed to release the spores. By capillary action, the drop was sucked into a sterile Pasteur pipette, which was then released into a Petriplate containing malt extract agar, which was gently tapped so that the drop flows downwards in a straight line. The malt extract agar was prepared in 50 % seawater fortified with 10 % antibiotic solution and 1 % (w/v) benomyl. The plates were examined under the microscope for the presence of isolated spores, the area on the plates on which isolated spores were present were marked and incubated at room temperature and examined daily for germination under the light microscope. The germinated spores were excised and transferred onto fresh malt extract agar prepared with half-strength seawater and again incubated at room temperature and observed for growth.

For non-sporulating fungi as well as fungi which produce spores that tightly adhere to each other, the hyphae or the cluster of spores were directly spotted / transferred onto plates containing malt extract agar in 50 % seawater fortified with streptomycin and penicillin.

2.3.3 SCREENING FOR LIGNIN-DEGRADING FUNGI

The fungi obtained from the above methods were qualitatively screened for laccase.

2.3.3.1 Qualitative assay for laccase.

The fungal isolates were screened for laccase production by growing them on plates of Boyd & Kohlmeyer (B&K) medium (Appendix 8.1.2) (Kohlmeyer & Kohlmeyer, 1979) containing 4 mM guaiacol / 2 mM ABTS / 0. 02 % Poly-R 478. The production of an intense brown colour under and around the fungal colony in guaiacol-supplemented agar and a deep green colour in ABTS-supplemented plates was considered as a positive reaction for the presence of laccase activity. The transformation of the Poly-R 478 plate from an initial pink colour to yellow indicated the presence of peroxidase activity.

2.3.3.2 Quantitative assay for lignin-degrading enzymes.

The fungal isolates, which were laccase-positive as well as showed dye decolourization ability, were grown in various liquid media like Malt extract broth (MEB) (Appendix 8.1.3), B&K and Low nitrogen (LN) media (Appendix 8.1.1) (Tien & Kirk, 1988), prepared with half strength sea-water. The week-old cultures were filtered through Whatman GF/C filter paper. Laccase and the peroxidases; lignin peroxidase (LiP) and manganese peroxidase (MnP) were estimated in the mycelia-free culture filtrate (Niku-Paavola *et al.*, 1988; Tien & Kirk, 1988). Their assay procedure and the definition of the enzyme unit are given in Appendix 8.2.2.1.1, 8.2.2.2 and 8.2.2.3 respectively.

2.3.3.3 Qualitative assay for dye decolourization ability.

The laccase-positive fungal isolates were grown in malt extract agar (MEA) and low nitrogen (LN) agar prepared with half strength sea-water containing various dyes. Trypan blue, aniline blue and remazol brilliant blue R (RBBR) at 0.04 %, methylene blue, crystal violet, brilliant green, congo red and Poly-R 478 at 0.02 % and reactive orange 16 (RO 16) at 0.015 % concentration were used. The decolourization of the dye under and around the fungal colony indicated the dye decolourization potential of the isolate.

2.3.4 FUNGAL IDENTIFICATION METHODS

2.3.4.1 Classical method.

The classical method of identifying fungi is by using light microscopy. The fungi with their telomorphic stages were identified based on the morphology of their spores and fruiting bodies. This was carried out by mounting them in sterilized seawater and identification were made with the help of the keys of Kohlmeyer & Volkmann-Kohlmeyer (1991).

2.3.4.2 Molecular identification.

The most efficient laccase producing strain was an anamorph. Hence, molecular techniques like rDNA sequencing were used for its identification.

A) *Isolation of genomic DNA*:

The biomass of the selected fungal strain in its log phase of growth (2 - 3 day-old culture) was lyophilized and the DNA was extracted by the method described by van Burik *et al.* (1998) (Appendix 8.3.1).

The purity of the pure DNA was calculated from 260 / 280 nm ratios. This was used as the template for further polymerase chain reactions (PCR) using the DNA Engine Thermal Cycler (BioRad, NSW, Australia).

B) *PCR amplification*:

1. **a)** The NS_1- NS_4 (~1030 bps) region of the 18S region of the rDNA of this strain was amplified using the forward and reverse primers (NS_1F and NS_4R) (White *et al.*, 1990). The PCR was carried out using the high fidelity proof reading enzyme Vent DNA Polymerase (0.3 U) in 25 µL reaction mixture containing 1X PCR buffer, 1.5 mM $MgSO_4$, 0.2 mM dNTP mix, 0.5 µM each of NS_1F and NS_4R and 50 ng of fungal genomic DNA as template. The PCR was standardized to get maximum amplification with least non-specificity. A negative control reaction (without DNA template) was also set up. PCR conditions are given in Table 1. The reaction was stopped by chilling the mixture at 4°C.

b) The NS_3- NS_8 (~1230 bps) region of the 18S region (Table 1) was amplified using identical concentrations of chemicals as well as PCR conditions mentioned above with the exception of the primers, which, in this case were NS_3F and NS_8R (forward and reverse primers respectively) used at a final concentration of 0.5 µM.

Table 1: PCR parameters & primers used for rDNA amplification

PRIMER PAIRS	SEQUENCE (5' → 3')	PCR PROFILE		PRODUCT SIZE	PRODUCT
		Parameters	Cycles		
NS_1F NS_4R	GTAGTCATATGCTTGTCTC CTTCCGTCAATTCCTTTAAG	94°C; 4 min	1	~ 1033 bps	NS_1- NS_4 region of 18S rDNA
		94°C; 1 min 62.7°C; 30 sec 72°C; 1 min	35		
		72°C; 10 min	1		
NS_3F NS_8R	GCAAGTCTGGTGCCAGCAGCC TCCGCAGGTTCACCTACGGA	94°C; 4 min	1	~ 1230 bps	NS_3- NS_8 region of 18S rDNA
		94°C; 1 min 62.7°C; 30 sec 72°C; 1 min	35		
		72°C; 10 min	1		
ITS 1 ITS 4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	94°C; 5 min	1	~ 650 bps	ITS1-5.8S-ITS2 rDNA
		94°C; 1 min 55°C; 30 sec 72°C; 1 min	35		
		72°C; 10 min	1		

2. A ~650 bps region of the ITS1-5.8S-ITS2 region of the rDNA of the fungus was amplified using ITS1 and ITS4 primers (Table 1). polymerase chain reaction was carried out using the high fidelity proof reading enzyme Vent DNA Polymerase (0.5 U) in 25 µL reaction mixture containing 1X PCR buffer, 1 mM $MgSO_4$, 0.2 mM dNTP mix, 0.5 µM each of the primers (mentioned above) and 25 ng of the fungal genomic DNA as template. The PCR was standardized to get maximum amplification with least non-specificity. A negative control reaction (without DNA

template) was also set up. PCR conditions are given in Table 1. The reaction was stopped by chilling the mixture at 4°C.

The PCR products along with the appropriate DNA ladder were electrophoresed on a 1 % agarose gel.

C) *Purification of PCR products from gel*:

The gel slice containing the amplified PCR products was excised out with a sterile blade and the DNA was eluted using the Sigma Gel Extraction Kit as per the manufacturer's protocol. The eluted DNA was then checked for purity by agarose gel electrophoresis. This pure DNA was lyophilized and used for sequencing.

For the large subunit (LSU) rDNA gene sequence data acquisition, the genomic DNA of the selected fungal strain was lyophilized and sent to MIDI Labs, Newark, USA. The MicroSeq™ D2 rDNA Fungal Kit was used for this purpose. Here the primers used correspond to positions 3334 and 3630 in the *Schizosaccharomyces japonicus* LSU rRNA gene. These primers amplify a portion of the D2 region of the LSU rDNA.

D) *Analysis of sequence data*:

All the sequences obtained were edited using the algorithm 'ChromasPro'. Two sets of sequences were obtained per PCR amplicon, one sequence obtained using the forward primer and the other using the reverse primer. For the reverse primer sequence, the reverse complement of this was derived using the algorithm 'Nucleic Acid Sequence Massager available at the web site: http://www.attotron.com/cybertory/analysis/seq Massager.htm. The sequence for each rDNA was processed to remove the overlap area obtained from sequencing using forward and reverse primers. A comparative study of other rDNA sequences with the rDNA from the selected fungus was accomplished using the BLAST (Altschul *et al.*, 1997) algorithm, at the website: http://www.ncbi.nlm.nih.gov. Phylogenetic and molecular evolutionary analyses in relation to NIOCC #2a were conducted using MEGA v. 4 (Tamura *et al.*, 2007).

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2.4 RESULTS

The sampling site for the isolation of lignin-degrading fungi was the mangrove swamps of Chorao Island in Goa, India. The physico-chemical conditions prevalent at the site at the time of sampling are shown in Table 2. The physico-chemical parameters of the mangrove sites at the sampling times varied greatly between the pre-monsoon (sampling I) and monsoon periods (sampling II & III).

Table 2: Physico-chemical conditions of the estuarine water prevalent at the sampling site (mangrove swamps) of Chorao island in Goa, India.

				pH	DISSOLVED OXYGEN CONTENT ($mg\ L^{-1}$)	CHLORIDE CONTENT ($g\ L^{-1}$)	SALINITY ($g\ L^{-1}$)
I	29.04.2003	4.00 pm	34°C	7.8	9.6	19.99	36.68
II	03.07.2003	7.45 am	27°C	7.2	13.44	0.175	0.321
III	13.08.2003	6.15 am	28°C	7.1	8.24	0.154	0.238

Fungi were isolated from mangrove detritus by the single-spore-isolation and particle-plating methods (Table 3). Leaves were chosen due to their high lignocellulosic content (Kumaresan *et al.*, 2002). Although the particle-plating method using mangrove leaves yielded maximum number of fungal isolates, none of them were found to be laccase-positive. Mangrove wood, however with their greater lignin content yielded laccase positive fungal strains. The single spore isolation yielded several laccase-positive isolates. Wherever possible, morphology of fruiting bodies and spores was used in the identification of some of fungal isolates using classical light microscopy. The spores of

the obligate marine fungus, *Halocyphina villosa* had been isolated only during the first sampling but could not be maintained *in-vitro*.

Table 3: Fungi obtained by screening for laccase production, their source and method of isolation.

S. No	SOURCE	ISOLATION METHOD	LACCASE PRODUCTION	ISOLATES & IDENTIFICATION
1	Decaying wood	Hyphal isolation	Positive	NIOCC #1, **NIOCC #2a**, NIOCC #7a & NIOCC #Xa (non-sporulating fungi)
2	Decaying wood	Single spore isolation	Positive	NIOCC #2, NIOCC #6, NIOCC #11, NIOCC #13 & NIOCC #15 are *Cirrenalia pygmea* (Kohlm); NIOCC #X (*Cirrenalia pseudomacrocephala*), NIOCC #4 (*Thielavia terricola* [Gilman & Abbot Emmons]) & NIOCC #8, NIOCC #5, NIOCC #9 & NIOCC #10
3	Decaying leaves	Particle plating	Negative	57 isolates

The identification of isolates was carried out by the classical method using their teleomorphic state.

Table 4: Qualitative tests for dye decolourization by the laccase-positive fungi.

ISOLATE	DYE	LOW NITROGEN MEDIUM		MALT EXTRACT MEDIUM	
		GROWTH	DECOLOURIZATION	GROWTH	DECOLOURIZATION
NIOCC #2a & NIOCC #Xa	Brilliant Green	++++	++++	++++	++++
	Crystal Violet	++++	++++	++++	++++
	Congo Red	++++	++++	++++	++++
	Poly-R 478	++++	++++	++++	++++
	Remazol Brilliant Blue R	++++	++++	++++	++++
	Methylene Blue	++++	++++	++++	++++
	Aniline Blue	++++	++++	++++	++++
	Trypan Blue	++++	++++	++++	++++
NIOCC #7a	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	++	++	++	+
	Poly-R 478	++	++	++	+
	Remazol Brilliant Blue R	++	++	++	++
	Methylene Blue	++	++	++	+
	Aniline Blue	++	++	++	++
	Trypan Blue	++	++	++	++
NIOCC #1	Brilliant Green	-	-	-	-
	Crystal Violet	+	-	+	-
	Congo Red	+	+	+	+
	Poly-R 478	+	+	+	++
	Remazol Brilliant Blue R	+++	+++	+++	+++
	Methylene Blue	++	+	++	++
	Aniline Blue	+	+	++	++
	Trypan Blue	++	++	++	++
NIOCC #4	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	++	++	++	++
	Methylene Blue	++	++	++	+
	Aniline Blue	++	++	++	+
	Trypan Blue	++	++	++	++
NIOCC #9	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	+	-	-
	Poly-R 478	+	+	+	-
	Remazol Brilliant Blue R	+	+	+	+
	Methylene Blue	+	-	+	-
	Aniline Blue	+	+	+	+
	Trypan Blue	+	+	+	+

Table 4 (contd): Qualitative tests for dye decolourization by the laccase-positive fungi.

ISOLATE	DYE	LOW NITROGEN MEDIUM		MALT EXTRACT MEDIUM	
		GROWTH	DECOLOURIZATION	GROWTH	DECOLOURIZATION
NIOCC #5 & NIOCC #10	Brilliant Green	+	-	-	-
	Crystal Violet	+	-	+	+
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	+	+	+	+
	Aniline Blue	+	-	+	+
	Trypan Blue	+	-	+	-
NIOCC #3	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	-	++	-
	Poly-R 478	+	-	++	+
	Remazol Brilliant Blue R	+	-	++	-
	Methylene Blue	+	-	++	-
	Aniline Blue	+	-	++++	++++
	Trypan Blue	+	-	++	-
NIOCC #8	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	-	-
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	-	-	+	+
	Aniline Blue	+	-	+	-
	Trypan Blue	+	-	+	+
NIOCC #2, NIOCC #6, NIOCC #11, NIOCC #13 & NIOCC #15	Brilliant Green	-	-	-	-
	Crystal Violet	-	-	+	-
	Congo Red	+	-	+	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	+	-	+	+
	Aniline Blue	+	-	+	-
	Trypan Blue	+	-	+	-
NIOCC #X	Brilliant Green	-	-	-	-
	Crystal Violet	+	-	-	-
	Congo Red	+	-	-	-
	Poly-R 478	+	-	+	-
	Remazol Brilliant Blue R	+	-	+	-
	Methylene Blue	+	-	-	-
	Aniline Blue	+	-	-	-
	Trypan Blue	+	-	-	-

All the laccase-positive isolates have been prefixed with 'NIOCC' and deposited at the National Institute of Oceanography Culture Collection (NIOCC). Table 4 shows qualitatively, the decolourization potential of all the laccase positive isolates when grown on two types of solid media in the presence of various dyes. The solid media included the low nitrogen (LN) mineral medium and high nitrogen medium such as malt extract agar (MEA).

Table 5: Production of laccase, MnP & LiP by 6 day-old cultures of the laccase-positive fungi, when grown in LN liquid medium prepared in half strength seawater.

ISOLATE	LACCASE ($U L^{-1}$)	MnP ($U L^{-1}$)	LiP ($U L^{-1}$)
#2a	30857	77	0.7
#Xa	16000	68	0
#7a	5714	2	0.4
#1	66	39	0.2
#4	51	2	0
#X	46	2	0
#5	40	0	0
#9	23	8	0
#10	23	0	0
#3	17	106	0.2
#15	17	2	0
#11	11	0	0.2
# 8	11	0	0
#13	6	3	1.1
# 2	6	0	0.2

Table 5 shows the quantitative production of the LDEs, laccase, MnP & LiP of all the laccase positive isolates when grown in LN medium prepared in half strength sea water. An intense brown colour obtained from the oxidation of guaiacol (Fig. 2A) and deep green colour from ABTS oxidation (Fig. 2B) indicated laccase activity. Transformation of the pink coloured Poly-R 478 dye (Fig. 2C) by NIOCC # 2a to brown, was indicative of

peroxidase activity. Figures 2 C-E displays qualitatively, the dye decolourization / transformation potential of NIOCC #2a.

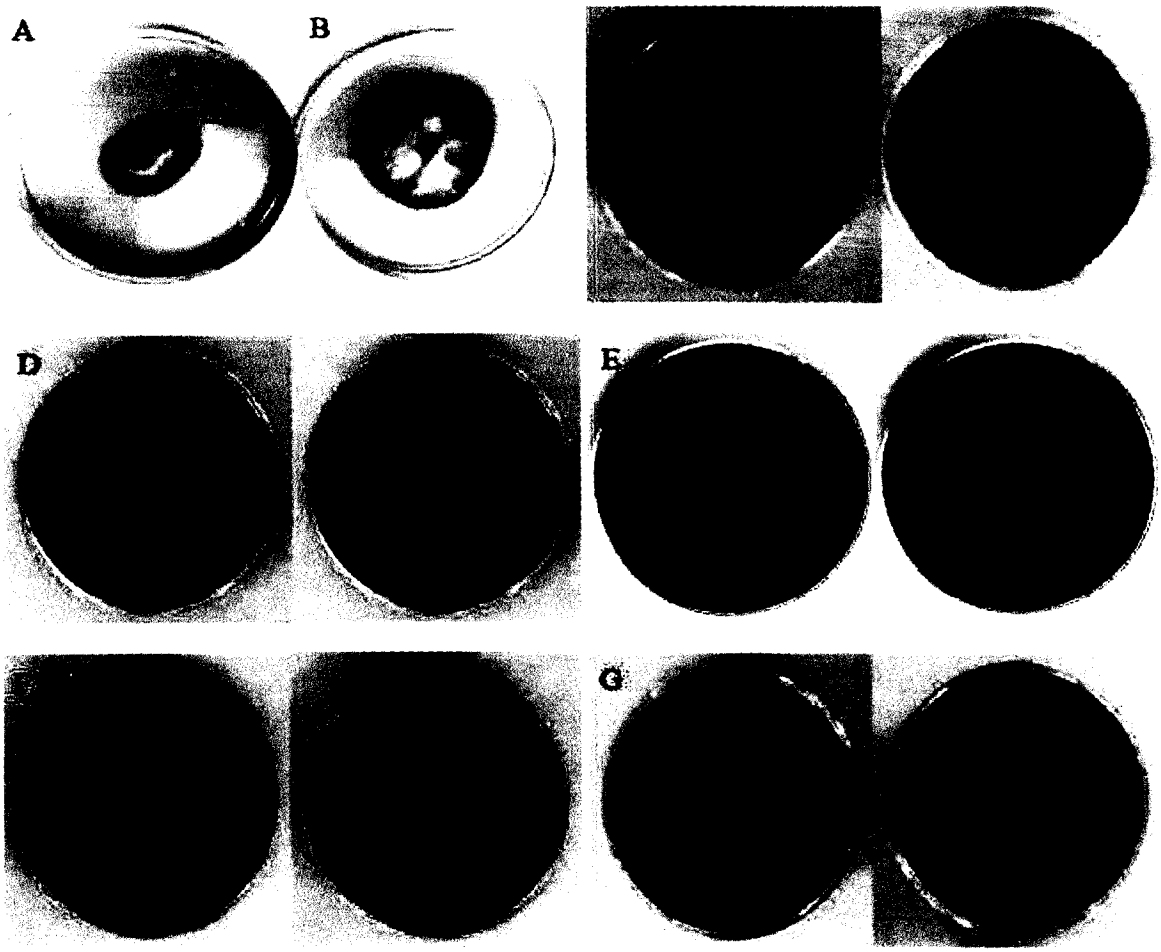

Fig. 2: Qualitative determination of laccase activity with (A) Guaiacol at 4 mM and (B) ABTS at 2 mM. Qualitative determination of dye decolourization potential with (C) Poly-R 478, (D) Methylene blue and (E) Congo red at 0.02 %; (F) Trypan blue and (G) Aniline blue at 0.04 % concentration.

A 7 day-old culture of NIOCC #2a was grown in LN medium prepared in half strength seawater containing the indicators at the above given concentrations. For the dyes (Fig. 2 C-G), the corresponding control plate, without culture is on the right hand side of the test plate.

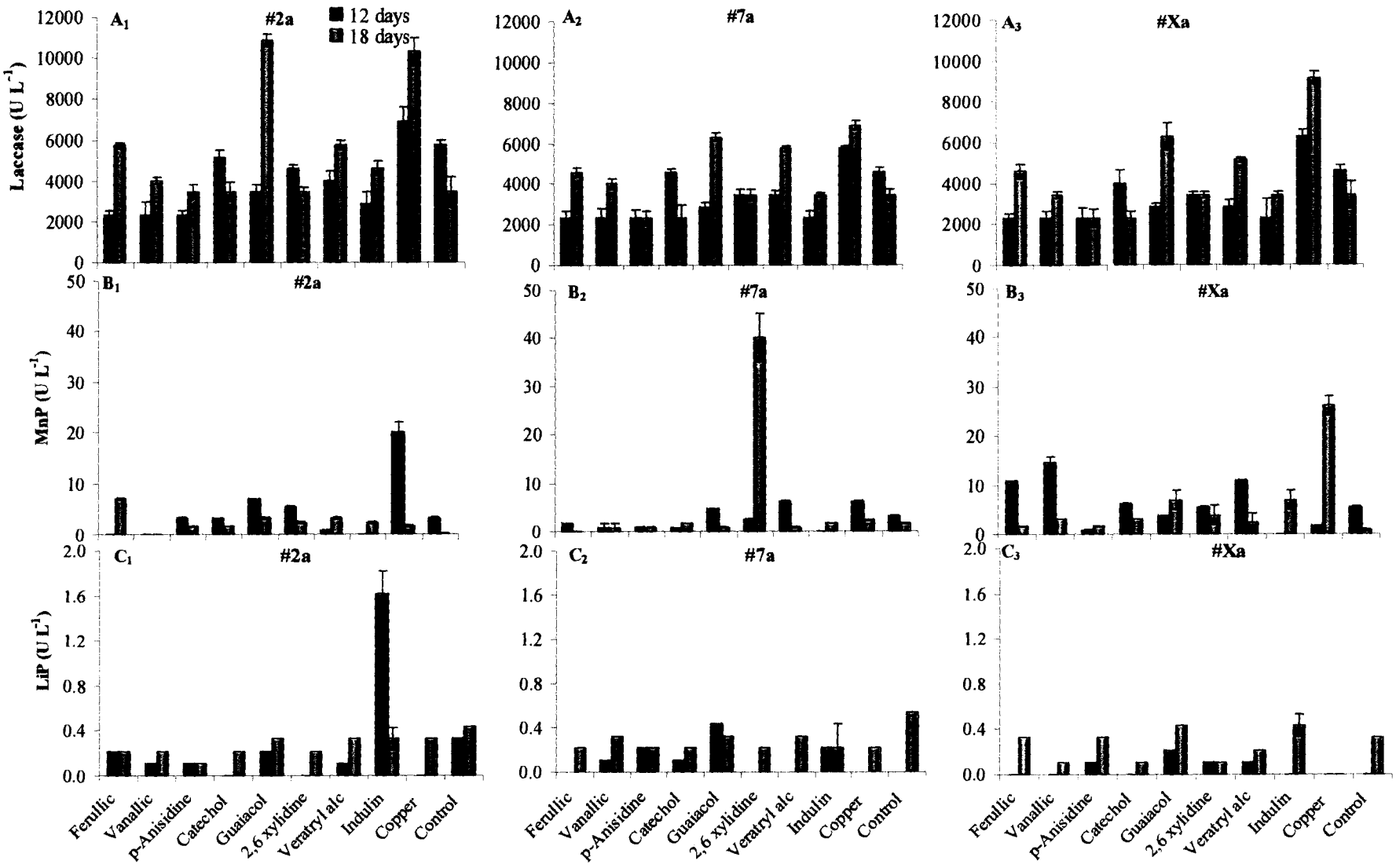

Fig. 3: LDE production by NIOCC #2a, #7a & #Xa in LN medium supplemented with various inducers estimated on day 12 & 18.

The best laccase-producers; NIOCC #2a, #7a & #Xa were all anamorphs. Growth of these fungi in LN medium in the presence of various inducers, was compared and the production of the LDEs was determined as shown in Fig. 3. Figure $3A_1$ - A_3 represented laccase production by NIOCC #2a, #7a and #Xa respectively; Fig. $3B_1$- B_3 represented MnP production by NIOCC #2a, #7a and #Xa respectively whereas Fig. $3C_1$ - C_3 represented LiP production by NIOCC #2a, #7a and #Xa respectively.

Results show that one the whole NIOCC #2a performed better than NIOCC #7a and NIOCC #Xa, especially with regard to laccase production.

Day	*Source of Variation*	*df*	*F*	*P-value*	*F critical*
	Inducer	9	59	1.7E-11	2
12	Culture	2	13	0.00037	4
	Inducer	9	17	5E-07	2
18	Culture	2	6	0.00954	4

Since laccase was the focus of study and the peroxidase titer as compared to laccase in all cases was negligible, a two-way ANOVA was carried out for only laccase production on day 12 and 18 separately between the different inducers used and the 3 isolates (NIOCC #2a, #7a & #Xa). From the above *p*-values, it was clear that both, the type of inducer as well as the isolate used, showed significant difference in laccase production. From the average values from Fig. 3 as well as from Table 4, it is obvious that NIOCC #2a was better than the other two anamorphs and thus was selected for further investigation in this thesis.

The temporal production of biomass and the LDEs of NIOCC #2a was determined in B&K medium prepared in varying salinities (calculated in terms of parts per thousand, ppt) (Fig. 4). The MnP (Fig. 4 B) and LiP (Fig. 4 C) production was very low. However, from Fig. 4 A, it is observed that maximum laccase production occurred on day 12 in 25 ppt seawater and maximum biomass (Fig. 4 D) was attained in full strength seawater for most of the days sampled.

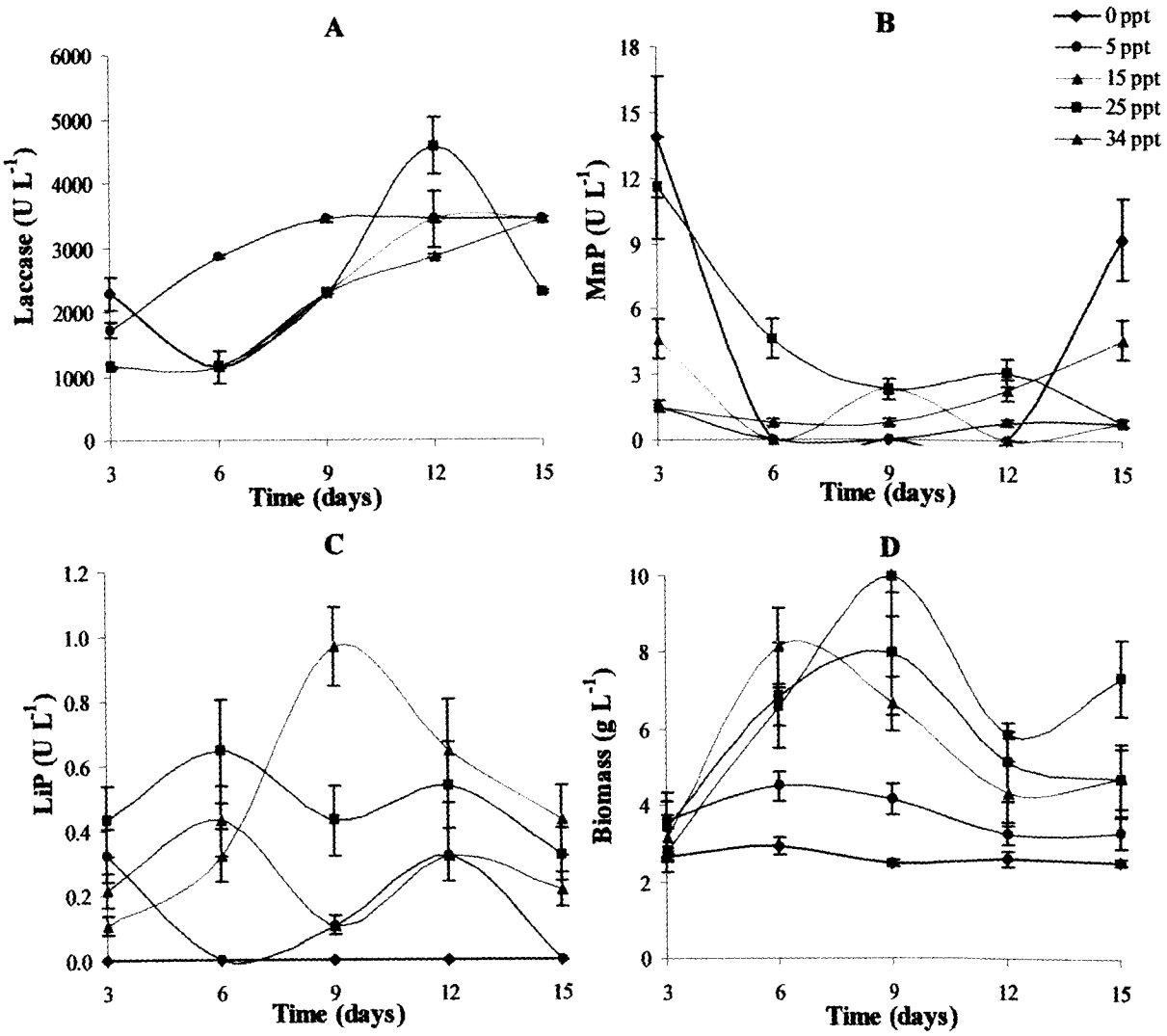

Fig. 4: Temporal production of (A) Laccase; (B) MnP; (C) LiP and (D) Biomass.

NIOCC #2a was grown in B&K medium prepared in seawater of varying salinities from 0 to full strength sea water (34 ppt).

Having confirmed that the marine-derived NIOCC #2a was a good laccase producer, it was deposited in the Microbial Type Culture Collection (MTCC, Chandigarh, India) under the accession number MTCC 5159. It was also deposited under the Budapest treaty for patent culture deposition (Raghukumar & D'Souza, 2004). **Henceforth, NIOCC #2a is referred to as MTCC 5159.**

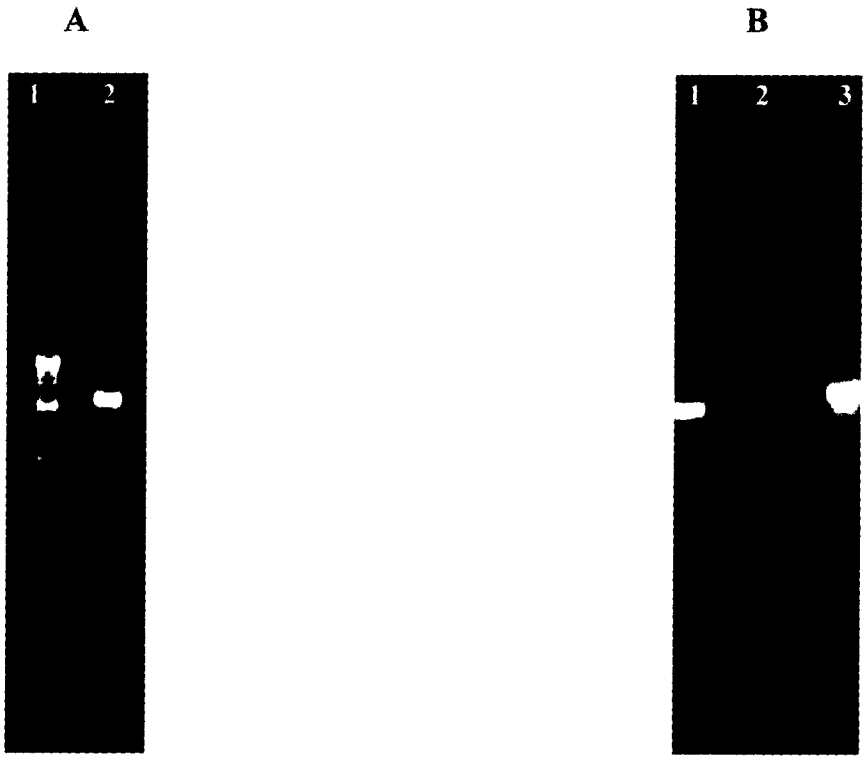

Fig. 5: PCR amplicon of the region of (A) ITS 1 - 5.8 S - ITS 2 and (B) 18 S rDNA of MTCC 5159

Lane 1: 100 bps – 1 kb marker

Lane 2: ITS 1-5.8S-ITS 2 rDNA PCR amplicon (~ 650 bps)

Lane 1: 'NS_1- NS_4 region' of 18S rDNA amplicon (~1033 bps)

Lane 2: 150 bps – 2.2 kb marker

Lane 3: 'NS_3- NS_8 region' of 18S rDNA amplicon (~1230 bps)

Pure cultures of MTCC 5159 on MEA were all of identical appearance. The colony consisted of rich white cottony aerial mycelium. The reverse of the colonies was colourless; no pigments were produced even after 1 month of cultivation. Several attempts to bring MTCC 5159 to sporulate such as the use of carrot juice agar and wood pieces in carrot juice agar (Appendix 8.1.4), were not successful. As the morphology was uninformative, rDNA sequence comparison was used for the identification. (Fig. 5A & B). Here the rDNA sequence of MTCC 5159 was compared with the rDNA sequences of other fungi present in the database. To confirm the identification, three separate stretches of rDNA from MTCC 5159 were used namely, the 18S, the D1/D2 region of the 25-28 S and the ITS1-5.8S-ITS2 rDNA regions. The sequences for the same have been deposited in the gene bank under the following accession numbers; EF059806, AY939878 & AY939879 respectively.

The phenograms reflecting the phylogenetic relationship between MTCC 5159 constructed using data from the BLAST analysis of the various rDNA regions of MTCC 5159 are shown separately in Figs. 6A, B & C and the details along with the accession numbers of the sequences used to construct the trees are given in Tables 6A, B & C.

In Tables 6A, B & C, some hits have not been listed due to the following reasons:

1. Once the closest hit (both genus & species name required) to MTCC 5159 was listed, the other hits denoting the same genus but different species were not included.

2. When the species name was missing. (An exception is *Sclerotinia* BF 105, since the species name is not available for this genus and this belongs to only the out-group). Note the out-group genera have been kept common within the 18S, ITS1-5.8S-ITS2 & D1/D2 phenograms. Another exception was the phenograms drawn with ITS1-5.8S-ITS2 rDNA sequence data, since unidentified fungi showed greater homology than the first positively identified hit, *Cerrena unicolor*, these fungi have been listed.

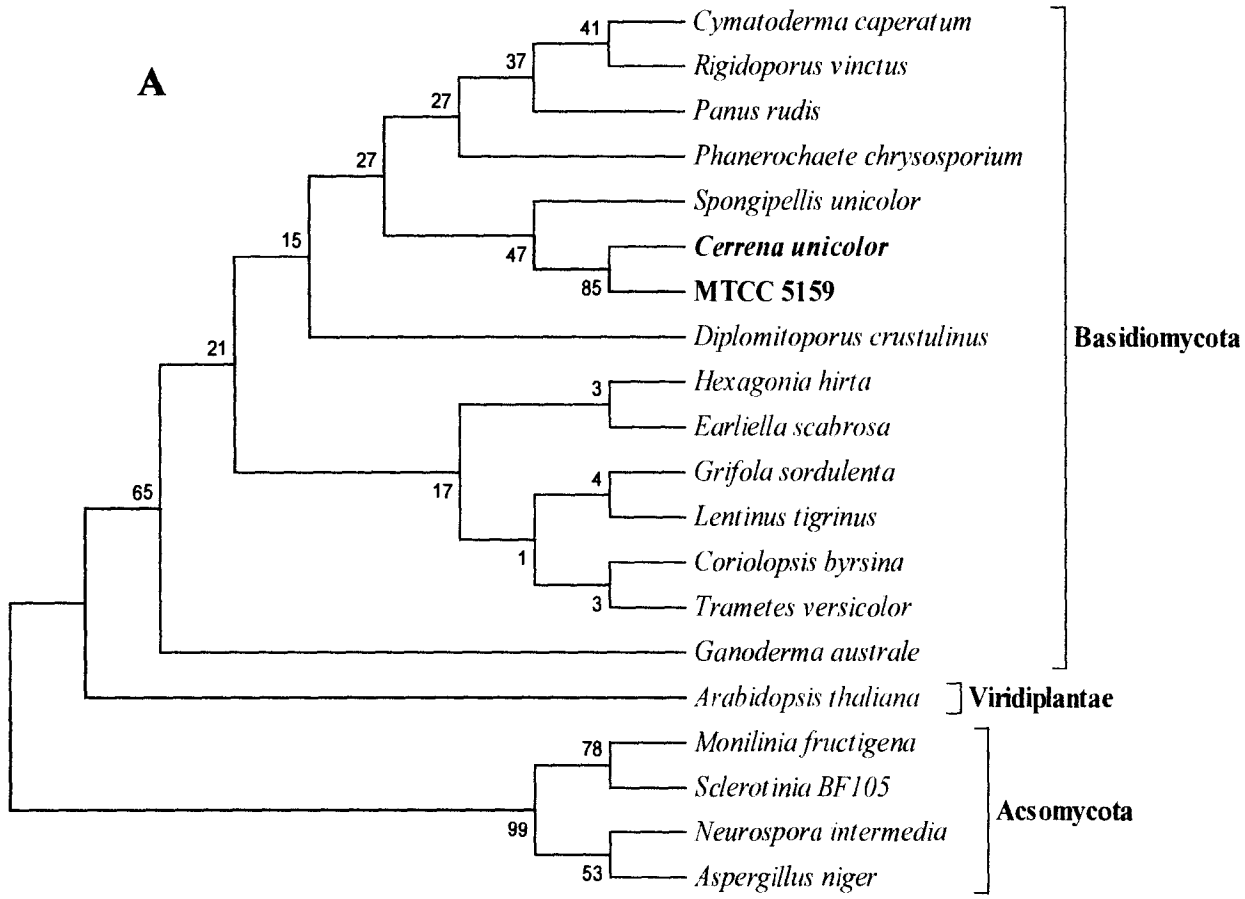

Fig. 6: (A) Phenogram constructed from 18S rDNA sequence homologues of MTCC 5159.

The accession numbers along with other details of the members belonging to Class Basidiomycota in the above figure are given in Table 6A. The accession numbers of the out-group members are given below:

Viridiplantae: *Arabidopsis thaliana* (X16077)

Ascomycota: *Monilia fructigena* (EF207429)
Sclerotinia BF 105 (AM901701)
Neurospora intermedia (EF197071)
Aspergillus niger (AB305102)

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are not significant. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 359 positions in the final dataset.

Table 6A: Sequences producing significant alignments with MTCC 5159 18S rDNA (EF059806.1)

Accession No.	Description	Max score	Total score	Query coverage	E value	Max identity
AY850007.1	***Cerrena unicolor***	2058	2058	100 %	0.0	99 %
M59760.1	*Spongipellis unicolor*	2025	2025	100 %	0.0	98 %
AF082849.1	*Cymatoderma caperatum*	2025	2025	100 %	0.0	98 %
AY665780.1	*Grifola sordulenta* isolate AFTOL-ID 562	2013	2013	100 %	0.0	98 %
AF026569.1	*Panus rudis*	2010	2010	100 %	0.0	98 %
	:					
AY336766.1	*Earliella scabrosa* strain Wu 9704-83	2008	2008	100 %	0.0	98 %
	:					
AY336773.1	*Coriolopsis byrsina* strain CRM-46	1997	1997	100 %	0.0	98 %
AY336770.1	*Diplomitoporus crustulinus* strain FP 101824	1997	1997	100 %	0.0	98 %
	:					
AY336763.1	*Ganoderma australe* strain Wu 9302-56	1997	1997	100 %	0.0	98 %
AY336760.1	*Hexagonia hirta* strain CBS 515.96	1997	1997	100 %	0.0	98 %
	:————:————:————:————:————:					
AY946269.1	*Lentinus tigrinus*	1997	1997	100 %	0.0	98 %
AY336751.1	*Trametes versicolor* strain Wu 9507-7	1980	1980	100 %	0.0	97 %
AY336752.1	*Rigidoporus vinctus* strain Chen 674	1953	1953	100 %	0.0	97 %
AF026593.1	*Phanerochaete chrysosporium*	1947	1947	100 %	0.0	97 %

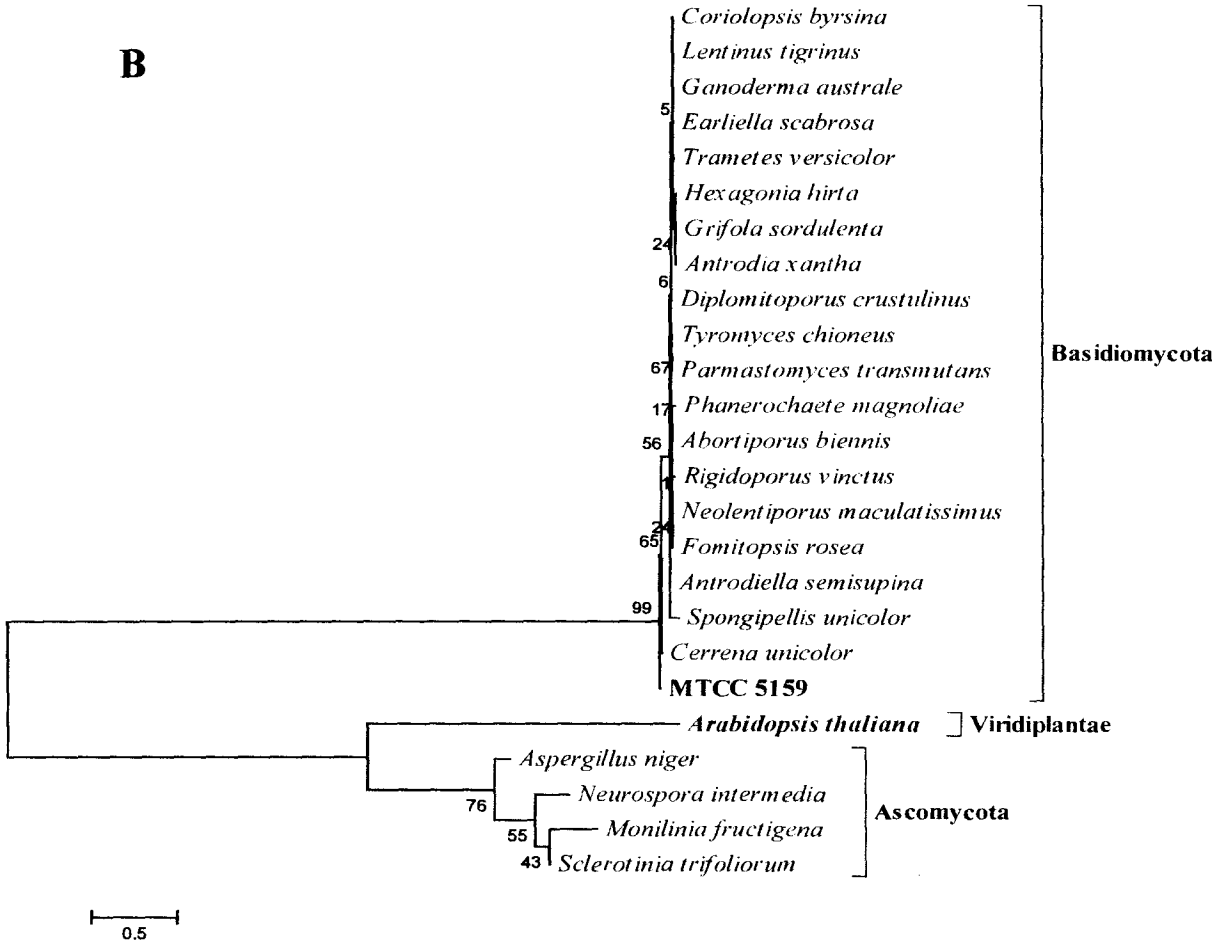

Fig. 6: (B) Phenogram constructed from D1/D2 domains of the 25-28S rDNA sequence homologues of MTCC 5159

The accession numbers along with other details of the members belonging to Class Basidiomycota in the above figure are given in Table 6B. The accession numbers of the out-group members are given below:

Viridiplantae: *Arabidopsis thaliana* (X52320)

Ascomycota: *Aspergillus niger* (AB305102)
Neurospora intermedia (EF197071)
Monilia fructigena (EF207429)
Sclerotinia trifoliorum (DQ904361)

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are not significant. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 409 positions in the final dataset.

Table 6B: Sequences producing significant alignments with MTCC 5159 25-28S rDNA (AY939878.2)

Accession No.	Description	Max score	Total score	Query coverage	E value	Max identity
AY850007.1	***Cerrena unicolor***	1875	1875	100 %	0.0	98 %
AF334899.1	*Abortiporus biennis*	1851	1851	100 %	0.0	98 %
	:					
M59760.1	*Spongipellis unicolor*	1842	1842	100 %	0.0	98 %
AF334902.1	*Antrodia xantha*	1840	1840	100 %	0.0	98 %
AY336770.1	*Diplomitoporus crustulinus*	1829	1829	100 %	0.0	98 %
	:					
AY336766.1	*Earliella scabrosa* strain Wu 9704-83	1829	1829	100 %	0.0	98 %
AY336764.1	*Fomitopsis rosea* strain RLG 6954	1829	1829	100 %	0.0	98 %
AF334938.1	*Tyromyces chioneus*	1829	1829	100 %	0.0	98 %
	:					
AY336773.1	*Coriolopsis byrsina* strain CRM-46	1823	1823	100 %	0.0	97 %
AY665780.1	*Grifola sordulenta* isolate AFTOL-ID 562	1823	1823	100 %	0.0	97 %
AY293143.1	*Parmastomyces transmutans*	1821	1821	100 %	0.0	97 %
	:					
AY336774.1	*Antrodiella semisupina* strain FCUG 1216	1818	1818	100 %	0.0	97 %
	:					
AY336760.1	*Hexagonia hirta* strain CBS 515.96	1818	1818	100 %	0.0	97 %
AF334921.1	*Neolentiporus maculatissimus*	1816	1816	100 %	0.0	97 %
AY336763.1	*Ganoderma australe* strain Wu 9302-56	1812	1812	100 %	0.0	97 %
	:------:-------:---------:---------:------:					
AB084596.1	*Phanerochaete magnoliae*	1812	1812	100 %	0.0	97 %
AY946269.1	*Lentinus tigrinus*	1812	1812	100 %	0.0	97 %
AY336752.1	*Rigidoporus vinctus* strain Chen 674	1807	1807	99 %	0.0	97 %
AY309019.1	*Trametes versicolor* strain BCRC 36387	1807	1807	100 %	0.0	97 %

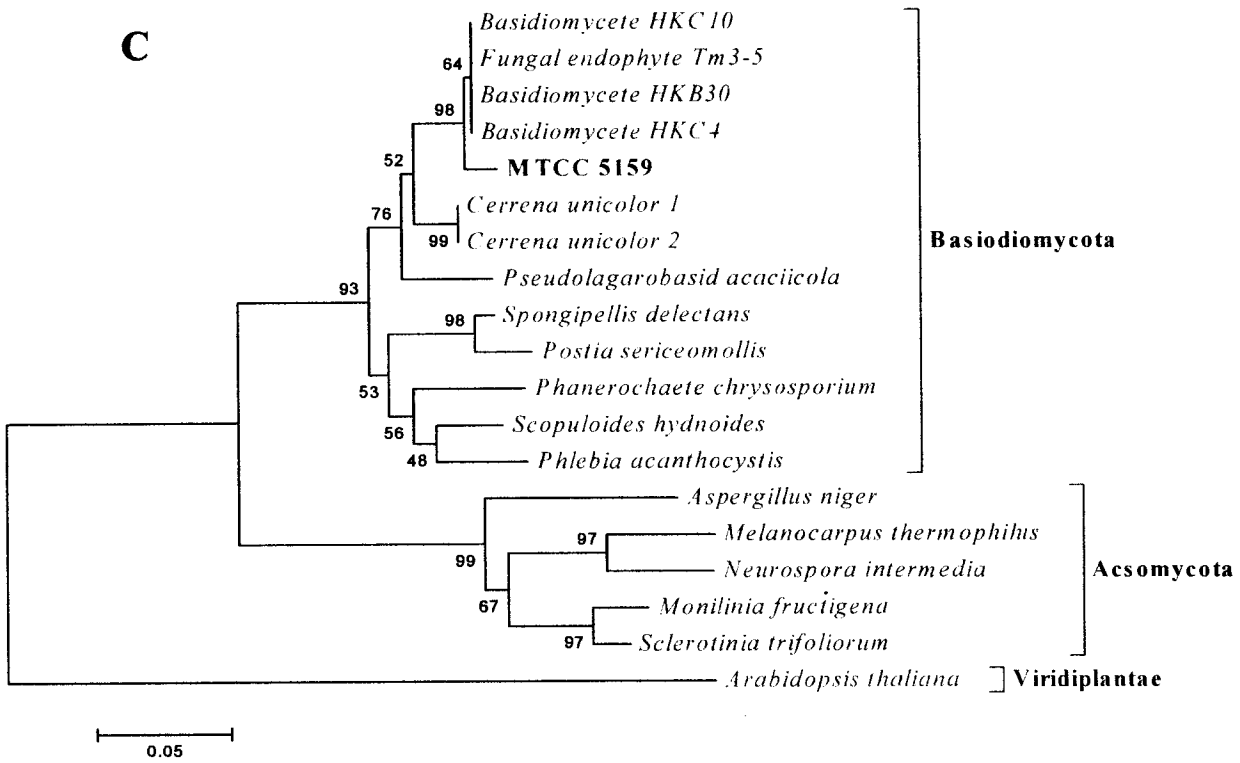

Fig. 6: (C) Phenogram constructed from ITS1-5.8S-ITS2 rDNA sequence homologues of MTCC 5159.

The accession numbers along with other details of the members belonging to Class Basidiomycota in the above figure are given in Table 6C. The accession numbers of the out-group members are given below:

Viridiplantae: *Arabidopsis thaliana* (U43225)

Ascomycota: *Aspergillus niger* (AB305102)
Melanocarpus thermophilus (AJ271586)
Neurospora intermedia (EF197071)
Monilia fructigena (EF207429)
Sclerotinia trifoliorum (DQ904361)

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 1.04232160 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 212 positions in the final dataset.

Table 6C: Sequences producing significant alignments with MTCC 5159 ITS1-5.8S-ITS2 rDNA (AY939879.1)

Accession No.	Description	Max score	Total score	Query coverage	E value	Max identity
EF029829.1	Basidiomycete sp. HKC10 (from marine sponges)	586	586	100 %	2e-164	99 %
EF029823.1	Basidiomycete sp. HKC4 (from marine sponges)	586	586	100 %	2e-164	99 %
EF029817.1	Basidiomycete sp. HKB30 (from marine sponges)	586	586	100 %	2e-164	99 %
	:					
AY456192.1	Fungal endophyte Tm3-5	586	586	100 %	2e-164	99 %
	:					
EF577058.1	***Cerrena unicolor*** (from *Tremex fuscicornis*)	448	448	100 %	9e-123	91 %
DQ056858.1	***Cerrena unicolor***	442	442	100 %	4e-121	91 %
	:					
DQ517883.1	*Pseudolagarobasidium acaciicola* strain CBS 115543	416	416	100 %	3e-113	89 %
	:					
AY089734.1	*Postia sericeomollis* isolate Brunn-75	366	366	100 %	3e-98	87 %
AJ006670.1	*Spongipellis delectans* Sample 26	357	357	67 %	2e-95	95 %
AB210074.1	*Phlebia acanthocystis* TMIC 35095	342	342	97 %	4e-91	86 %
	:					
EU118665.1	*Scopuloides hydnoides* voucher KHL 11916 (GB)	339	339	66 %	6e-90	94 %
	:———:———:———:———:———:———:					
AY854086.1	*Phanerochaete chrysosporium* isolate AFTOL-ID 776	298	298	66 %	1e-77	91 %

MTCC 5159 18S rDNA (EF059806) and D1/D2 of 25-28S rDNA (AY939878) showed 99 % and 98 % homology respectively with *Cerrena unicolor* (AY850007). MTCC 5159 (AY939879) showed 91 % identity with two *Cerrena unicolor* sequences (EF577058.1 & DQ056858.1), which were the closest completely identified sequences, from ITS1-5.8S-ITS2 rDNA homology.

2.5 DISCUSSION

Mangroves harbor a rich diversity of mycota. Fungi that live in these environments are mostly saprophytic in lifestyle; some of them however also live as endophytes. Lignicolous fungi are best isolated from substrates rich in lignin and in a state of decay. Mangrove detritus was collected during the low tide at all times since it has been shown that slightly higher number and type of fungal species are present compared to during high tides (Besitulo *et al.*, 2002). Detritus from such sites would have been submerged for longer periods resulting in a more diversified mycota. Presence of fouling organisms, especially the remains of calcareous shells and bore-holes on the wood samples indicate that they had been subjected to a considerable period of marine submersion. This translates into a greater chance of isolating marine-derived liginicolous fungi (Vrijmoed, 2000).

The mangrove swamps are regularly flushed with fresh water from the rivers. This causes a great variation in salinity and other physico-chemical parameters in the estuarine areas. These parameters also varied greatly due to the varying conditions brought by the pre-monsoon and monsoon periods. Together, these conditions tend to support a rich diversity of marine and marine-derived fungi and affect the abundance and diversity of species in the mangroves. Species abundance and diversity also have been influenced by other factors such as the nature of the substrata (e.g. host plant species, age of plant parts), the condition of the substrata (e.g. duration period of submergence) and the maturity and diversity of the mangrove vegetation itself (Abdel-Wahab & El-Sharouny, 2002).

Leaves were chosen due to their high lignocellulosic content (Kumaresan *et al.*, 2002) Although the particle-plating method using mangrove leaves yielded

maximum number of fungal isolates, none of them were found to be laccase-positive. Wood of tree trunks however with their greater lignin content yielded the laccase producing fungal strains. Wherever possible, morphology of fruiting bodies and spores (sexual and asexual) were used in the identification of some of fungal isolates using classical light microscopy. *Halocyphina villosa* is considered to be the most commonly occurring tropical obligate marine fungus in mangroves (Hyde & Jones, 1988). Its spores had been isolated during the first sampling in the presence of highly saline conditions but could not to be maintained *in-vitro*. Being an obligate marine form, salinity obviously has a great impact, which could account for its absence in the last two sampling when salinities were low.

From Table 4 and Table 5, it was clear that the laccase production and decolourization potential of the sporulating laccase-positive fungal isolates were much lower than the three anamorphic isolates, namely MTCC 5159 (NIOCC #2a), #7a & #Xa. Hence, no further emphasis had been given to the identification of the sporulating forms. From Table 5 it was evident that MTCC 5159 was superior to the other two anamorphs; NIOCC #7a & NIOCC #Xa. However, to alleviate the doubt whether the comparatively poor performance of these two anamorphs was only due to the medium composition, the LDE production of all the three anamorphs in LN medium, which is known to support high laccase production (Raghukumar *et al.*, 1994) prepared in half-strength seawater, in the presence of various inducers was determined (Fig. 3). The results further emphasized the superiority of MTCC 5159.

However, it was required to confirm that MTCC 5159 isolated during the 3rd sampling is actually marine-derived fungus and not a terrestrial one. Since in the last two samplings, the salinity of the estuarine area was closer to fresh water than seawater due to the combined effects of the monsoons as well as the low tide conditions prevalent at that time. For this MTCC 5159 was tested for growth and LDE production at different time intervals, in varying salinities in B&K medium. Maximum biomass in full strength seawater and maximum laccase production in 25 ppt seawater confirmed that MTCC 5159 was in fact a marine-derived or salt tolerant fungus.

MTCC 5159 was identified using rDNA homology. The 18S and D1/D2 of 25-28S rDNA of MTCC 5159 showed maximum identity with *Cerrena unicolor*

(AY850007) at 99 % and 98 % respectively. This because in the case of EF059806 as well as AY939878, the first BLAST hit was AY850007, which was an 18S rDNA sequence.

From ITS1-5.8S-ITS2 rDNA homology, the closest two completely identified sequences were both *Cerrena unicolor*. Here MTCC 5159 (AY939879.1) had 91 % identity with both the *Cerrena unicolor* sequences (EF577058.1 & DQ056858.1). Since the ITS1-5.8S-ITS2 rDNA is a hyper variable region, i.e. this region reflects more variation than either the 18S or the 25-28S rDNA. The evolutionary distance resolved by ITS, is usually restricted to demarcating within the species level and cannot be completely relied above genus level, like 18S or 25-28S rDNA. Organisms showing more than 90 % similarity can be considered as belonging to the same genus (Sugita & Nishikawa, 2003). Whereas for 18S rDNA sequence homology, ≥ 99 % sequence identity can safely be considered as belonging to the same genus (Guarro *et al.*, 1999). The 18S, 25-28S and ITS1-5.8S-ITS2 rDNA sequence homology leads to the conclusion that MTCC 5159 belongs to the genus *Cerrena* and species *unicolor*. **MTCC 5159 is designated as *Cerrena unicolor* MTCC 5159, henceforth.**

The taxonomical hierarchy for *Cerrena unicolor* MTCC 5159 is as follows: Eukaryota, Fungi, Dikarya, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetes incertae sedis, Polyporales, Coriolaceae, *Cerrena*, *unicolor* MTCC 5159.

A few other facts also corroborate with the source of isolation and identification of *Cerrena unicolor* MTCC 5159:

1. ***Cerrena unicolor* MTCC 5159 is an anamorphic lignicolus marine-derived fungus.** Mangicolous fungi are adapted to grow and reproduce in a wide range of salinities; this allows them to inhabit the intertidal brackish water in mangroves. This point has been observed in the genus *Halophytophthora*, some species of this genus reproduce asexually in response to fluctuating salinities and/or temperatures and to a change of dry and wet conditions. This teleomorphic to anamorphic adaptation of this species had allowed them to flourish in the mangrove

environments, where conditions fluctuate greatly according to the daily tidal rhythm (Nakagiri, 2002).

2. ***Cerrena unicolor* MTCC 5159 has maximum homology with the marine sponge-derived fungi.** From the ITS1-5.8S-ITS2 rDNA homology studies with other fungi, it is observed that MTCC 5159 is taxonomically closest (99 % identity) to fungal strains; HKC 4, HKB 30 & HKC 10, all of which are known to be associated with marine sponges. With the exception of some diseased states and the genus *Koralionastes*, fungi have not been regularly observed in marine sponges. It is probable that sponges, by their filter-feeding process, trap fungal particles/spores in their tissues. If these fungi flourish in their new habitat, they can be called 'marine-derived' since they would have had enough time to adapt to the marine environment. Marine sponge-derived fungi are mostly anamorphic (Jensen & Fenical, 2002) and are the single most significant source of new marine fungal metabolites. A terrestrial fungus *Coriolus consors*, (related to *Cerrena*) as well as an unidentified sponge-derived / associated fungus were shown to be capable of unusual modifications of hirsutane sesquiterpenes (Wang *et al.*, 1998).

3. ***Cerrena unicolor* MTCC 5159 has highest homology with a fungal endophyte.** Fungal endophytes are fungi that reside in living leaf tissues. One cannot positively conclude that MTCC 5159 is an endophyte since it was isolated from decaying wood. However, it showed 99 % identity to a fungal endophyte, Tm3 - 5 (Table 6C). This high homology to a fungal endophyte suggests the possibility of MTCC 5159 having an alternate life style as a fungal endophyte. This has been shown to be the case for many anamorphic mangicolous fungi, which are endophytic (Kumaresan *et al.*, 2002) yet retain the capacity to live like saprophytes. This is in concurrence with the source of isolation of *Cerrena unicolor* MTCC 5159.

The genus *'Cerrena'* has been known for its symbiotic relationship with both plants and animals such as the horntail *Tremex fuscicornis* (wood wasp). Woodwasps maintain a

close association with a fungal symbiont such as *Cerrena unicolor*, 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 & Martin, 1983). The fungal symbiont is indispensable for larval development especially beyond the first instar (Pazoutova & Srutka, 2007). These authors isolated a fungus from *T. fuscicornis*, the fungus had a very similar morphology to *Cerrena unicolor* MTCC 5159 and was also unable to form basidiocarps *in vitro*. It too was identified using ITS1-5.8S-ITS2 and 18S rDNA homology. The closest match was *Cerrena unicolor* (EF577058.1). The original, *Cerrena unicolor* (Bull) Murrill was identified from basidiospores. This was successfully mated with the fungus isolated from the mycangia of *T. longicollis* (Tabata & Abe, 1995), using the dikaryotization technique, indicating that this fungus too, was a *Cerrena unicolor* strain. The above indicates that *Cerrena unicolor* is capable of living as an endosymbiont, an endophyte as well as a 'free-living form'. The reason for this symbiosis between fungus and animal is the large amount of lignin-degrading enzymes (such as laccases) and proteases (data not shown) 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*.

D'Souza *et al.*, (2006) have shown that MTCC 5159 produces a high laccase titer. Thus, *Cerrena unicolor* MTCC 5159 is a mangicolous, lignicolous marine-derived saprophytic basidiomycete probably capable of retaining an endophytic as well as endosymbiotic life style. This is in concordance with the source of isolation of this fungus which was decaying mangrove wood (with holes made by wood borers) at the intertidal region. Besides, this fungus shows greater genetic homology with the marine sponge-derived fungal strains than the terrestrial endosymbiont *C. unicolor* from *Tremex*. This implies that *Cerrena unicolor* MTCC 5159 may have been subjected to the marine influence long enough to alter its genetic makeup. This makes the strain lucrative, it terms of isolation of novel marine metabolites.

Chapter 3

Characterization of Lac IId, a Laccase isozyme from Cerrena unicolor MTCC 5159

ABSTRACT

The marine-derived, laccase-hyper-producing *Cerrena unicolor* MTCC 5159 was the source of laccase used in the present study. Among several methods tested to concentrate laccase, ultrafiltration using positive pressure was found to be most efficient. Partially purified laccase showed optimum activity at pH 3 and 60°C and contained a consortium of laccase isozymes.

Out of these, an isozyme, 'Lac IId' was purified with a final yield of 17 % and characterized in detail. It has a molecular mass of 59 kDa, a pI of 5.3 and 17 % *N*-linked glycosylation. The UV-visible spectrum showed a shoulder at 330 nm and peak at 610 nm which is characteristic of blue laccases. The N-terminal and an internal peptide sequence near the C-terminal are both deposited under the accession number P85430 in the 'Uniprot' database.

Lac IId showed optimum activity at pH 3 and 70°C. It has a half life of 90 min at 70°C and retained more than 60 % activity up to 180 min at 60°C at pH 9. Even after 1 year of storage at -20°C, no loss in activity was observed. Its energy of activation value of 2.5 kJ mol^{-1} between 60° to 70°C at pH 3, attested to its thermostability. Lac IId could catalyze the oxidation of a wide range of substrates and its substrate range increased by the inclusion of mediators. Of all the inhibitors tested, only azide was able to effectively inhibit it up to 95 % of its original activity. Sodium chloride up to 0.3 M did not inhibit it above which, Lac IId was only reversibly inhibited. From the metal ions tested, Lac IId was maximally inhibited by chromium and retained 44 % of its original activity, whereas the other metals resulted in even lesser inhibition.

3.1 INTRODUCTION

Laccases (EC 1.10.3.2); benzenediol:oxygen oxidoreductase), are copper-containing lignin-degrading enzymes, often extracellular in nature. They use molecular oxygen to oxidize a wide range of aromatic compounds. Laccases are superior to other lignin-degrading enzymes, lignin-peroxidase and manganese-dependent peroxidase in that, the isolated enzyme can be used *in vitro* for depolymerization of lignin or lignin-related compounds. Laccases do not have oxidation potentials as high as those of the peroxidases but in the presence of a suitable redox mediator 2,2'-azino-bis-(3-ethyl benzothiazoline-6-sulphonic acid) (ABTS), their effective redox potential is increased i.e. they can oxidize nonphenolic lignin model compounds. Besides fungi, laccases are also found in plants, bacteria and insects. Among fungi, the white rot fungi are the major laccase producers (Baldrian, 2006).

Obligate and facultative (marine-derived) fungi from lignocellulose substrates in the marine environment, particularly mangroves and sea-grasses are an important source of lignin-degrading enzymes (LDEs) (Bucher *et al.*, 2004; Pointing & Hyde, 2000) . A majority of fungi from mangroves were shown to produce cellulase, xylanase and laccase enzymes (Pointing *et al.*, 1998; Pointing & Hyde, 2000; Raghukumar *et al.*, 1994). Most of these studies have been qualitative or semi-quantitative. The LDEs from marine fungi have hardly been characterized in detail. The marine-derived fungus *Cerrena unicolor* MTCC 5159, a white-rot basidiomycete isolated from mangrove wood produced laccase as the dominant LDE in seawater medium (D'Souza *et al.*, 2006). The purification and characterization of laccase from this marine-derived basidiomycete is presented here.

A characteristic of laccase, besides being a blue copper protein is that it is glycosylated. The glycosylation of laccase has also served as a criterion in their classification. Laccases produced by plants often have 20 - 50 % glycosylation and are more involved in polymerization reactions. Whereas fungal laccases have 5 - 25 % glycosylation and are more involved in depolymerization reactions (Baldrian, 2006). Glycosylation of proteins is the enzymatic process that links saccharides (from an activated nucleotide sugar) to proteins, to produce glycoproteins. Three classes of glycans are produced which specifically attach to proteins: *N*-linked glycans attach to the amide

nitrogen of asparagine side chains, *O*-linked glycans attach to the hydroxy oxygen of serine and threonine side chains whereas glycosaminoglycans attach to the hydroxy oxygen of serine. *N*-linked glycosylation is important for the folding of some eukaryotic proteins. This process occurs in eukaryotes and widely in archaea but very rarely in bacteria.

Proteins can be glycosylated by both types of oligos at different sites on the protein. In laccases, the glycans are *N*-linked to the polypeptide chain (Brown *et al.*, 2002; Ko *et al.*, 2001; Saparrat *et al.*, 2002). Glycosylation serves various functions, for instance, some proteins do not fold correctly unless they are glycosylated first. Also, polysaccharides linked at the amide nitrogen of asparagine in the protein confer stability on some secreted glycoproteins. Glycosylation may not be a strict requirement for proper folding but the unglycosylated protein degrades quickly. It was proposed that in addition to the structural role, glycosylation can also participate in the protection of laccase from degradation by proteases (Yoshitake *et al.*, 1993), which are often present in the harsh extracellular environment into which laccases are secreated in.

In general, laccase can catalyze the oxidation of a wide array of substrates due its low specificity for the reducing substrate. The greater the range of substrates it can oxidize, greater is its commercial and biotechnological impact. The substrates range from metal complexes like ferrocyanide to phenolic and aromatic compounds to inorganic, organic and even biological compounds. The range of the substrates that can be oxidized by laccase is determined by the redox potential of the T1 Cu site. As long as the redox potential of a substrate is lower than that of the T1 Cu site, the laccase will be capable of catalyzing its oxidation (Gianfreda *et al.*, 1999). Also, crystallographic studies on the substrate-binding pocket of *Coprinus cinereus* laccase indicate that this binding-pocket is shallow and thus has limited steric hindrance on simple phenolic substrates (Ducros *et al.*, 1998; Gianfreda *et al.*, 1999; Hyde *et al.*, 2000; Xu, 1996) resulting in easy access of substrate to the active site. Physical methodology such as circular dichroism (CD) and electron paramagnetic resonance (EPR) have been used to study the metal centers of fungal laccases, showing that they are relatively stable compared to the secondary structure of the protein (Bonomo *et al.*, 2001). The secondary structure can undergo changes to alter substrate specificity. Numerous substrates have been used to study laccase activity. The use of syringaldazine or ABTS as a substrate can be flawed, since

both these substrates are also oxidized by manganese dependant peroxidase and lignin peroxidase which are often produced along with laccase. Special precautions involving removal of hydrogen peroxide is frequently omitted in work in which the presence of laccase is supposedly shown. Similarly, laccase and tyrosinase share a large number of substrates. To differentiate between laccase and peroxidase on the one hand and between laccase and tyrosinase on the other requires several proofs. One of the difficulties is the fact that the immediate product of laccase activity is a semi-quinone i.e. a free radical, which is extremely reactive and able to interact with numerous compounds which are present particularly in inadequately purified enzyme preparations. The complexity of such reactions has been shown by Ferrari *et al.* (1997). The only conclusive proof of laccase activity according to Mayer & Staples (2002) is that it is able to oxidize quinol as determined by oxygen uptake no matter what other substrates are attacked.

Tyrosinases, catechol oxidases and laccases were earlier summarized as phenoloxidases and share a common substrate range. This often leads to misidentification. There are structural differences between the two enzymes. Tyrosinases have a coupled binuclear copper complex at their active site i.e. only two T3 copper atoms are present and are best known for their polymerization reactions often leading to the formation of melanin. Laccase on the other hand, contains one T1 copper at the substrate binding site and a trinuclear copper center which contains one T2 and two T3 coppers at the co-substrate-binding site. Laccases are capable of both polymerization as well as depolymerization reactions, the direction of the reaction depends on laccase molecular structure and the type and quantity of substrate as well as product build up, in the reaction environment.

Although their end-products are often the same and both enzymes catalyze the four-electron reduction of O_2 to H_2O, the mechanisms by which they achieve their goal are different (Fig. 1). Tyrosinases (EC 1.14.18.1) comprise two reactions; mono- and di-phenolase activities i.e. hydroxylation followed by oxidation reactions. For the hydroxylation reaction, one atom of O_2 is incorporated into the aromatic ring of the monophenol, resulting in *o*-diphenol (mono-phenolase / cresolase activity). This *o*-diphenol is then converted into its benzoquinone by the oxidation reaction (diphenolase / catecholase activity). In this respect, tyrosinase is a 'mixed-function oxidase'. The oxidation step does not require the exclusive presence of tyrosinase, auto oxidation also

works. The catechol oxidases (EC 1.10.3.1) exert only the diphenolase activity but not the chemically fastidious hydroxylase step. Whereas Laccases (EC 1.10.3.2) have no hydroxylase activity but oxidize mono- and diphenols by the generation of radicals of the substrate they are attacking.

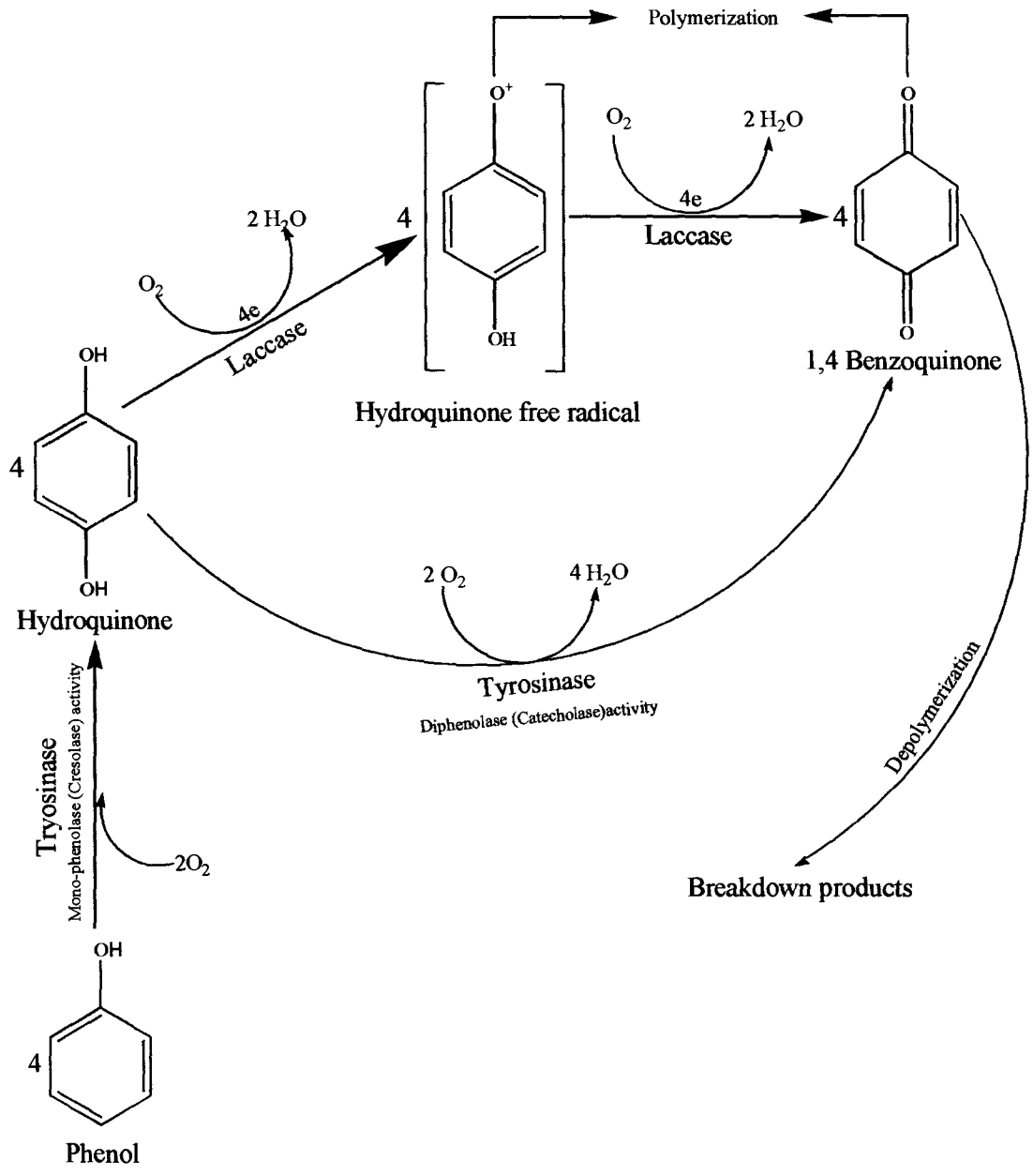

Fig. 1: Difference in mechanism of action between tyrosinase and laccase.

For tyrosinase the pH optima ranged from 3 (*Vanilla planifolia*) to 9.6 (*Pinus densiflora*), could even work at pH 11 (*Thermomicrobium roseum*). Whereas for laccase, the pH optima ranged from 2 (*Daedalea quercina*), to 8 (*Coriolus versicolor*) *Myrothecium verrucaria* laccase can work at even pH 11.5. On the whole tyrosinases often have the optimum working pH in the alkaline range whereas laccases in the acidic range.

Based on their catalytic properties, tyrosinases and laccases can be also differentiated. Cetyltriammonium bromide (CTAB), an inhibitor of laccase does not have much effect on tyrosinase. The specificity of tyrosinases for substrates like tyrosine and selective inhibition by inhibitors such as carbon monoxide, 4-hexylresorcinol or salicylhydroxamic acid (Allen & Walker, 1988; Dawley & Flurkey, 1993; Petroski *et al.*, 1980) facilitate their differentiation from laccases when protein purification is not successful.

Presented below are a few techniques regularly used in purifying and characterizing laccases.

Size exclusion chromatography is a chromatographic method wherein particles like proteins are separated based on their size in more technical terms, their *hydrodynamic volume* (Lathe & Ruthven, 1956). It is known as 'gel filtration' when an aqueous solution is used as the mobile phase and polyacrylamide, dextran or agarose is used as the gel medium or the stationary phase. The underlying principle is that proteins of different sizes will elute (filter) through the stationary phase of a fixed pore size (cut off determined according to the protein to be purified) at different rates. The elution volume decreases roughly linearly with the logarithm of the molecular hydrodynamic volume (for globular proteins this is proportional to their molecular mass). The collected fractions are detected by spectrocopic techniques like ultraviolet (UV) absorbance, at 280 nm for protein.

The advantage of this method is that various solutions can be applied without interfering with the filtration process, while preserving the biological activity of the soluble proteins to be separated. This technique is generally combined with others that further separate molecules by other characteristics, such as affinity (lectins for glycoproteins such as laccase are used) and charge (Wilson & Walker, 2000).

Ion exchange chromatography relies on the interactions between the charges of the sample protein and the charge of the immobilized on the resin which is used for separation. It is of two types; *cation exchange chromatography*, where positively charged proteins bind to a negatively charged resin and *anion exchange chromatography* where the reverse occurs. Once the solutes are bound, the column is equilibrated with a low ionic strength starting buffer and the bound proteins are eluted off using a gradient of eluent buffer, with steadily increasing ionic strength. Alternatively a buffer with steadily increasing/ decreasing pH values may also be used as eluent in some cases (Wilson & Walker, 2000).

Two-Dimensional PolyAcrylamide Gel Electrophoresis (2-D PAGE) separates proteins according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to size (Mr) by SDS PAGE in the second dimension (O' Farrell, 1975).

In IEF, the protein is run on a pre-formed (by ampholytes) pH gradient. The protein stops moving in the pH gradient corresponding to its isoelectric point (pI) or Zwitter ionic state. Problems of pH gradient instability and reproducibility were resolved using Immobilized pH Gradients (IPG) (Bjellqvist *et al.*, 1982). This is based on the principle that the pH gradient is generated by a limited number (6 - 8) of well-defined chemicals (the 'Immobilines') which are co-polymerized with the acrylamide matrix. Thus, cathodic drift is eliminated, reproducibility enhanced and pattern matching and inter-laboratory comparisons simplified. The IPGs allows for the generation of pH gradients of any desired range (broad, narrow or ultra-narrow) between pH 3 and 12 and offers high sample loading capacity as well.

The second dimension (traditional SDS PAGE) can be run on horizontal or vertical systems (Görg *et al.*, 1998). In the vertical setup, the equilibrated IPG gel strips are placed on top of the vertical SDS gels and embedded in agarose. After termination of the second dimension run, the separated proteins in the gel are immobilized and interfering non-protein components removed by fixing. The separated polypeptides can be visualized by 'general protein' stains like Coomassie blue, silver, fluorescence or autoradiography, or by 'specific protein' stains such as Schiff staining for glycoprotein or immunochemical detection methods. Silver staining methods are about 10 - 100 times more sensitive than various Coomassie blue staining techniques. General protein stains

are carried out directly in the gel whereas immunochemical detection methods are usually performed after electrophoretic transfer 'blotting' of the separated polypeptides from the electrophoresis gel onto an immobilizing membrane (Kyhse-Andersen, 1984).

It is preferable to gain N-terminal or even internal amino acid sequence information of proteins separated by 2D-PAGE. The stained spots excised from the gel or the blotting membrane can be subjected directly or after cleavage, to amino acid sequencing, amino acid composition analysis, or mass spectrometry (Wilm *et al.*, 1996).

N-terminal sequencing is a chemical process based on the technique developed by Pehr Edman in the 1950's. The N-terminal amino acid (the end of the protein which is terminated by a free amine, $-NH_2$ group) is derivativatized with phenylisothiocyanate (PITC). The derivatizing process results in a phenylthiohydantoin (PTH)–amino acid. This amino acid is then sequentially removed, while the rest of the peptide chain remains intact. Each derivatization process is a cycle. Each cycle removes a new amino acid. The amino acids are sequentially analyzed to give the amino acid sequence of the protein or peptide. These sequentially removed amino acid residues from the N-terminus of the protein are then identified by reversed phase HPLC. Pure proteins (> 90 %) usually generate easily interpreted data. Long sequences of 50 amino acids or more are possible with this technique.

Matrix-Assisted Laser Desorption / Ionization (MALDI) is a soft ionization technique used in mass spectrometry (Karas *et al.*, 1987). This allows the analysis of biomolecules such as proteins, peptides and sugars which tend to be fragile and fragment when ionized by more conventional ionization methods. In proteomics, MALDI is used for the identification of proteins isolated through 1D or 2D SDS PAGE. One method used is peptide mass fingerprinting by MALDI-MS, carried out by digests of the protein in question.

In MALDI, the ionization is triggered by normally a nitrogen laser. A matrix is used to protect the biomolecule from being destroyed by direct laser beam and to facilitate vaporization and ionization. The matrix consists of crystallized molecules, of which the three most commonly used are sinapinic acid (for glycoproteins and glycopeptides), α-cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid

(DHB). A solution of one of these molecules is made often in a mixture of highly purified water and an organic solvent such as acetonitrile or ethanol, trifluoroacetic acid may also be added. The matrix solution is mixed with the analyte (e.g. protein-sample). The organic solvent allows hydrophobic molecules to dissolve into the solution while the water allows for water-soluble (hydrophilic) molecules to do the same. This solution is spotted onto a MALDI plate. The solvents vaporize, leaving only the recrystallized matrix but now with analyte molecules spread throughout the crystals. The matrix and the analyte are co-crystallized in a MALDI spot. The laser is fired at the crystals in the MALDI spot. The matrix absorbs the laser energy and then transfers part of its charge to the protein thus ionizing it while still protecting it from the disruptive energy of the laser. Ions observed after this process consist of a neutral molecule [M] and an added or removed ion. Together, they form a quasimolecular ion, for example $[M+H]^+$ in the case of an added proton, $[M+Na]^+$ in the case of an added Na ion, or $[M-H]^-$ in the case of a removed proton.

The type of mass spectrometer most widely used with MALDI is the TOF MS (Time-Of-Flight Mass Spectrometer), mainly due to its large mass range. The TOF measurement procedure is also ideally suited to the MALDI ionization process since the pulsed laser takes individual 'shots' rather than working in continuous operation. Proteins / peptides can be sequenced using MALDI -TOF-MS with a ladder sequencing technique, which consists of either a time-dependent or concentration-dependent chemical degradation from either the N- or C-terminus of the protein / peptide into fragments, each of which differs by one amino acid residue. The mixture is mass analyzed in a single MALDI-TOF-MS experiment with mass differences between adjacent mass spectral peaks corresponding to a specific amino acid residue. This type of analysis determines the masses of a series of peptides / proteins that are present in a single MALDI sample. The order of occurrence in the mass spectrum defines the sequence of amino acids in the original protein / peptide. The sequence information for an unknown peptide or protein sequence is deduced from its metastable ion decay mass spectrum.

Protein identification by MALDI-TOF is carried out by digesting the protein with trypsin (cleaves at R-X and K-X except when X is Pro) and a peptide mass fingerprint produced by analyzing the digested protein with a MALDI-TOF MS generating monoisotopic masses of the peptides. The most abundant peptide ions are

subjected to MALDI-TOF / TOF analysis, providing information that can be used to determine the sequence. The results from both types of analysis are combined and searched using algorithm e.g. Mascot (http://www.matrixscience.com) against protein or translated DNA / EST database. The database is then theoretically digested with trypsin and the experimentally generated mass list is compared to theoretically digested database. The match is scored on number of factors, depending on the search program utilized. This method is recommended for the identification of known purified proteins. However there is limited success in identification of proteins that are not in the database, it is generally not suitable for proteins <15 kDa in size, the matches are based on peptide masses and not sequence information and generally only able to suggest post-translational modifications. Amino acid sequencing conclusively confirms the identity of the protein.

3.2 OBJECTIVE

The objective of this chapter is to characterize lignin-degrading enzymes, especially laccase, from the selected fungal isolate *Cerrena unicolor* MTCC 5159, which produced the maximum laccase titer.

3.3 MATERIALS AND METHODS

3.3.1 ORGANISM AND CULTURE CONDITIONS

Cerrena unicolor MTCC 5159, a basidiomycete was the culture used in this study. Modified Low nitrogen (mod LN) medium (Appendix 8.1.1) was prepared with half-strength seawater. The culture was raised in the same medium for 6 days, homogenized in Omni Macro-homogenizer (Model No.17505, Marietta, GA, United States) for 5 sec and the mycelial suspension was used as inoculum. Erlynemeyer flasks (250 mL) containing 20 mL mod LN medium were inoculated with 2 mL mycelial inoculum (10 % v/v). Cultures were incubated at 30°C under static conditions, in the dark. On day 6, $CuSO_4$ at 2 mM final concentration was added to the cultures under aseptic conditions to stimulate laccase production. For large-scale cultivation, 3-L Haffkine flasks with 1 L mod LN medium were inoculated with 100 mL of mycelial suspension and incubated as described above.

3.3.2 PRODUCTION OF ENZYME

On day 12 of cultivation when laccase activity reached its maximum, the cultures were vacuum-filtered to separate the mycelium. The culture filtrate was collected after passing through a series of filter papers namely; Whatman no. 1, GF/C, 0.45 µm and finally 0.22 µm (GV, Millipore, USA). The filtrate was used as the starting material for further experiments. The fungal biomass was estimated as mycelial dry weight (Appendix 8.2.1.5).

The fungus is known to produce exopolymeric substances (EPS) to up to 50 % of its dry weight (D'Souza *et al.*, 2006). During protein purification through chromatographic columns, this EPS, if not removed can lead to their blockage. Therefore, it was preferable to remove the EPS to prolong the life of the column. To remove the EPS, the culture filtrate was frozen at -20°C, thawed, and then centrifuged at ~ 22,000 *g* for 15 min at 4°C. The precipitated EPS was removed.

3.3.3 ENZYME ASSAY

Laccase activity was estimated spectrophotometrically using the ABTS method (Appendix 8.2.2.1.1). It was also estimated by syringaldazine and guaiacol, in specific experiments which warranted it, the estimation procedures are given in Appendix 8.2.2.1.2 & 8.2.2.1.3 respectively. An abiotic control without laccase had been used in every experiment and all the assay components had been brought to reaction temperature before assay. Laccase was expressed as either U L^{-1} or U mL^{-1} and has been defined in Appendix 8.2.2.1.1. Protein concentration was estimated spectrophotometrically using Bradfords reagent (Sigma, St. Louis, Montana, U.S.A), (Appendix 8.2.1.4). All spectrophotometric measurements were carried out using UV-Vis 2450 spectrophotometer (Shimadzu, Japan). All values represent the mean of three measurements of two independent experiments each.

3.3.4 COMPARISON OF ENZYME CONCENTRATION TECHNIQUES

Various techniques to concentrate laccase from 250 mL the culture filtrate were compared for obtaining the maximum laccase activity.

3.3.4.1 Concentration with Poly Ethylene Glycol (PEG) 6000: The culture filtrate was placed in pretreated (as per manufacturers protocol) dialysis bags (12 kDa cut off, Sigma). Both the ends were sealed with clips and covered with PEG. The PEG was removed by washing the outer surface of the bag with distilled water. The volume of the retanate was brought up to 10 mL using distilled water.

3.3.4.2 Vacuum Concentration: The culture filtrate was concentrated in a speed vacuum concentrator (Ecospin 314, Biotron, Korea) to 10 mL.

3.3.4.3 $(NH_4)_2SO_4$ Precipitation: Ammonium sulphate at 80 % saturation (140.25 g) was added slowly to the culture filtrate with stirring at 4°C. After the $(NH_4)_2SO_4$ had completely dissolved it was stored overnight in the cold, to allow the protein to precipitate. The solution was then centrifuged at ~ 22,000 *g* at 4°C for 30 min. The

precipitate was rinsed with distilled water and the final volume was brought to 10 mL using distilled water.

3.3.4.4 Ultra Filtration:

3.3.4.4.1 Centrifugal force (Centricon): The culture filtrate was centrifuged in a Centricon with a 5 kDa cutoff membrane at ~ 7,200 *g* at 4°C for 30 min till 10 mL of retentate was left behind.

3.3.4.4.2 Positive pressure (Amicon): The culture filtrate was concentrated to 10 mL, in an Amicon unit (350 mL capacity, with a 5 kDa cutoff membrane) with constant stirring at 4°C. The membrane cutoff was 5 kDa. Positive pressure was applied using nitrogen gas, to drive the ultrafiltration process.

3.3.5 PURIFICATION OF LACCASE

The Amicon concentrated culture filtrate was considered as the partially purified laccase source. This was filtered through 0.22 μm sterile filter (Whatman Asia Pacific, Singapore) and loaded on a High Load 16 / 60 Superdex 75 preparative grade column and eluted with Na-acetate 0.2 M buffer (pH 4.5) containing 1 M KCl at a flow rate of 1 mL min^{-1} using a fast protein liquid chromatography system (FPLC; Amersham Biosciences, Sweden). The molecular markers (Amersham Pharmacia, Sweden) used were bovine serum albumin (66 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa) and α-lactoalbumin (14.2 kDa). Out of the 3 laccase peaks obtained during this size exclusion chromatographic step, the fractions showing maximum absorbance at 280 nm and maximum laccase activity, at retention volume of 50 mL (mol mass ~56 kDa) were collected, pooled and concentrated using Centricon with a 5 kDa cutoff membrane (Millipore, MA, USA).

The concentrate was then applied to Mono Q^{TM} 10 / 100 GL (Amersham Biosciences, Sweden) column of 10 × 100 mm size and eluted with Tris-HCl buffer (pH 8) containing 0.5 M KCl at a flow rate of 0.3 mL min^{-1}. Out of several peaks showing laccase activity, the laccase fractions eluting at 0.185 - 0.197 M KCl gradient showing

maximum absorbance at 280 nm and maximum laccase activity were pooled and concentrated as above. This laccase fraction was used for further characterization reported in the present study.

3.3.6 CHARACTERIZATION OF LACCASE

3.3.6.1 Non-denaturing SDS PAGE & 2-D PAGE: Non-denaturing SDS PAGE is identical to SDS PAGE (Appendix 8.3.2.1) with the exception that the sample was not heat treated thereby retaining enzyme activity, which could be stained for activity (Appendix 8.3.2.3.1). The molecular mass of the protein could also be determined since SDS was used in the gel and in the sample buffer. For determining the molecular mass, standard protein molecular markers of the medium range 14.3 - 97.4 kDa (Protein molecular mass marker; PMWM, Bangalore Genei, India) were used and protein staining by the silver staining method (Appendix 8.3.2.3.2.1) was performed. The gels were stained for glycoprotein (Appendix 8.3.2.3.3) using the Schiff's reagent (Sigma, MA, USA).

A 2-D PAGE (Bio-Rad, CA, USA) was run for confirming the pI (using IEF strips, pH gradient: 4 - 7) and molecular mass of purified laccase. In case of both non-denaturing PAGE and 2-D PAGE, a 12 % polyacrylamide gel was used.

3.3.6.2 Iso Electric Focussing (IEF): Isozyme pattern of laccase in partially purified mycelia-free culture filtrate was determined by non-denaturing IEF using IEF strips with a pH gradient of 3 - 10 and 4 - 7 in an IEF Cell (BioRad, CA, USA). The strips were stained for activity with guaiacol.

For the purified laccase, denaturing IEF was performed with IEF strips with pH gradient of 4 - 7 and pI was determined by protein (silver) staining.

3.3.6.3 Determination of glycosylation status of purified laccase: For evaluating the *N*-linked glycan content, 0.6 µg purified laccase was treated with 60 mU of Endoglycosidase H (Endo H from *Streptococcus plicatus*, Sigma, MA, USA) overnight at 37°C. A non-denaturing SDS-PAGE of the Endo H-treated and untreated

purified laccase was then carried out. The gel was stained for protein and glycoprotein. The difference in molecular mass of the Endo H-treated and untreated purified laccase, gave the mass (kDa) of the purified laccase that was contributed by its *N*-linked glycan content.

3.3.6.4 Spectral profile of purified laccase: The UV-visible spectrum of purified laccase at 0.7 mg mL^{-1} was spectrophotometrically determined.

3.3.6.5 Determination of N-terminal amino acid sequence of purified laccase: The purified laccase was electro blotted (by over night transfer at 4°C) from an SDS-PAGE gel onto a PVDF membrane (BioTrace, Pall Pharmalab Filtration, India) using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, CA, USA). The membrane was stained for protein by the Coomassie blue staining method (Appendix 8.3.2.3.2.2). The band corresponding to the purified laccase was excised and loaded directly onto the automated protein sequencing system (Procise HT - 491, Applied Biosystems, CA, USA), to determine the N-terminal sequence.

3.3.6.6 Determination of internal peptide sequence of purified laccase: Trypsin is a highly active and specific protease, which cleaves the C-terminal to lysine and arginine residues of a protein to generate peptides that fall within the mass range of 500 to 5000 Da, which is suitable for peptide mass mapping. After the *in-gel* digestion of purified laccase, by trypsin, the peptides were extracted by sonication and the spotted on the α-cyano-4-hydroxycinnamic acid (HCCA) matrix for a MALDI TOF analysis. This was carried out at the Proteomics Facility, TCGA, New Delhi with Ultra Flex MALDI TOF, (Germany).

3.3.6.7 Physical properties of laccase:

3.3.6.7.1 Determination of pH and temperature assay optima for laccase: To determine the pH and temperature optima of laccase, its activity was measured with 1mM ABTS prepared in varying pH using different buffer systems. The buffers included glycine-HCl (pH 2.5 and 3), sodium citrate-phosphate (pH 3 - 6),

Tris-HCl (pH 7 - 9) and glycine-NaOH (10 and 11), all of which were prepared at 0.2 M. Laccase activity was assayed at different temperatures for each pH tested. For partially purified laccase assay, the pH and temperature range determined was from 3 - 11 and 5° – 90°C whereas, for purified laccase it was from 2.5 - 9 and 20° – 90°C, respectively using ABTS. For purified laccase, the optimum pH for assay was also determined using different substrates (ABTS, syringaldazine & guaiacol) at 30°C, in the pH range of 2.5 - 9.

3.3.6.7.2 Determination of pH and temperature stability of purified laccase: To determine the pH stability, purified laccase was preincubated at pH 2.5 – 9 for 1 h at 30°C, the residual activity was estimated with ABTS at pH 3 and 70°C (optimum pH and temperature for activity). For testing temperature stability, it was incubated in Tris-HCl buffer, pH 9 (at its maximum stability) at 50°, 60° and 70°C for various incubation periods. Activity was then estimated as above. It was presented as % relative activity, considering activity at 0 h to be 100 %.

3.3.6.8 Kinetic properties of purified laccase:

3.3.6.8.1 Determination of temperature coefficient (Q_{10}) & Energy of Activation (E_{act}) values: The Q_{10} & E_{act} values of purified laccase were determined based on the pH & temperature assay results. The formulae used for calculations are given in Appendix 8.2.3.2.5 & 8.2.3.2.6 respectively.

3.3.6.8.2 Determination of maximum velocity (V_{max}), Michaelis constant (K_m), catalytic constant (K_{cat}) & specificity constant: The V_{max} and K_m were determined from the Lineweaver Burke plot pH 3 at 30° & 70°C for the substrate ABTS and at pH 6 (optimum pH) and 30°C for the substrate syringaldazine. The enzyme assay was carried out at varying substrate concentrations; 10 - 500 µM for ABTS and at 10 - 200 µM for syringaldazine. The K_{cat} and specificity constant were determined from the K_m and V_{max} values. The calculations for V_{max}, K_m , K_{cat} and specificity constant are given in Appendix 8.2.3.2.1 - 8.2.3.2.4.

3.3.6.9 Catalytic properties of purified laccase:

3.3.6.9.1 Substrate specificity and inhibitors: Oxygen uptake during the reaction of purified laccase with various substrates, non-substrates, mediators and inhibitors was measured for 15 min using an oxygen electrode (Oxygraph, Hansatech, Norfolk, England) at 30°C in a 1 mL reaction chamber. The total volume was made to 1 mL with 0.2 M citrate phosphate buffer (pH 4), such that there was no air gap between the under surface of the lid and the surface of the reaction mixture. The reaction mixture for the testing of substrates consisted of 1 U purified laccase and the substrate. Whereas for the testing of non-substrates, mediators and inhibitors, the reaction mixture contained 1 U purified laccase, I mM ABTS and the compound under test. In case of inhibitors, the purified laccase was pre-incubated with the inhibitor for 1 min before the addition of 1 mM ABTS, after which oxygen consumption was monitored. The rate of oxygen uptake was measured in terms of nmol O_2 min^{-1} U^{-1} laccase. The activity was expressed in terms of % relative activity, considering laccase activity in the presence of only 1 mM ABTS as 100 %. The composition of the control for both substrate specificity and inhibitor effect was identical to that of the test sample and was subjected to identical conditions, however here purified laccase was absent.

3.3.6.9.2 Effect of metal ions: Effect of various metal ions at 1 mmol on the activity of purified laccase was measured spectrophotometrically using 1 mM ABTS as substrate, at its optimum pH (3) and temperature (70°C). The purified laccase was pre-incubated for 1 min with the metal ion before assaying with ABTS. The activity was expressed as % relative activity considering the activity of purified laccase in the presence of only ABTS as 100 %. The control contained the metal ion dissolved in glycine-HCl buffer (pH 3) in the same volume used to dissolve laccase and was pre-incubated at 70°C for 1 min and was assayed without the presence of purified laccase.

3.4 RESULTS

Cerrena unicolor MTCC 5159 showed growth and laccase production in mod LN medium prepared with sea water (Fig. 2). Laccase activity and fungal biomass (growth) reached a maximum on day 12 (~12,600 U L^{-1}) hence, the culture was regularly harvested for laccase on day 12 of growth, for purification and characterization purposes.

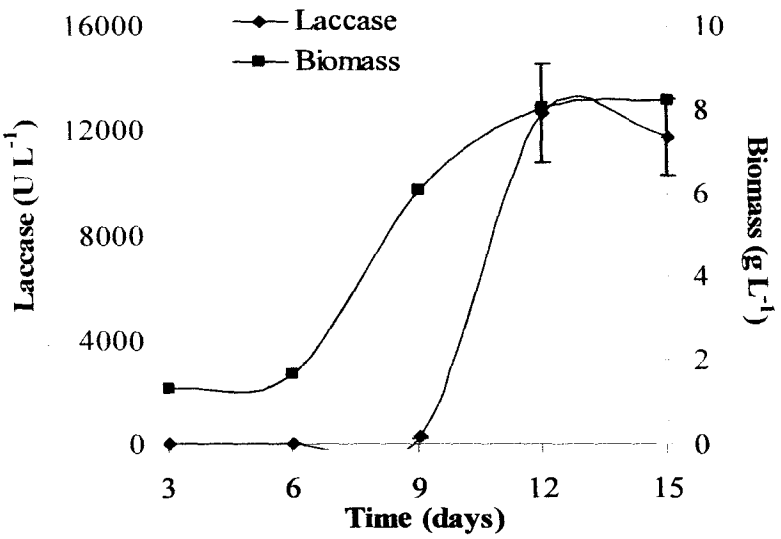

Fig. 2: Temporal production of laccase and biomass.

Table 1: Efficiency of different concentration techniques.

Concentration Technique	Laccase Activity (U mL^{-1})	Protein Concentration (mg mL^{-1})	Specific Activity (U mg^{-1})	Fold
Culture Filtrate	24	0.1	330	1
PEG Concentration	775	1.5	2260	33
$(NH_4)_2SO_4$ Precipitation	515	2	1445	22
Ultrafiltration (centrifugal)	430	1.5	1700	18
Ultrafiltration (positive pressure)	**3245**	**0.3**	**12981**	**137**
Vacuum Concentration	735	2.5	1590	31

Various methods to concentrate the culture filtrate were compared. These included concentration by polyethylene glycol (PEG) 6000, ammonium sulphate [$(NH_4)_2SO_4$], ultrafiltration by positive pressure (Amicon) and by centrifugal force (Centricon) and by vacuum concentration (Table 1). The culture filtrate was concentrated exactly 25 times for all the concentration techniques used. It is clear that Amicon concentration was the most efficient method for the concentration of laccase, it also yielded maximum specific activity. Henceforth, Amicon concentrated culture filtrate was used as the source of partially purified laccase for all other experiments.

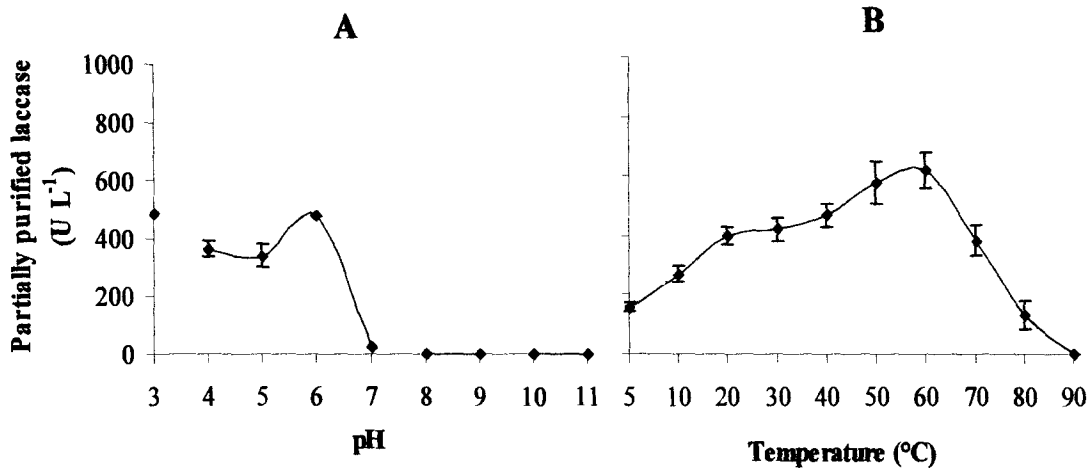

Fig. 3: Effect of (A) pH and (B) Temperature on assay of partially purified laccase.

The pH and temperature optima (Fig. 3A & B) for assay of partially purified laccase were determined spectrophotometerically, using ABTS as substrate. Laccase showed a bimodal pH profile with activity peaks at both 3 and 6. However with slightly greater activity at pH 3 (Fig. 3A) and maximum activity at 60°C (Fig. 3B). Partially purified laccase preparations were estimated at pH 3 and 60°C, henceforth.

Non-denaturing IEF was first carried out in the pH range of 3 - 10 (data not shown) the laccase bands obtained were within the pH range of 4 – 7. Due to the broad pH range, the bands obtained were close to each other and not very distinguishable. Hence a non- denaturing IEF in the pH range of 4 – 7 (Fig. 4) was carried out and the strip stained for activity with guaiacol. Two very distinct laccase bands were observed

besides several other laccase bands, which were not clearly distinguishable. This suggested the presence of several laccase pI isozymes (seen as fine bands due to their relative lower concentration) to the tune of over 20, in partially purified laccase.

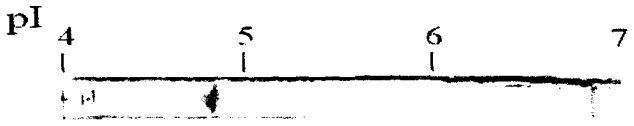

Fig. 4: Activity staining by guaiacol after non-denaturing IEF (pH 4 - 7) of partially purified laccase.

Due to the presence of a large number of laccase isozymes based on pI, a size exclusion chromatographic step was carried out prior to anion exchange chromatography. When partially purified laccase was subjected to size exclusion chromatography (Superdex-75 column), it yielded 3 distinct laccase peaks corresponding to molecular mass of 82, 56 and 45 kDa termed as Lac I, Lac II and Lac III (Fig. 5) respectively. Compared to the other isozymes, Lac II was present in maximum abundance.

Hence it was selected for further resolution by anion exchange chromatography (Mono Q column). This resolved into 4 major peaks (Fig. 6), indicating that at least 4 different isozymes having the same molecular mass but differing in surface charge were present. Lac I & III were also resolved into 4 peaks each, by anion exchange chromatography (data not shown). This indicates that all the three laccases have isozymes differing in their surface charges. Due to significantly low recovery, these laccases were not characterized.

From the anion exchange chromatography of Lac II, the peak showing maximum absorbance at 280 nm also corresponded to maximum laccase activity (eluted between 0.185 and 0.197 M KCl gradient) was designated '**Lac IId**'. This laccase isozyme was selected for further characterization.

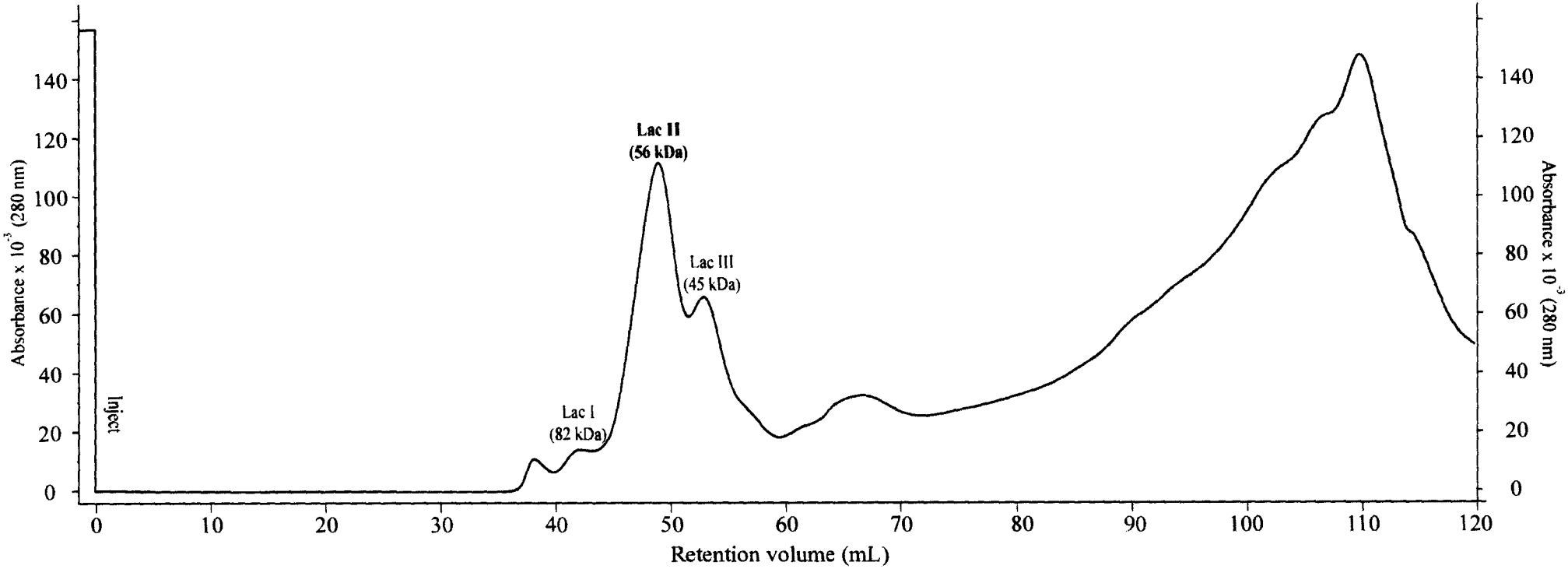

Fig. 5: Size exclusion chromatographic profile of crude laccase using Superdex 75 column.

Culture filtrate of a 12 day-old culture of *Cerrena unicolor* MTCC 5159 grown in mod LN medium was concentrated by ultrafiltration (positive pressure) and used as the source of crude laccase. 3 major peaks Lac I, Lac II and Lac III with differing molecular masses were obtained.

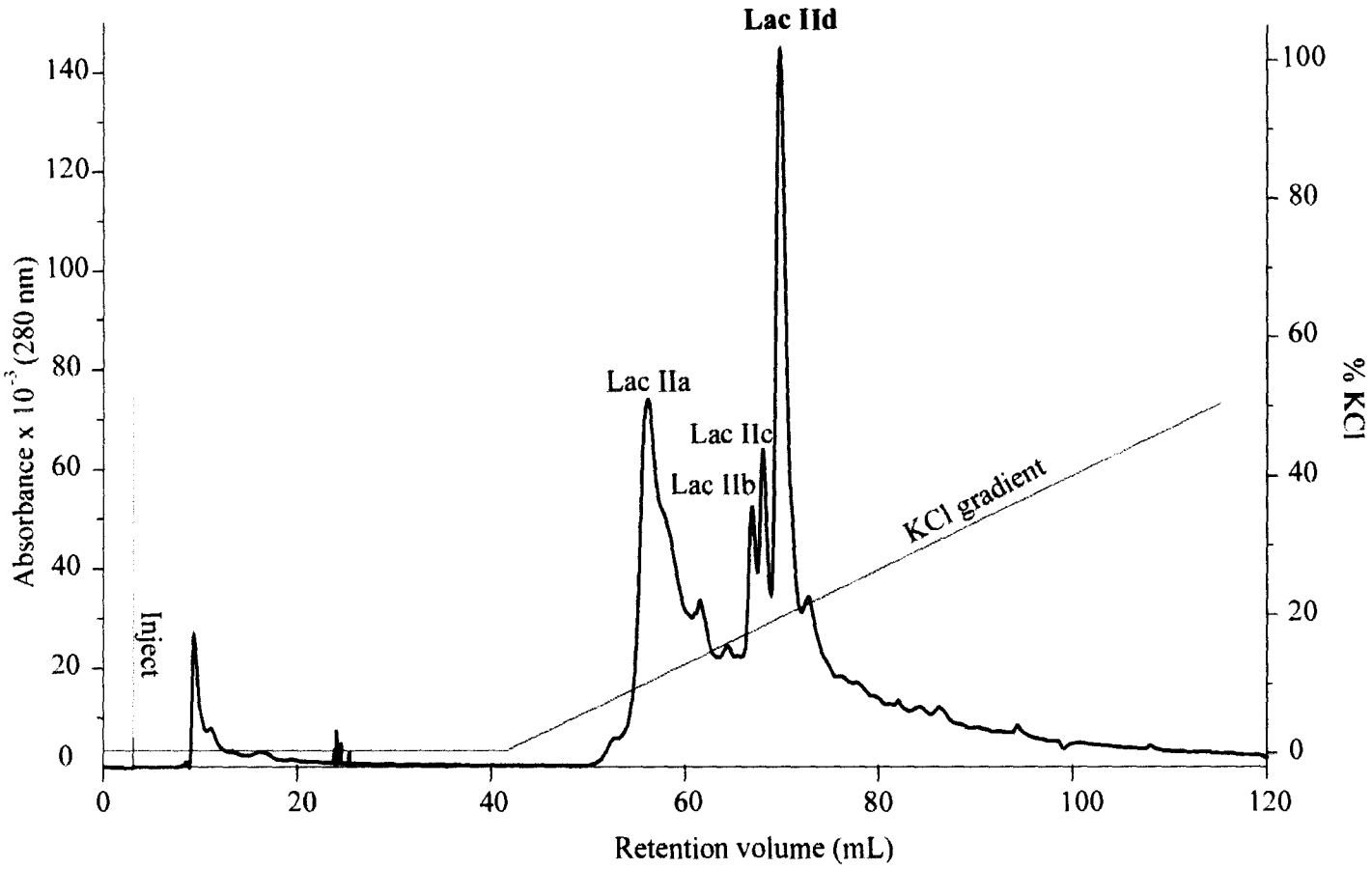

Fig. 6: Anion exchange chromatographic profile of Lac II fraction from the size exclusion chromatographic step, using Mono Q column. The KCl gradient was maintained between 0 to 0.5 M. Lac II resolved into 4 peaks with differing surface charges namely Lac IIa, IIb, IIc and IId.

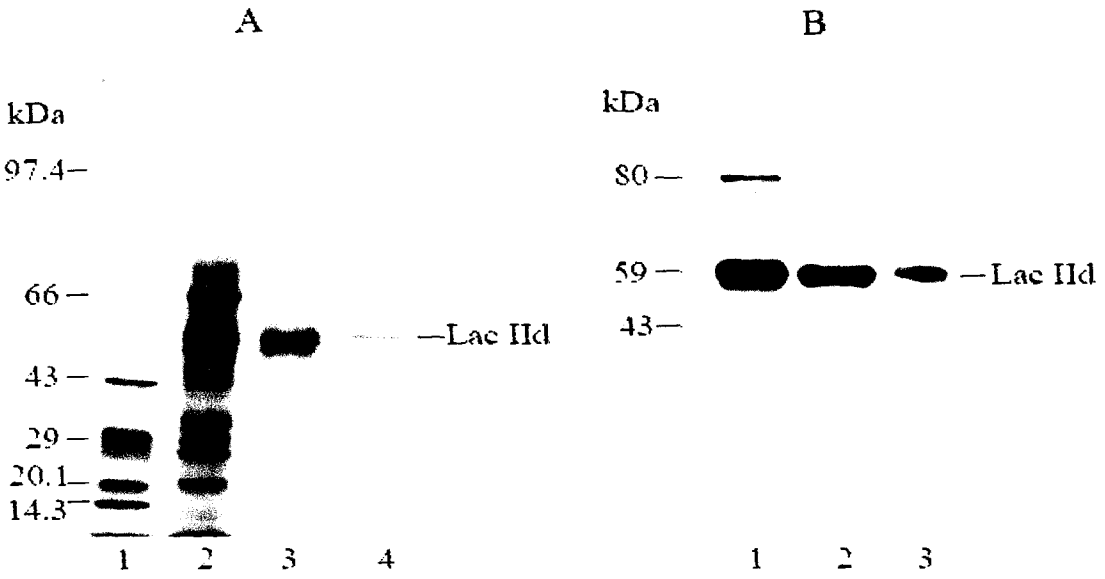

Fig. 7: Non-denaturing SDS-PAGE of *Cerrena unicolor* MTCC 5159 laccase. (A) Silver and (B) Guaiacol staining of the 12 % gel.

Lanes 1, 2 & 3 of (B) correspond with lanes 2, 3 & 4 of (A), respectively

Lane 1: Protein molecular mass marker; lane 2: concentrated partially purified laccase; lane 3: Lac II (from size exclusion chromatography); lane 4: Lac IId (from anion exchange chromatography).

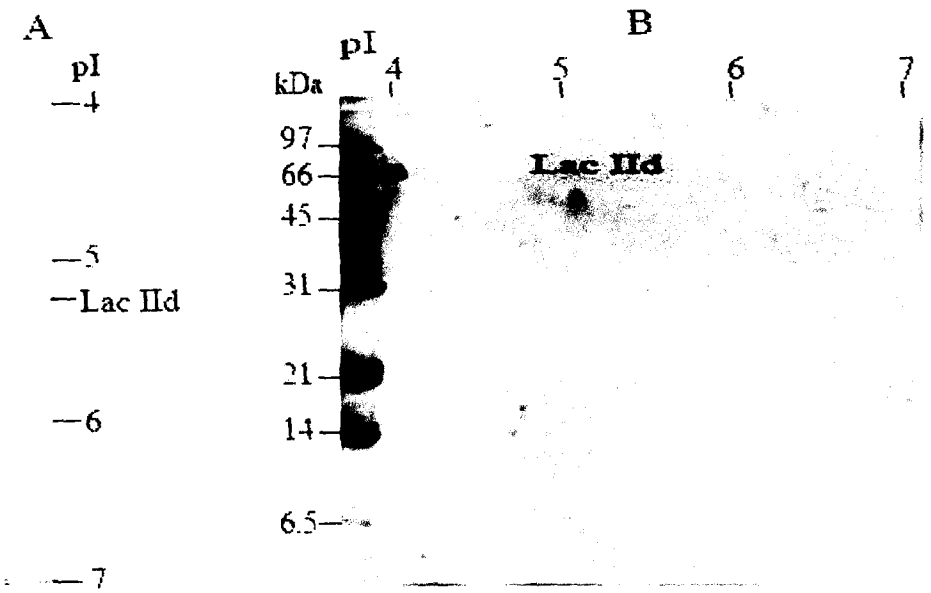

Fig. 8: Confirmation of Lac IId homogeneity. Silver staining of (A) IEF strip, pH 4 - 7 and (B) Gel from 2D PAGE.

A non-denaturing SDS PAGE for the laccase samples obtained at each purification stage was performed. The gel was stained for total protein (Fig. 7A) and activity (Fig. 7B). Activity staining of the gel confirmed that at least 3 laccase molecular mass isozymes were present in partially purified laccase with masses of 80, 59 and 43 kDa which corresponded to the 82, 56 and 45 kDa laccase peaks obtained during size exclusion chromatography. The Lac II fraction further resolved into several laccase isozymes including Lac IId, which had a mass of 59 kDa from Fig. 7A.

The pI was determined to be 5.3 by IEF (Fig. 8A). Besides confirming the molecular mass and pI of Lac IId, 2D PAGE (Fig. 8B) which had been carried out under denaturing conditions (involving heat) revealed that Lac IId was monomeric in nature.

Table 2: Purification of Lac IId from the culture filtrate of *C. unicolor* MTCC 5159.

Purification Step	Total Laccase (U)	Total Protein (mg)	Specific Activity (U mg^{-1} protein)	Yield (%)	Purification Factor (fold)
Culture Filtrate	23714	72	330	100	1
UltraFiltration (5 kDa cut off)	129810	12	11126	547	34
Size Exclusion Chromatography (Superdex 75)	3039	0.92	3315	2	10
Anion Exchange Chromatography (Mono-Q)	531	0.05	10918	17	33

A summary of the purification level obtained at each step is given in Table 2. A 33 - fold purification with a final yield of 17 % for Lac IId was achieved

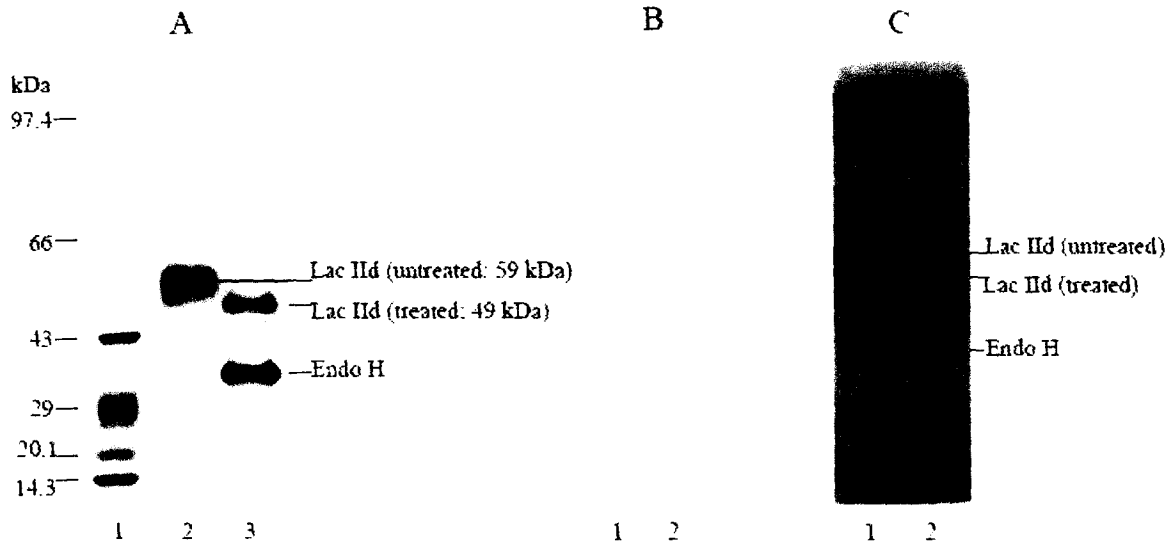

Fig. 9: Glycosylation status of Lac IId; Non-denaturing SDS-PAGE (12 %) for Endo H-treated Lac IId; (A) Silver staining; (B) Glycoprotein (Schiff's) staining and (C) Silver staining of the glycoprotein-stained gel.

Lane 1: Protein molecular mass marker; lane 2: Lac IId (untreated); lane 3: Lac IId treated with Endo H. Lane 1 & 2 of (B) and (C) correspond to lane 2 & 3 of (A) respectively.

In order to determine the percentage of molecular mass contributed by *N*-linked glycosylation, Lac IId was treated with Endoglycosidase H (Endo H) that selectively removes *N*-linked glycosylation. The molecular mass of Lac IId was reduced to 49 kDa (Fig. 9A) revealing that Lac IId contained an *N*-linked glycan content of 17 %. The untreated Lac IId showed positive reaction for glycosylation with Schiff's reagent (Fig. 9B), whereas, the Endo H-treated sample tested negative, thus Endo H was successful in removing most of the glycosylation of Lac IId, as seen in Fig. 9B. Almost the entire glycosylation was of the *N*-linked type. Silver staining of the Endo H-treated Lac IId, after it had been stained for glycosylation was carried out to confirm the presence of Endo H-treated Lac IId in the gel (Fig. 9C).

The UV-visible spectrum of Lac IId (0.7 mg mL^{-1}) demonstrated a peak at 610 nm and a shoulder at 330 nm (Fig. 10) which is typical of blue laccases.

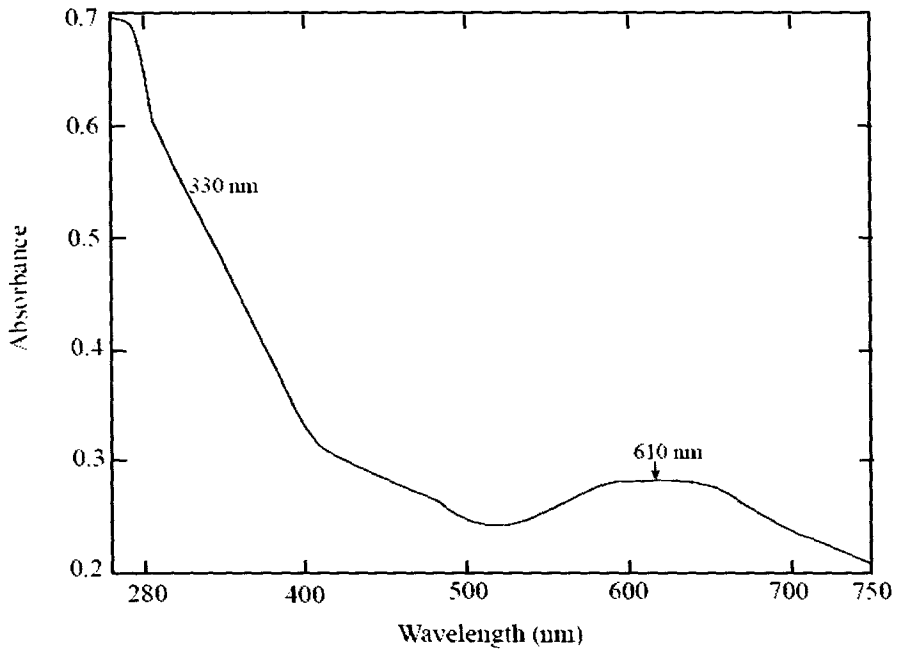

Fig. 10: UV-visible spectrum of Lac IId.

Table 3: Alignment of the Lac IId N-terminal sequence with other fungal laccases.

	1 10 20 30	Similarity (%)	E value
Cerrena unicolor MTCC 5159 (P85430)	GTGPVADLHIINKDLSPDGFQRPTVVAGGG	100	6e-19
Schizophyllum commune (BAA31217)	ALGPVGNLPIVNKEIAPDGFSRPTVLANGK	85	8e-06
Spongipellis FERMP 18171 (BAE96003)	AIGPVADLHIVDVSIAPDGFSRPAVLAGGT	85	5e-06
Panus rudis (AAW28932)	AIGPVTDLHIVNDNIAPDGFTRAAVLAGGT	81	8e-06
Rigidoporus microporus (AAO38869)	AIGPVADLHISNANISPDGFTRAAVLAGGS	81	6e-06
Volvariella volvacea (AAR03582)	AIGPVTELQIVNDEIAPDGFSRGSVLANGA	77	0.018
Trametes I-62 (AAQ12270)	AVGPVTDLTISNANVSPDGFQRAAVVANGG	75	3e-05
Ganoderma lucidum (AAR82930)	AIGPVANLTISDADIAPDGFTRAAVVVNGV	70	0.25
Cerrena maxima (2H5U)	GVGPVADNTITNAATSPDGFSRQAVVVNGV	58	1.7
	. *** : * : :**** * :*:..*		

The N-terminal amino acid sequence of Lac IId was deposited in the universal protein resource database, 'Uniprot' (http://www.pir.uniprot.org) under the accession number P85430. A comparative study of homology of various laccase N-terminal sequences with P85430 using the BLASTP algorithm, at the website: http://blast.ncbi.nlm.nih.gov/Blast, was conducted. This was then aligned using the Clustal version T-Coffee v. 5 (www.tcoffee.org). Similarity values were determined by algorithm 'bl2seq' (Tatusova &

Madden, 1999) where each of the N-terminal sequences given in Table 3 were aligned with P85430.

Comparison with other laccases showed 70 - 85 % similarity with basidiomycetous fungi (Table 3). Maximum similarity was observed with laccases from *Schizophyllum commune* and *Spongipellis* sp. that are white-rot basidiomycetous fungi.

From the MALDI TOF analysis of Lac IId, the sequence of only one peptide could be positively identified. This internal peptide sequence was deposited in 'Uniprot' under the accession number P85430 (same as the N-terminal sequence). A comparative study of various internal peptide sequences homologous to P85430 internal peptide using the HSSP (Homology Derived Secondary Structure of Proteins, v. 1.1, 2001) was conducted, after digesting the databases (protein, translated DNA & EST) with trypsin *in-silico*. P85430 matched with several known laccases. The maximum score of 51 % at p = 0.05; using the algorithm, 'MASCOT Search' of Matrix Science (available at http://www.matrixscience.com/) was with a peptide (Q69FX1_9AGAR) present in 'Laccase 2' of *Volvariella volvacea* (AAR03581, from NCBI database). The scoring is based on the 'Probability based Mowse Score' which is the 'Ion score' being equal to 10 x Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 42 indicate identity or extensive homology at p <0.05. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. The internal peptide, P85430 of *Cerrena unicolor* MTCC 5159 had 100 % homology with Q69FX1_9AGAR peptide of Laccase 2 of *Volvariella volvacea* (AAR03581) at position 471. However, AAR03581 has 545 amino acids (AAs) and P85430 has only 17 AAs, resulting in sequence coverage of only 2.75 %, which is not enough. The predicted molecular mass and pI for AAR03581 was 59367 Da (~59 kDa) and 5.4, respectively, similar to Lac IId of *C. unicolor* MTCC 5159 values for molecular mass and pI.

These sequences were aligned using the Clustal version T-Coffee v. 5 and presented in Table 4. Varying colours denote varying kinds of amino acids (AAs), those which are presented in an identical colour, have similar functions. Only the sequences showing maximum similarity to P85430 were considered, three exceptions were made; *Trametes* sp. I-62 (AAQ12270) which was chosen since it was also used in Table 3, *Ganoderma lucidum* (AAG17009) was chosen on the same basis although the accession

number differed with that of the one used in Table 3. *Cerrena maxima* (2H5U) was chosen for N-terminal as well internal peptide homology studies, since it belonged to the same genus as that of MTCC 5159.

Table 4: Alignment of the Lac IId internal peptide sequence with other fungal laccases.

	1 5 10 15 |...|... .|....|..	Signal peptide (AA)	Start Position (AA) including Signal peptide	Similarity (%)	E value
Cerrena unicolor MTCC 5159 (P85430)	RDVVSIGR--AGDNVTIRF			100	
Volvariella volvacea (AAR03581)	RDVVSIGR--AGDNVTIRF	1-22	471	100	3e-07
Rigidoporus microporus (AAO38869)	RDVVSIGN--AGDNVTIRF	1-21	444	94	2e-05
Flammulina velutipes (BAE91880)	RDVVSIGA--AGDNVTIRF	--	423	94	2e-05
Laccaria bicolor (XP_001886681)	RDVVSIGG--AGDNVTIRF	1-17	439	94	4e-05
Pholiota nameko (ABR24264)	RDVVSIGG--AGDNVTIRF	1-18	444	94	4e-05
Panus rudis (AAW28932)	RDVVSTGT--AGDNVTIRF	1-21	445	88	3e-04
Trametes sp I-62 (AAQ12270)	RDVVSTGTPAAGDNVTIRF	1-25	448	--	0.008
Cerrena maxima (2H5U)	RDVVSTGTPAAGDNVTIRF	--	423	--	0.008
Ganoderma lucidum (AAG17009)	RDVVSTGTPAAGDNVTIRF	1-21	444	--	0.008
	***** * *********				

Comparison of the internal peptide, P85430 with the correspondingly-positioned internal peptides of other basidiomycete laccases showed 88 - 100 % similarity (Table 4). Similarity values were determined in the same way as for the N-terminal sequence. 100 % similarity was observed with the Q69FX1_9AGAR (*in-silico* tryptic digest), an internal peptide of 'Laccase 2' from *Volvariella volvacea* (AAR03581). *Panus* had a homology of 88 % with the internal peptide, P85430 (Table 4) and 81 % with the N-terminal, P85430 (Table 3). *Trametes*, *Ganoderma* and *Cerrena* did not show significant homology with the internal peptide P85430, enough to be listed, whereas they showed the lowest homology of 75, 70 and 58 %, respectively with the N-terminal, P85430 (Table 3).

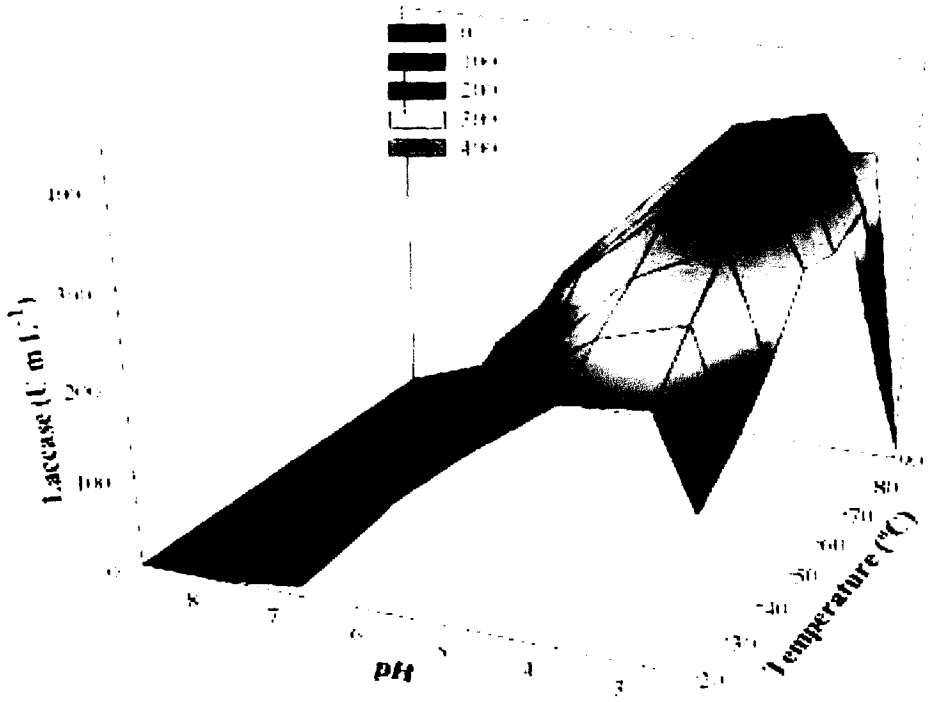

Fig. 11: Effect of pH and temperature on assay of Lac IId.

Lac IId (purified laccase isozyme) showed optimum activity at pH 3 and 70°C (Fig. 11). Whereas with guaiacol and syringaldazine, Lac IId had optimum activity at pH 6 (Fig. 12A).

The enzyme retained 100 % of its activity after 1 h at pH 9 and 30°C and thus was most stable at this pH (Fig. 12B). When Lac IId was incubated at pH 9 in Tris-HCl buffer for 1 h at various temperatures, it retained about 60 % of its activity at 70°C (Fig. 12C). At this pH, more than 60 % of activity was retained at 50° and 60°C for up to 180 min whereas about 50 % residual activity was detected up to 90 min at 70°C (Fig. 12D).

The shelf life of Lac IId is presented in Fig. 12E. Lac IId retained 67 % and 100 % of its activity at 4° and -20°C respectively for over 35 days and complete activity even after 1 year of storage at -20°C.

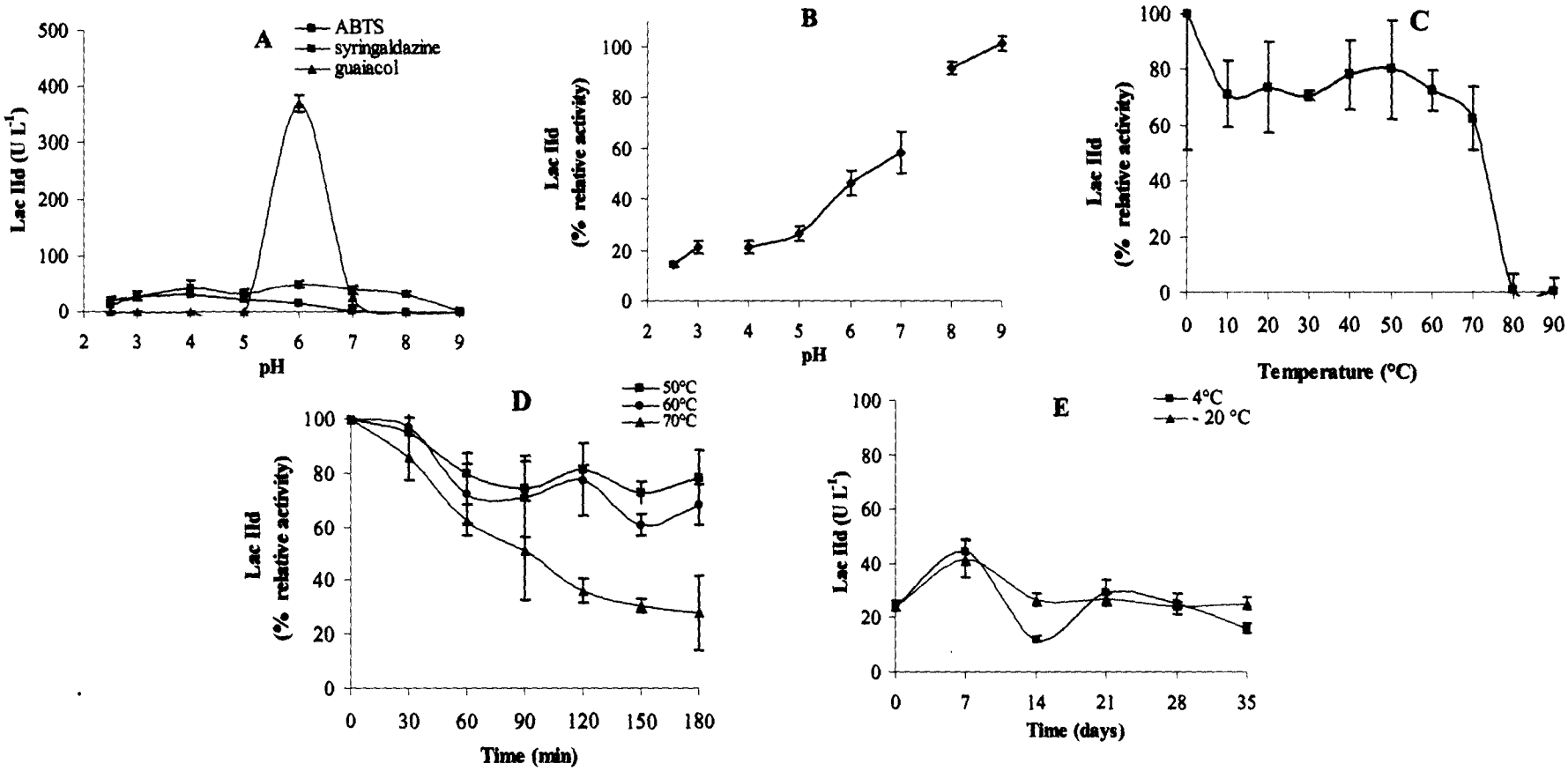

Fig. 12: Properties of Lac IId. (A) Effect of pH on assay (using various substrates at 30°C); **(B) pH stability** (after incubation at 30°C for 1 h at varying pH); **(C) Thermostability between 0° – 90°C** (after incubation at pH 9 for 1 h); **(D) Thermostability between 50 °- 70°C** (after incubation for various time intervals at pH 9); **(E) Shelf life at 4° and -20°C** (when stored at pH 9).

Table 5: Q_{10} values for Lac IId at temperatures (20° - 90°C) and pH (2.5 - 8).

pH	2.5	3	4	5	6	7	8
Q_{20-30}	1.21	1.20	1.14	1.20	1.13	1.42	ND#
Q_{30-40}	1.24	1.15	1.12	1.05	1.10	1.35	ND#
Q_{40-50}	1.09	1.09	1.14	1.22	1.43	1.52	ND#
Q_{50-60}	0.97	1.05	1.06	1.03	1.00	1.03	2.14
Q_{60-70}	1.07	1.02	1.05	1.07	1.14	1.31	3.20
Q_{70-80}	0.39	0.85	0.75	0.87	0.77	0.81	0.94
Q_{80-90}	0.11	0.95	1.05	0.72	0.83	0.66	0.71

Where the activity was zero, it was not possible to calculate the Q_{10} value (hence ND, not determined). Since showed no activity was observed at pH 9, at any temperature it was not included in the table.

The Q_{10} values were found to be >1 between 20° and 70°C in the pH range of 3 - 7 for Lac IId (Table 5). The E_{act} for Lac IId was found to be 13.4 k J mol^{-1} at pH 3 between temperature 20° and 30°C which decreased to 2.5 kJ mol^{-1} between 60° and 70°C, at its optimum pH (3).

Table 6: Kinetic parameters of Lac IId. *

Substrate	K_m (μM)	V_{max} (μmol min^{-1})	K_{cat} (min^{-1})	Specificity Constant (K_{cat} / K_m) (min^{-1} μM^{-1})
ABTS# (70°C)	54	2	6507	120
ABTS# (30°C)	57	2	5327	93
Syringaldazine# (30°C)	19	1	2112	110

*The amount of Lac IId was held constant (0.0196 μg) in all the assays.
#Assay carried out at optimum pH.

The K_m, V_{max} and K_{cat} of Lac IId were compared using the substrates ABTS and syringaldazine; maximum specificity constant (K_{cat} / K_m) of 120 min^{-1} μM^{-1} was observed with ABTS at 70°C and pH 3 (Table 6).

Table 7: Oxidation of various substrates (1 mM) by Lac IId.

Substrates	% Relative Activity (SD)
ABTS	100 (27)
Ferulic acid	64 (9)
Guaiacol	53 (7)
Syringaldazine	42 (9)
L-ascorbic acid	33 (13)
N,N-dimethyl-*p*-phenylenediamine	21 (2)
Pyrocatechol	15 (3)
Indulin (0.25 %)	15 (7)
p-anisidine	0 (0)
2,5-dimethyl aniline	0 (0)
Violuric acid	0 (0)
*L (-) tyrosine	0 (0)
Vanillic acid	0 (0)

*L (-) Tyrosine is a substrate of tyrosinase and not laccase.

SD = Standard deviation

Among the substrates (Table 7), ABTS was found to be the most preferred substrate followed by ferulic acid and guaiacol. Tyrosine, vanillic acid, 2,5-dimethyl aniline, *p*-anisidine and violuric acid were not oxidized by Lac IId.

Table 8: Oxidation of various non-substrates (1 mM) by Lac IId, in presence of ABTS (1 mM) as mediator.

Non Substrates	% Relative Activity (SD)
Control (only ABTS)	100 (11)
p-anisidine	161 (12)
2,5-dimethyl aniline	132 (16)
Violuric acid	122 (16)
L (-) tyrosine	82 (7)
Vanillic acid	67 (8)

SD = Standard deviation

However in the presence of ABTS with the exception of vanillic acid and tyrosine, most of the compounds which previously did not get oxidized (Table 8) got oxidized.

Table 9: Oxidation of various known mediators (1 mM) by Lac IId, in presence of ABTS (1 mM) as substrate.

Mediators	% Relative Activity (SD)
Control (only ABTS)	100 (11)
Hydroquinone	160 (21)
3,5-dimethoxy-4-hydroxyacetophenone	128 (23)
Syringic acid	122 (10)
Veratryl alcohol (3,4-dimethoxy benzyl alcohol)	109 (13)
4-hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl	100 (15)
1-hydroxybenzotriazole	86 (11)

SD = Standard deviation

When ABTS (1 mM) was used as substrate, four of the known mediators; hydroquinone, 3,5-dimethoxy-4-hydroxyacetophenone, syringic acid and veratryl alcohol (Table 9) were effective in increasing the activity of laccase whereas 4-hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl and 1-hydroxybenzotriazole were not effective.

Table 10: Effect of inhibitors on Lac IId, in presence of ABTS (1 mM) as substrate.

Inhibitors	% Relative Activity (SD)
Control (only ABTS)	100 (11)
DTT	160 (7)
#4-hexyl resorcinol	117 (10)
Cysteine	115 (9)
EDTA	104 (4)
Cysteine (0.1 mM)	104 (7)
o-coumaric acid	101 (5)
DTT (10 mM)	80 (3)
8-hydroxyquinoline	77 (10)
4-nitro phenol	73 (7)
CTAB	69 (5)
Kojic acid	62 (3)
Tropolone	58 (9)
Sodium chloride (1M)	30 (4)
Potassium chloride (1M)	30 (6)
2-mercapto ethanol	23 (5)
SDS (1%)	23 (0)
Sodium azide (0.1 mM)	5 (3)

#4-hexyl resorcinol is an inhibitor of tyrosinase and not laccase.

Among the various inhibitors tested for Lac IId, sodium azide followed by SDS and mercaptoethanol inhibited its activity (Table 10). It was not inhibited by 4-hexyl resorcinol, a differential inhibitor of tyrosinase but not laccase. The enzyme was neither inhibited by L-cysteine nor DTT, which are known inhibitors of laccase.

Table 11: Effect of metal ions (1 mmol) on Lac IId, in presence of ABTS (1 mM) as substrate.

Metal ions	% Relative Activity (SD)
Control (only ABTS)	100 (11)
#NH_4^+	104 (9)
Mo^{+5}	103 (4)
Na^+	98 (5)
Ni^{+2}	97 (4)
Zn^{+2}	95 (1)
Cd^{+2}	95 (3)
Co^{+2}	94 (5)
Al^{+3}	94 (3)
Ca^{+2}	93 (2)
Cu^{+2}	93 (5)
Ba^{+2}	92 (2)
Mg^{+2}	90 (12)
As^{+3}	89 (7)
Pb^{+2}	84 (12)
Mn^{+2}	81 (6)
Li^{+2}	75 (3)
K^+	72 (2)
V^{+5}	72 (7)
Fe^{+3}	71 (2)
Hg^{+2}	68 (2)
Ag^{+2}	66 (15)
Sn^{+2}	63 (5)
W^{+6}	52 (1)
Cr^{+4}	44 (11)

#Is not a metal ion.

Activity of Lac IId was maximally inhibited by Cr and W by 56 and 48 % respectively, whereas in the presence of Sn, Ag and Hg the inhibition was only ~32 – 37 % (Table 11). The other metal ions did not show significant inhibition.

3.5 DISCUSSION

When comparing various enzyme concentration methods, it was realized that in vacuum concentration inhibiting substances present in seawater (Luo *et al.*, 2005) do not get removed but rather get concentrated. This combined with its low capacity and the long time taken to concentrate, deters one from using this as a method for concentration of laccase on a large scale. Concentration with PEG requires the use of dialysis bags, this method besides being expensive and having a low capacity also requires the pretreatment of the dialysis tubing. The process is also arduous and time consuming. Ammonium sulphate precipitation method also suffers from similar drawbacks, especially the post processing where large volumes of liquid require to be centrifuged and the salt also requires to be completely removed for further processing. Ultrafiltration by centrifugal pressure (Centricon) is relatively easy to use but its low capacity, expensive centrifugal tubes and the rapid blockage of its membranes which cannot be regenerated, hinders its use on a large scale. This however is ideal for laccase concentration during the chromatographic steps where very small volumes of relatively pure laccase fractions are generated and have too low a volume to be used by ultrafiltration with positive pressure (Amicon) concentration method.

It is clear that Amicon concentration was the most efficient among all the methods tested for the concentration of laccase, it also yielded maximum specific activity i.e. several small molecular mass extraneous proteins and inhibiting substances present in the medium were removed. For both Centricon and Amicon, the material used was regenerated cellulose with identical cutoff values yet Amicon was more efficient in concentrating laccase and the membrane although expensive, could also be regenerated several times over. The large capacity of the Amicon unit (can concentrate up to 350 mL at a time) and non-requirement of expensive chemicals, makes it the method of choice to concentrate laccase from the culture filtrate and was selected for all further work. The Amicon concentrated culture filtrate was the source of partially purified laccase for all further experiments.

When partially purified laccase was subjected to non- denaturing IEF (pH range of 4 – 7) and stained for activity, two very distinct and several indistinct laccase bands were observed. This suggested that several laccase pI isozymes (seen as fine bands

due to their relative lower concentration) to the tune of over 20, were present in the concentrated culture filtrate. Since it was a non-denaturing IEF, urea was not used; this to a certain extent affects the determination of the exact pI, since electro-endosmosis could not be avoided, nor the resolution (http://www.weihenstephan.de/blm/deg/manual/manfrm. htm). However, the non-denaturing IEF served its purpose in detecting the presence of several laccase isozymes.

Non-denaturing SDS PAGE confirmed that more than one molecular mass laccase isozymes were present. Since there were many more isozymes based on surface charge than molecular mass, a size exclusion followed by an anion exchange chromatographic step was carried out in an attempt to purify laccase.

The size exclusion chromatography corroborated the non-denaturing SDS PAGE results demonstrating the presence of more than one molecular mass laccase isozyme. At least four different isozymes based on surface charge were obtained for each molecular mass isozyme. These results are in concurrence with the non-denaturing IEF (Fig. 4), which shows numerous laccase isozymes based on pI.

Lac IId has 17 % *N*-linked glycosylation, the average range for glycosylation of laccase is between 10 % and 25 % (Baldrian, 2006). *N*-linked glycosylation, plays a structural role in laccase and to a certain extent protects it from extracellular proteases (Yoshitake *et al.*, 1993). This is essential if Lac IId or even the crude or partially purified laccase preparation is to be used in the wild / in-situ, for bioremediation purposes where provision for protection from proteases would be prohibitively expensive.

When *Cerrena unicolor* MTCC 5159 was grown in mod LN medium prepared in seawater, in presence of $CuSO_4$ the pathway was routed to laccase production with negligible quantities of the peroxidases, which are normally produced albeit in low quantities. This fungus was demonstrated to grow and produce 85,829 U L^{-1} laccase in high nitrogen medium containing peptone and yeast extract prepared with sea water in the presence of 1 % textile mill effluent (D'Souza *et al.*, 2006). However, a mineral medium like modified LN medium was used since it supported laccase production yet did not increase the load on the purification column. Modified LN medium was ideal for purification and characterization of laccase even though the overall laccase production was lower. It produced a moderately high laccase titer of ~23,700 U L^{-1} with a specific

activity of 330 U mg^{-1} protein. This is significant since laccase activity is normally inhibited in the presence of seawater (Luo *et al.*, 2005). This would signify that either the laccases produced are tolerant to the consortium of salts present in sea water or that the actual laccase titer was much higher than that which was estimated by the laccase assay, i.e. the laccase titer would have been actually underestimated. This may provide an explanation for the total laccase, (Table 1) in the culture filtrate was lower than that after ultrafiltration. The inhibiting salts present in the culture filtrate, due to its small size, get removed during the course of ultrafiltration. Thus in the absence of the inhibiting salts, the laccase activity estimated was much higher than in the original culture filtrate. This is in concordance with Lou *et al.*, (2005) who have experimentally proven that inhibition by sea water is reversible, once the inhibiting ions have been removed.

Another *Cerrena* strain; *Cerrena unicolor* strain 137 was reported to produce 18,700 U L^{-1} (Michniewicz *et al.*, 2006). Our strain produces a higher titer of laccase than this strain (Gianfreda *et al.*, 1998; Michniewicz *et al.*, 2006; Stepanova *et al.*, 2002) even in the presence of inhibiting salts of seawater. Isolates of *Pycnoporus* from various geographical regions of China were reported to produce lower titers of laccase (11,000 - 17,000 U L^{-1}) (Lomascolo *et al.*, 2002). Thus, *Cerrena unicolor* MTCC 5159 can be acknowledged as marine-derived laccase hyper-producing fungal strain.

For the yield experiment, EPS was not removed since its removal results in great loss of laccase activity due to the entrapment of laccase in the EPS matrix and the subsequent freezing and thawing steps required to remove it. Even though several molecular mass and surface charge laccase isozymes were present in the partially purified culture filtrate, a 33-fold purification with a final yield of 17 % of a single isoform (Lac IId) was achieved. In comparison, *C. unicolor* strain 137 which was reported to produce Lacc I and Lacc II, which together gave a total yield of 22 % (Michniewicz *et al.*, 2006), on purification.

Laccases have characteristic spectral properties. Lac IId exhibited a peak at 610 nm, typical for the T1 Cu responsible for the blue colour of the enzyme, a characteristic typical of blue laccases. It also exhibited a shoulder at 330 nm which suggests the presence of the T3 binuclear Cu pair, which forms a part of the tri-nuclear cluster T2 Cu.

Comparison of N-terminal sequence of Lac IId with other N-terminal sequences showed 70 - 85 % similarity with basidiomycetous laccases. Maximum similarity was observed with laccases from the white-rot basidiomycetes *Schizophyllum commune* and *Spongipellis* sp. Even though the N-terminal sequence of *Volvariella* did not have very high homology to the N-terminal of Lac IId it was still included in Table 3 since an internal peptide of a *Volvariella* laccase had maximum homology with an internal peptide of the Lac IId.

The internal peptide of 17 AAs, (generated by trypsin) from Lac IId demonstrated complete homology to an internal peptide (Q69FX1_9AGAR) of 'Laccase 2' of *Volvariella volvacea* which had a predicted molecular mass and pI of 59367 Da (~59 kDa) and 5.4, respectively. This was almost identical to the experimentally determined molecular mass and pI of Lac IId which was 59 kDa and 5.3 respectively, further endorsing the results.

The inability to identify more peptides may have been due to the HCCA matrix used instead of a matrix that is specific for glycoproteins such as 2, 5-dihydroxybenzoic acid (DHB), super DHB (sDHB), 2,4,6-trihydroxyacetophenone (THAP) or DHAP. It could also be due to the novelty of this laccase considering its marine source and that the current MALDI database is not comprehensive enough to account for it since not much work has been done for marine-derived laccases, in terms of sequence determination.

Laccase from *Pycnoporus sanguineus* (SCC 108) had an optimum temperature at only 55°C (Litthauer *et al.*, 2007). Whereas using ABTS as substrate, partially purified laccase had a bimodal pH optima at 3 and 6 and a temperature optima at 60°C, the bimodal pH profile indicates the presence of at least 2 laccase isozymes as observed in Fig. 4. For pure Lac IId, the pH and temperature optimum was at 3 and 70°C, respectively. This is consistent with data that pH optima for the oxidation of ABTS are generally lower than 4 (Baldrian, 2006).

Lac IId had pH optima at 6 when syringaldazine and guaiacol were used as substrate. This is in concordance with known higher pH optima values for phenolic compounds which range between 4 and 7 (Baldrian, 2006). The pH optima of laccases are highly dependant on the type of substrate. If the substrate is phenolic in nature, the pH profile is bell-shaped, which is formed by two opposing effects. The oxidation of phenols

depends on the redox potential difference between the phenolic compound and the T1 copper (Xu, 1996). The E° of a phenol decreases when pH increases due to the oxidative proton release. At a rate of $\Delta E / \Delta pH = 59$ mV at 25°C i.e. a pH change from 2.7 to 11 would result in a 490 mV decrease in E° of the phenol. However, over the same pH range, the E° decrease for the fungal laccases studied (*T. villosa*, *R. solani* and *M. thermophila*) were much smaller ($\leq$ 100 mV) (Xu, 1997). The enzyme activity at higher pH, decreases due to the binding of a hydroxide anion to the trinuclear coppers of laccase. This interrupts the internal electron transfer from T1 to trinuclear centre (Munoz *et al.*, 1997). Not only the rate of oxidation but also the reaction products may differ with varying pH since pH may affect abiotic follow-up reactions of primary radicals resulting from laccase catalysis. Laccases from *Rhizoctonia praticola* and *T. versicolor* catalyzed the formation of different products from syringic and vanillic acids at varying pH values but both enzymes generated the same products at a particular pH (Xu, 1997).

ABTS is electron-rich like phenolic compounds such as guaiacol or syringaldazine but is not phenolic in nature. Hence the oxidation potential of ABTS is not pH-dependent within the range 2 – 11 and proceeds in a single step (Terrón *et al.*, 2004). Hence we can conclude that Lac IId has optimum activity at pH 3 excluding interference from the substrate.

It is common for basidiomycete laccases to have optimum pH of activity in the acidic range and stability at neutral or alkaline pH (Xu *et al.*, 1996). In concordance, Lac IId was most stable at pH 9 where it showed no activity. Similar results were obtained with laccase of *Peniophora* sp. (Niku-Paavola *et al.*, 2004) and *C. unicolor* strain 137 (Michniewicz *et al.*, 2006). Lac IId retained 67 % of its activity at 4°C for over 35 days and 100 % activity when stored at -20°C for over 1 year. This makes it a potential commercial candidate.

Lac IId's half-life of 90 min at 70°C is much superior to other laccases such as, Lacc I from *C. unicolor* strain 137 which completely lost its activity after less than 10 min at 70°C and Lacc II which had a half life of only 10 min at 70°C (Michniewicz *et al.*, 2006). Laccase from *Trametes* was reported to have a half-life of 27 min at 75°C (Xiao *et al.*, 2003) while the laccase from an ascomycete, *Mauginiella* displayed a half-life of 3 min at the same temperature (Palonen *et al.*, 2003). Thermostable laccase from *Peniophora* sp. displayed a half-life of 15 min at 75°C (Niku-Paavola *et al.*, 2004),

whereas Jordaan and Leukes (2003) reported a fully active laccase at 60°C for 9 h from an unidentified basidiomycete. In terms of thermostabiltiy, Lac IId occupies a superior position.

The Q_{10} values were found to be >1 between 20° and 70°C in the pH range of 3 - 7 for Lac IId (Table 5), indicating its temperature dependency for activity in this pH and temperature range. In contrast, the Q_{10} values <1 for temperatures above 70°C suggest the negative effect of these temperatures on the enzyme activity. The Q_{10} value of above 1 between 20° – 70°C also lends support to the conclusion that Lac IId is a high-temperature active enzyme.

The energy of activation (E_{act}) for Lac IId between 60° and 70°C at its optimum pH (3) was 2.5 kJ mol^{-1}. This was lower than 3.9 kJ mol^{-1} of *Pleurotus florida* laccase (Das *et al.*, 2001) and 12.4 kJ mol^{-1} of *P. sajor-caju* laccase (Lo *et al.*, 2001) besides the temperature optima for these laccases were much lower at 50° and 45°C, respectively. The temperature profile of Lac IId was more of plateau for a wide range of temperature, indicating that its activity was not adversely affected by high temperature. This, along with the high turnover numbers for oxidation of ABTS by Lac IId at 70°C (K_{cat} and K_{cat} / K_m values) further attest its thermostable character.

Some low molecular weight compounds that can be oxidized to stable radicals by laccase, can act as redox mediators oxidizing other compounds that in principle are not substrates of laccase. In addition, to enable the oxidation of non-substrates e.g. the nonphenolic lignin moiety, the mediators can diffuse far away to sites that are inaccessible to the fungus of the enzyme itself. When ABTS was used as substrate, the activity of Lac IId increased with four of the known mediators (hydroquinone, 3, 5-dimethoxy-4-hydroxyacetophenone, syringic acid and veratryl alcohol), confirming their role as mediators. Lac IId was non-responsive in the presence of 4-hydroxy-2, 2, 6, 6-tetra-methylpiperidin-1-oxyl. The combination of 1-hydroxybenzotriazole and ABTS proved to be antagonistic resulting in an overall decrease in laccase activity.

Although ABTS does not occur naturally in nature, most laccases have great affinity for it as a substrate. Among the substrates tested with Lac IId, ABTS was found to be the best followed by ferulic acid and guaiacol. Whereas tyrosine, vanillic acid, 2, 5-dimethyl aniline, *p*-anisidine and violuric acid were not oxidized by Lac IId. Tyrosine

was used as a negative control, since a true laccase should be unable to catalyze the oxidation of tyrosine. Besides acting as a substrate, ABTS is also known to act as a mediator as well when another compound was used as substrate. In the presence of ABTS, most of the compounds got oxidized which, previously did not get oxidized (with the exception for vanillic acid and tyrosine). This indicates the mediating role of ABTS in the oxidation of these compounds when exposed to Lac IId.

Laccases can be inhibited when the inhibitor binds strongly to it stopping further catalysis of the reaction. This occurs when the Cu at the catalytic center is removed / chelated or by competing for O_2, which is the specific co-substrate of laccase. Laccases have been known to be inhibited by diethyl dithiocarbamate and thioglycolic acid probably due to their effect on copper at the catalytic centre of laccase and by several sulfhydryl compounds such as dithiothreitol, thioglycolic acid, cysteine and diethyldithiocarbamic acid (Baldrian, 2006). This is true for laccase from *P. cinnabarinus* which was totally inhibited by 1 mM L-cysteine and DTT (Eggert *et al.*, 1996a). However, Lac IId from *Cerrena unicolor* MTCC 5159 was neither inhibited in the presence of 0.1 and 1 mM L-cysteine nor with 1 mM DTT. Experiments with *T. versicolor* laccase showed that the inhibitory effect found with these compounds does not occur in the same way as inhibition by azide which decreases the oxygen consumption by laccase during the catalysis by competing for its essential co-substrate which is molecular oxygen (Johannes & Majcherczyk, 2000).

In presence of 4-hexyl resorcinol, Lac IId showed activity, this was because it is a known inhibitor of only tyrosinase and not laccase (Dawley & Flurkey, 1993). However, CTAB, a known inhibitor of laccase was able to inhibit Lac IId by only ~30 %. The broad range of substrates accepted by laccase as hydrogen donors, oxidation of syringaldazine in combination with the inability to oxidize tyrosine and ability to remain active in the presence of 4-hexyl-resorcinol, has been taken to be an indicator of laccase activity (Thurston, 1994). Unambiguous determination of laccase activity was best achieved by purification of the protein to electrophoretic homogeneity followed by determination of K_m with multiple substrates which include substrates such as syringaldazine and ABTS, for which laccase has a high affinity, and tyrosine for which laccase has no affinity (Edens *et al.*, 1999; Shin & Lee, 2000) as seen with Lac IId.

Heavy metals are reported to have a significant negative effect on the action of laccases in general (Baldrian, 2003), however Lac IId doesn't comply with this. Only chromium and tungsten were able to maximally inhibit it by 56 and 48 % respectively, whereas the other metals showed even lesser inhibition.

The wide spectrum of metal ions that Lac IId is resistant to, suggests that it is metal-tolerant and has a great bioremediation potential in heavy metal contaminated environments, even though a heavy metal like Cu is a well known inducer for laccase (Baldrian, 2003; Galhaup & Haltrich, 2001). For all the metals tested, chloride was present as the chloride salt of the metal. Thus, whether the inhibitory effect of the metal salt was due to the combined effect of the metal and the chloride or due to the metal alone, could be ascertained by testing the activity of Lac IId in the presence of ammonium chloride. Ammonium, not a heavy metal was used in the medium optimization studies for laccase production. Ammonium was used as its chloride salt. Here the effect of chloride could be tested independently, if any inhibition had to occur, it would have to be due to chloride alone. As seen in Table 11, chloride shows no inhibition, since its electro-negativity is lower than that of oxygen (co-substrate of laccase), it probably cannot competitively inhibit it. In addition, the amount of chloride tested was lower than which is present in seawater.

The Lac IId from *Cerrena unicolor* MTCC 5159 has all the characteristics of a typical blue laccase: (i) blue colour due to its absorbance at 610 nm, (ii) a shoulder at 330 nm which is representative of T3 binuclear copper pair, (iii) N-terminal amino acid sequence showing 70 – 85 % homology to other basidiomycete laccases, (iv) Internal peptide showing 100 % homology to *Volvariella volvacea* (basidiomycete) laccase, (v) its inability to oxidize tyrosine and high affinity for syringaldazine and ABTS and (vi) its non inhibition by 4-hexyl-resorcinol, a selective inhibitor of tyrosinase.

The tolerance of Lac IId to heavy metals, its high optimum temperature for activity and thermostability, as well as high laccase titer even in the presence of sea water, indicating its salt tolerance, makes the marine-derived *Cerrena unicolor* MTCC 5159 a suitable candidate for bioremediation.

Chapter 4

Optimization of Laccase production by Cerrena unicolor MTCC 5159

ABSTRACT

To maximize laccase production, *Cerrena unicolor* MTCC 5159 was subjected to medium optimization. For laccase production, it was found that carbon and inorganic nitrogen act antagonistically with each other. A combination of low inorganic nitrogen and high carbon levels, favour biomass and laccase production. However, organic nitrogen at much higher concentrations also supported good laccase production. Buffering action of citrate-phosphate buffer is required to decrease the temporal variation in laccase production. Inducible laccases are produced in the idiophase only after addition of an appropriate inducer. Known inducers such as $CuSO_4$ induced maximum laccase activity however comparable activity had been obtained with 1 % raw textile effluent B which is economically lucrative. A single addition of inducer effectively aids in laccase induction, this is especially effective if added after sufficient growth has occurred. It has been observed that for growth of the fungus, pure sodium chloride cannot replace seawater and that oxygenation enhances laccase production. Addition of a surfactant such as Tween 80 at 0.25 % after sufficient growth had occurred positively impacted both biomass and laccase production. However at higher Tween 80 concentrations, laccase specific activity decreased. The surface to volume ratio of the culture vessel employed is critical for biomass and laccase production.

4.1 INTRODUCTION

Various factors influence laccase production, they include nutrients such as carbon, nitrogen, microelements and inducers. They also include cultivation conditions such as stationary or shake conditions, surface to volume ratio, pH of medium, temperature, light and oxygenation (Wesenberg *et al.*, 2003). Xavier *et al.*, (2001) have shown that laccase hyper-production was not dependent on high biomass yields since laccases are generally produced during the idiophase, in fungi.

D'Souza-Ticlo *et al.*, (2006) have shown that *Cerrena unicolor* MTCC 5159 under stationary conditions along with aeration produce a high titer of lignin-degrading enzymes. Fungi prefer acidic pH for growth, generally between 4.5 and 6; laccases are produced in abundance in this range. It has been found that maximal laccase production occurs when cultures are incubated at approximately 30°C in the dark (Thurston, 1994). Although high concentrations of an easily assimilable carbon source such as glucose have a positive impact on biomass accumulation, it showed an inhibitory effect or at least a delay in laccase production (Eggert *et al.*, 1996a). High levels of easily assimilable carbon source favour constitutive production of laccase but repress its induction (Bollag & Leonowicz, 1984).

The general consensus is that a high carbon to nitrogen ratio is required for laccase production. Laccase production occurs during secondary metabolic phase of fungi and is often triggered by nitrogen depletion (Keyser *et al.*, 1978). However, it was found that some strains were non-responsive to varying nitrogen concentrations (Leatham & Kirk, 1983) whereas, Buswell *et al.* (1995) have not only shown that laccases could be produced earlier with high nitrogen concentrations but also that these changes did not affect the biomass yield in anyway. The type of nitrogen also affects the types of laccase isozymes induced (D'Souza-Ticlo *et al.*, 2006). Hence, the nutritional requirements of the fungal strain under investigation were studied in detail before initiating medium optimization. Determination of the contribution of a particular nutrient without interference from other nutrients present in the medium can be achieved using mathematical modeling.

Main effects and interactions of the factors that play fundamental roles in the medium optimization for a particular response such as biomass or enzyme production can

be understood by applying statistical methods. Although several methods are found in literature, only a few such as the Placket Burman design and response surface methods have been used in biotechnological processes (Mundra *et al.*, 2007; Soni *et al.*, 2007).

The medium components critical to either response requires to be first identified before the optimization of varying levels/concentrations of the components. This is done by screening a large number of medium components for their relative significance in affecting a particular response. Screening is generally done when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or nearly optimal responses. According to the classical method, screening procedure for each category of sources will consume a lot of time as it is done for one category at a time while keeping the other parameters/components constant. Experimental design techniques present a more logical alternative to the one-factor-at-a-time approach for medium improvement.

All possible process factors, expected to be crucial in experimental design are identified by consulting relevant literature. Once the list of factors has been made, the settings for each factor are determined. In a screening experiment, two settings corresponding to two different concentrations of the same medium component are chosen.

The screening design contains only a sub unit of all possible factor-setting combinations, resulting in fewer required combinations which is ultimately economical and time saving. The Placket-Burman design is a type of two-level screening design which allows for the study of 'n' number of factors with 'n+1' + '2' Trials (Plackett & Burman, 1946). Moreover, the design is orthogonal in nature, thereby the effects of each component worked out are pure in nature and are not confounded with interactions among components for further optimization.

In the Placket-Burman design to make the factors computationally equivalent, low and high factor settings corresponding to the low and high concentrations of a particular medium component are coded as -1 and +1, respectively. Each factor is tested equal number of times at its low and high settings. Because of this equal allocation within each factor, balance exists between each and every pair of factors throughout the design. Equal allocation and balance characterize all two-level designs and make statistically designed experiments complete and efficient from the stand point of resources used and information gained.

The screening method of Placket-Burman frees the researcher from keeping 'other factors' at constant levels. The large number of components considered for experimentation is varied simultaneously but at different levels (as per a chosen design and ranges selected). The effects of individual components as well as their significance are conveniently computed mathematically:

$$Y = \beta_0 + \sum \beta_i X_{i\,(i = 1,\ldots\ldots,\, k)}$$

Where Y is the estimated response; β_0 the linear intercept; $\beta_{i\text{-}k}$ are the linear regression coefficients; $X_{i\text{-}k}$ are the experimental variables (i.e. medium components)

The coefficients obtained from this equation, give information about the impact of each factor individually on the response measured. A number of tools may be used to help access the significance of each factor. These include *p* values, normal plots and paretto charts. The most common means of assessing significance is the '*p*' value.

The *p* value is the probability that the magnitude of a parameter estimate is due to random process variability. A low *p* value indicates a "real" or significant effect and provides a base line for determining the relative criticality of the variables tested with respect to the response required. Out of these, generally the most effective components with positive effects are selected for further study, while those showing large negative effects may be dropped for all further experiments. This however is left to the discretion of the investigator depending upon the response studied. Therefore, dual purpose is served with such a systematic approach.

Once the critical factors have been identified via screening, one can then proceed to the optimization stage of experimental design. Information from screening experiments must first be used to fix certain factor settings and possibly revise the design space of the critical factors. All non-critical factors i.e. those that will not be tested further, must be fixed at a constant setting. Typically, the investigator will need to select either middle level of the factor setting range or level at which the factor provides the best response in light of individual effects and interactions observed earlier.

After the critical components have been identified via screening, their concentrations are required to be optimized to achieve maximum or minimum response as per the requirement. One way to achieve this is via a statistically designed optimized

study such as the Central Composite Design (CCD). This also confirms or rejects previous effects and interactions obtained from the screening experiment. The design can consist of two to five variables (Myers & Carter, 1973).

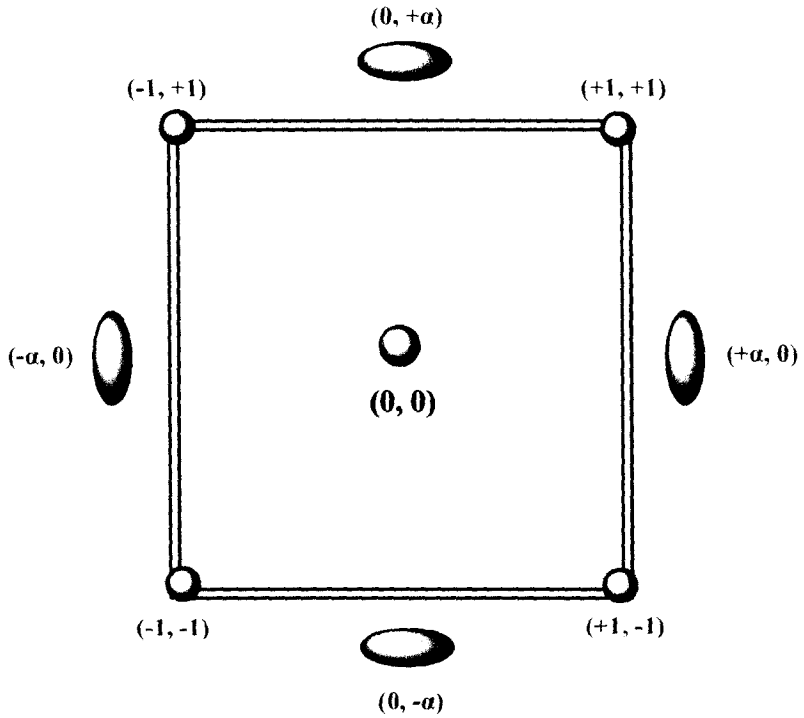

Fig. 1: Central Composite Design with two variables

In a Two Variable Composite design, variables can be presented as the two axis of a square (X_1, X_2). One end of the axis represents the high level and the other as the low level of the variable. Further, the center of the axis is presented as central level of variables according to the Central Composite design (Fig. 1). Complete randomization can be achieved by assuming all the levels as equally important and the central value as the optimum for maximum or minimum of response, as per the two experimental set-ups. Boundary limits of the design are considered as two levels. This requires a minimum of 12 experiments described as four corner points (high-low level coordination), 4 face / star points (boundary limits) and one center point (optimum level) of the square to achieve the goal of regression and estimation of error within experiments, the center point has to be repeated. The experimental data is then processed to fit the following second order polynomial equation: $Y = \beta_0 + (\beta_1 * A) + (\beta_2 * B) + (\beta_{11} * A^2) + (\beta_{22} * B^2) + (\beta_{12} * A * B)$

Where A and B are the experimental variables, β_0 is the constant coefficient, β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients whereas β_{12} is second order interaction coefficient.

Validation of the model developed is then carried out by comparison between the experimental and calculated values of response observed. If the calculated values fall close to the experimental value, the model is said to be fitted well. To validate a model, the lack of fit should be less than 50 % of the experiments.

After determining the critical nutrients which affect laccase production through screening experiments such as the Placket Burman design and then optimizing their concentrations for the particular fungal strain being investigated, laccase production can be further enhanced by the inclusion of inducers. Many of these compounds resemble lignin molecules or other phenolic chemicals (Farnet *et al.*, 1999). They include aromatic compounds such as xylidine, ferulic acid, guaiacol etc and a heavy metal such as copper (D'Souza *et al.*, 2006). These inducers activate response element sites in the promoter regions of laccase genes (Faraco *et al.*, 2003) and are known to increase the laccase titer ~400 times over the non-induced cultures (D'Souza *et al.*, 2006). These compounds can also affect the metabolism or growth rate hence indirectly, laccase production. White-rot fungi are very diverse in their response to laccase inducers (Eggert *et al.*, 1996a) and a large number of inducers have to be screened for their laccase-inducing ability with a particular fungus.

4.2 OBJECTIVES

The aim of this study was to determine the medium components critical to laccase production, optimize their concentrations and cultivation conditions that support high laccase yields from the selected hyper-secretory fungus, *Cerrena unicolor* MTCC 5159. The selected strain was evaluated for laccase production using different media, inducers and incubation conditions to support laccase production. Initial work was focused on the isolation, screening and identification of a laccase-producing marine-derived fungus (Chapter 2). Confirmation of the choice of hyper-secretory fungal strain was based on characteristics of the laccase produced by the selected hyper-secretory strain (Chapter 3).

4.3 MATERIALS AND METHODS

4.3.1. MICROORGANISM, CULTURE CONDITIONS AND RESPONSES

Cerrena unicolor MTCC 5159 was raised for 6 days in a medium identical to the one used in the subsequent experiment. Under sterile conditions, it was rinsed with DW then homogenized for 5 sec. The mycelial suspension was used as inoculum at 5 % v/v. The cultures were raised in 250 mL Erlenmeyer flasks with 20 mL total volume of medium i.e. surface to volume ratio of 2.5 and incubated at 30°C in the dark under static conditions. Various responses in terms of biomass accumulation, laccase activity, protein concentration and specific activity were determined, details of which are given in Appendix 8.2.1.5, 8.2.2.1.1, 8.2.1.4 and 8.2.3.4 respectively. In all experiments, the biomass obtained on any particular day had been subtracted from the initial biomass used as inoculum.

4.3.2 Effect of synthetic and organic media

Three types of media were employed viz. a synthetic medium such as low nitrogen (LN) medium, an organic low nitrogen medium such as Boyd and Kohlmeyer (B&K) medium and a nutritionally rich high nitrogen organic medium such as Malt extract broth (MEB). The composition for LN, B&K and MEB are given in Appendix 8.1.1 - 8.1.3 respectively. Laccase and its specific activity were determined every 3 days after inoculation until day 21.

4.3.3 LACCASE PRODUCTION MEDIUM

The laccase production medium always contained a mineral salts basal medium consisting of 5 % (v/v) basal salts solution (Appendix 8.1.1.1), 10 % (v/v) trace elements solution (Appendix 8.1.1.2) and 1 mL of 0.005 % (w/v) thiamine. The pH was adjusted to 4.5 using citrate-phosphate buffer and the final volume was made using distilled water. To this basal medium, glucose, ammonium chloride, copper sulphate, Tween 80 and sodium chloride were added at varying concentrations as per the requirement of the experiment.

4.3.4 Effect of glucose and citrate

To determine the type of interaction between the designated carbon source, glucose and citrate from the citrate-phosphate buffer since both contain assimilable carbon; three sets, G, C and B were prepared. In all sets, 0.2044 % ammonium chloride, 0.1 % NaCl, 0.05 % Tween 80 and 0.0375 M $CuSO_4$ were added to the mineral salts base. In addition to the above medium, in set 'G' only glucose at 4.5 %, in 'C' only citrate at 0.2 % and in 'B' both glucose and citrate at 4.5 and 0.2 % respectively were present.

4.3.5 Screening of significant components using Plackett Burman design

Screening of medium components was carried out using the Plackett-Burman design to determine their effect on biomass and laccase production. The five components used included glucose (carbon), ammonium chloride (nitrogen), $CuSO_4$ (inducer), Tween 80 (surfactant) and sodium chloride (salt). Low and high settings of each of these components were used to prepare a combination of eight Trials of production medium, whose compositions are specified in Table 1. Inoculation was carried out using 5 % (v/v) of the inoculum and the day of inoculation was considered as day 0. An additional set of experiment was carried out with a Cu spike (0.375 mM) on day 6. Both sets of experiments were monitored every 3 days from the day 0 to 15.

Table 1: Distribution of the low and high setting for five variables with the coded and uncoded values in eight experimental Trials of the Plackett-Burman design.

Trial	Variables									
	Coded					Uncoded				
	Glucose (%)	NH_4Cl (%)	$CuSO_4$ (mM)	Tween 80 (%)	NaCl (%)	Glucose (%)	NH_4Cl (%)	$CuSO_4$ (mM)	Tween 80 (%)	NaCl (%)
A	-	-	-	-	+	0.9	0.2044	0.0375	0.05	1
B	-	-	+	+	-	0.9	0.2044	0.375	0.25	0.1
C	-	+	-	+	-	0.9	1.022	0.0375	0.25	0.1
D	-	+	+	-	+	0.9	1.022	0.375	0.05	1
E	+	-	-	+	+	4.5	0.2044	0.0375	0.25	1
F	+	-	+	-	-	4.5	0.2044	0.375	0.05	0.1
G	+	+	-	-	-	4.5	1.022	0.0375	0.05	0.1
H	+	+	+	+	+	4.5	1.022	0.375	0.25	1

4.3.6 Optimization of medium components using Central Composite design

Glucose and ammonium chloride were used for medium optimization. The concentrations of glucose and ammonium chloride corresponding to the various coded levels including details elucidating the experimental design are given in Table 2.

Table 2: Central Composite design matrix for the experimental design with the coded and uncoded values chosen for the experimental variables.

Trial	Variables			
	Coded		Uncoded	
	Glucose (%)	NH_4Cl (%)	Glucose (%)	NH_4Cl (%)
1	-1	-1	1.25	0.25
2	-1	+1	1.25	0.75
3	+1	-1	3.75	0.25
4	+1	+1	3.75	0.75
5	0	0	2.5	0.5
6	- α	0	0.7	0.5
7	+ α	0	4.3	0.5
8	0	- α	2.5	0.14
9	0	+ α	2.5	0.86
10	0	0	2.5	0.5
11	0	0	2.5	0.5
12	0	0	2.5	0.5

Each Trial contains different combinations of glucose and ammonium chloride. Copper sulphate at 0.375 mM, was added on day 6. Tween 80 was excluded from the medium. Laccase and biomass production were determined on day 12.

4.3.7 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Screening of the significance of medium components for biomass and laccase production was carried out by the Plackett Burman design. Table 1 represents the low and high

settings for the five components under testing. A linear approach is considered sufficient for screening for the effect of these components.

$$Y = \beta_0 + \sum \beta_i X_i (i = 1, \ldots\ldots, k)$$

Where Y is the estimated biomass or laccase production and β_i is the regression coefficient.

The Central Composite design (CCD) was used, to study the interactions between glucose and ammonium chloride as medium components. The design of the experiment along with the coded and uncoded variables for the two experimental factors presented in Table 2 is based on the results obtained from the screening experiment (Table 3A & B and Table 4A &B). The predicted and experimental values for laccase and biomass production obtained from the CCD experiment are given in Table 5. Various measurements for each experimental set were carried out in triplicates. Each experimental set was carried out in duplicate. The yields reported are the mean of the duplicates. The relationship of the independent variables (A & B); and their response (Y) estimated biomass or laccase production, were calculated by the second order polynomial equation, given below:

$$Y = \beta_0 + (\beta_1 * A) + (\beta_2 * B) + (\beta_{11} * A^2) + (\beta_{22} * B^2) + (\beta_{12} * A * B)$$

Where β_0 is the constant coefficient, β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients whereas β_{12} is second order interaction coefficient.

The statistical analysis using multiple regressions and MANOVA were performed with Statistica v 6.0 (StatSoft, Tulsa, Okla) software package.

4.3.8 Impact of addition of $CuSO_4$ and Tween 80

Production medium with 3.75 % glucose, 0.25 % ammonium chloride and 0.1 % NaCl was employed. To this, 0.375 mM $CuSO_4$ was added along with varying Tween 80 concentrations (0 % - 0.5 %) on day 6. Laccase and biomass production were estimated on day 12.

4.3.9 Effect of surface to volume ratio

Metabolite production is known to be enhanced under stationary conditions where the fungal mycelium is in maximum contact with the atmospheric surface above the medium. To test this, the following experiment was performed. The production medium containing 3 % glucose and 0.1 % NaCl with either 0.1 or 0.2 % ammonium chloride were tested along with varying flask capacities. The flask sizes ranged from 100 to 2000 mL capacity Erlenmeyer flasks. The volume of the production medium was maintained at $1/5^{th}$ flask capacity. On day 6, 0.375 mM $CuSO_4$ was added along with 0.25 % Tween 80 and laccase and biomass production were measured on day 12.

4.3.10 Effect of oxygenation

The cultures were raised in 2000 mL capacity Erlenmeyer flasks in production medium containing 3 % glucose, 0.1 % ammonium chloride and 0.1 % NaCl at $1/5^{th}$ flask capacity. Two identical experimental sets were maintained. In one set, the flasks (in triplicates) were oxygenated under sterile conditions with pure oxygen for 1 min using tygon tubing and a Pasteur pipette. Oxygenation was carried out every 3 days including day 0 until the measurement of response. The other set was not oxygenated. On day 6, in both the sets, 0.375 mM $CuSO_4$ was added along with 0.25 % Tween 80. Laccase and biomass production were measured on day 12 for both the sets.

4.3.11 Effect of inducers

In order to enhance laccase production, various known inducers at 1 mM such as *p*-anisidine, catechol, guaiacol, ferulic acid, vanillic acid, veratryl alcohol and 2,5 dimethyl aniline were used. A model lignin compound such as indulin SS at 0.25 mg mL^{-1}, 2 mM $CuSO_4$ and a combination of 2 mM $CuSO_4$ and 1 mM guaiacol were also used. Besides the known inducers, industrial effluents such as textile effluents A and B (TeA and TeB), molasses spent wash (MSW) and black liquor (BL) were also screened for laccase-inducing properties at 1 % final concentration. The details of TeA, TeB, MSW and BL

are given in Appendix 8.4.1 - 8.4.4 respectively. The inducers were added on day 3 to B & K medium prepared in 25 ppt seawater.

4.3.12 Effect of various carbon sources

Various carbon sources at 1 % final concentration were tested for laccase production using a synthetic LN medium (Appendix 8.1.1) prepared in distilled water, without inclusion of an inducer. The carbon sources included monosaccharide hexoses such as glucose, fructose, galactose and rhamnose; monosaccharide pentoses such as ribose, arabinose and xylose; disaccharides such as trehalose, maltose, cellobiose, sucrose, lactose and melibiose; trisaccharides such as raffinose; sugar-alcohols such as inositol, mannitol, sorbitol and glycerol and the polysaccharide, starch. Glucosamine although containing nitrogen was also included as a carbon source. Laccase was estimated on day 12. The mean values from triplicate experiments were used for comparing the effect of various inducers.

4.4 RESULTS

Low nitrogen (LN) medium is a completely synthetic medium whereas B&K and MEB are complex organic media. The effect of synthetic and complex media on laccase production was determined (Fig. 2A).

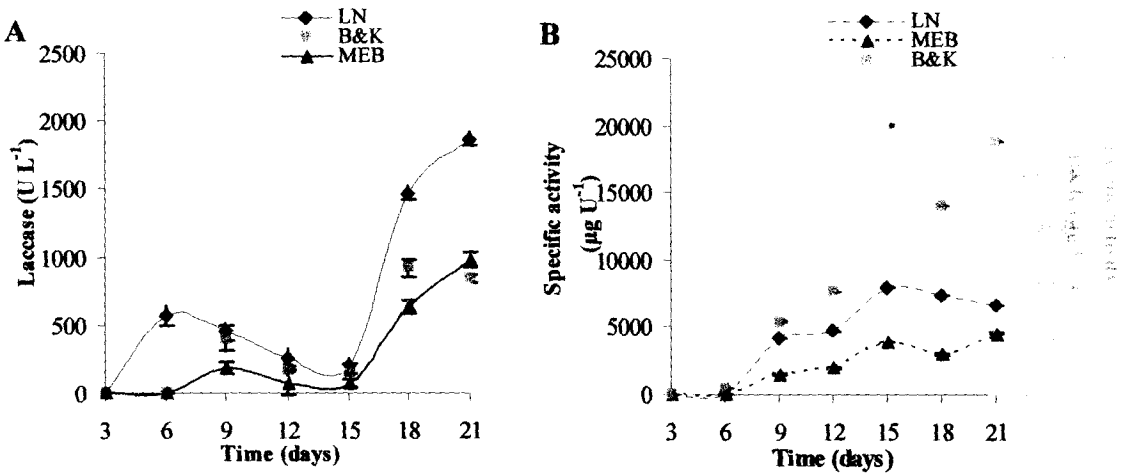

Fig. 2: Effect of medium types on (A) Laccase production and (B) Laccase specific activity.

The percent total nitrogen in LN, MEB and B&K are 0.0933 %, 3.256 % and 4.22 % respectively from calculations and carbon-nitrogen-sulfur (CNS) analysis. For all the sampling days, LN supported better laccase production than either B&K or MEB. However, maximum laccase specific activity was observed in B&K followed by LN and then MEB (Fig. 2B).

Low nitrogen medium was chosen to determine the effect of individual medium components on laccase production since it was a synthetic medium and supported maximum laccase production. This was performed using the Placket Burman design, a statistical screening method that requires the medium components (in this case) to be independent of each other in affecting laccase production.

However, the designated carbon source, glucose and the buffer, citrate-phosphate, both contain carbon. Hence it was required to determine whether citrate could

be used as a buffer. Figures 3A and 3B show that both glucose and citrate served as independent sources of carbon for both laccase and biomass production.

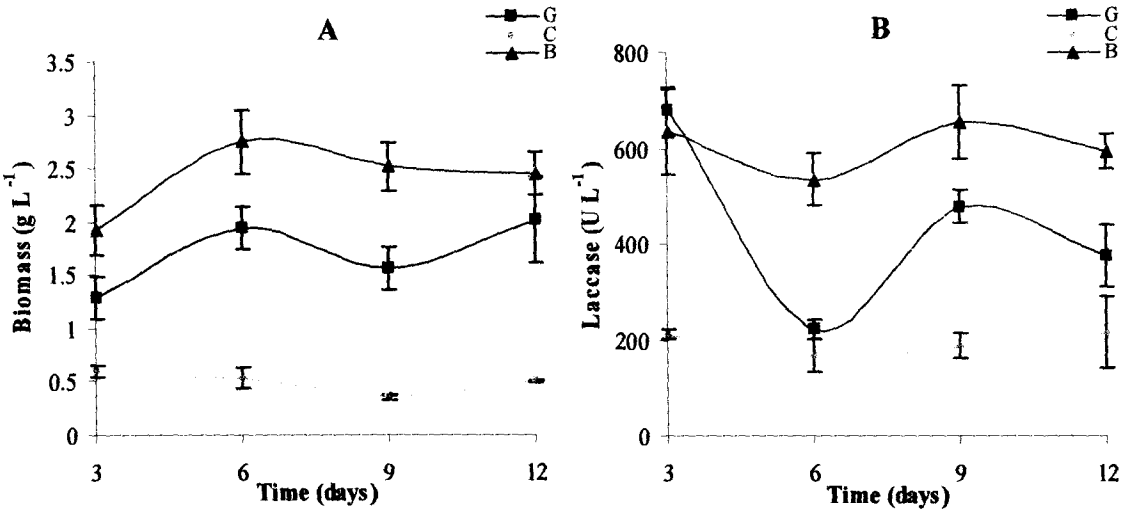

Fig. 3: Effect of glucose and citrate on production of (A) Biomass and (B) Laccase.

Here G = glucose only; C = citrate only and B = glucose and citrate both.

When only citrate was used, maximum laccase and biomass values were attained by day 3 and a low but stable biomass and laccase production was observed throughout. Whereas when only glucose was present, there was a large temporal variation in laccase production. When both glucose and citrate were present together, the effect was additive on biomass whereas for laccase besides an improvement in titer, temporal variation also decreased. No adverse interaction, between glucose and citrate for either laccase or biomass production was found. Thus, citrate-phosphate was used in the medium as a buffer and maintained at a constant level in all further experiments.

The screening of medium components for their significance was carried out using the Plackett Burman design. The experimental values of the Plackett-Burman sets determined at various sampling intervals for laccase and biomass are given in Table 3A. The effect on production of biomass and laccase due to a spike of 0.375 mM $CuSO_4$ on day 6 is presented in Table 3B.

Table 3A: Laccase and biomass production at 3-day sampling intervals, based on the eight Trials of the Plackett-Burman design.

Trial	Day 3		Day 6		Day 9		Day 12		Day 15	
	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)
A	0.96	807	1.21	901	1.30	888	1.08	48	1.70	3
B	0.48	17	2.59	2926	3.37	2358	3.36	1393	2.44	760
C	0.57	809	1.00	1651	1.25	792	1.32	3	1.55	220
D	0.38	8	0.51	33	0.62	908	0.34	3	1.15	84
E	0.89	6	1.85	1122	2.60	805	2.52	1070	2.12	156
F	1.00	10	2.52	1815	3.68	1744	3.47	1787	2.56	662
G	1.08	802	0.93	541	1.61	178	1.49	178	1.80	122
H	0.30	3	1.17	253	1.79	825	1.75	624	1.72	434

The medium composition of Trials A to H, are explained in Table 1. Here $CuSO_4$ was added only at the time of inoculation (day 0). This set of eight Trials is collectively referred to as the '*unspiked*' set. Here laccase and biomass were measured every 3 days from day 3 to 15.

Table 3B: Laccase and biomass production at 3-day sampling intervals, based on the eight Trials of the Plackett-Burman design after the $CuSO_4$ spike on day 6.

Trial	Day 9		Day 12		Day 15	
	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)	Biomass ($g L^{-1}$)	Laccase ($U L^{-1}$)
A'	0.93	502	1.21	254	1.50	145
B'	3.62	320	3.12	74	2.41	67
C'	1.08	288	1.10	82	1.24	72
D'	0.11	301	0.36	88	0.76	73
E'	2.44	302	2.26	62	2.09	65
F'	3.64	110	3.51	91	2.62	71
G'	1.15	100	1.62	102	1.55	82
H'	1.58	67	1.43	54	1.37	45

The medium composition of Trials A' - H' is identical to Trials A - H except besides day 0, 0.375 mM $CuSO_4$ was also to added each of the Trials on day 6. This set of eight Trials is collectively referred to as the '*spiked*' set. Here laccase and biomass were measured every 3 days from day 9 to 15.

In majority of the cases, Trial B showed greater laccase titer than other Trials (Table 3A & 3B).

Table 4A: Degree of positive and negative effects of medium components on biomass production according to the Plackett-Burman design.

Variables	Sampling Interval (Days)														
	3			6			9			12			15		
	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level
Intercept	*1.250*	*0.016*	*0.0000*	*1.916*	*0.016*	*0.0000*	*2.310*	*0.060*	*0.0000*	*2.107*	*0.092*	*0.0000*	*2.142*	*0.152*	*0.0000*
Glucose	0.061	0.003	0.0000	0.081	0.003	0.0000	0.217	0.010	0.0000	0.217	0.016	0.0000	0.095	0.026	0.0043
NH_4Cl	-0.305	0.012	0.0000	-1.393	0.012	0.0000	-1.739	0.045	0.0000	-1.692	0.069	0.0000	-0.794	0.113	0.0000
$CuSO_4$	-0.994	0.029	0.0000	1.327	0.030	0.0000	1.999	0.108	0.0000	1.854	0.167	0.0000	0.525	0.275	0.0849
Tween 80	-1.469	0.048	0.0000	1.798	0.050	0.0000	2.242	0.182	0.0000	3.213	0.282	0.0000	0.780	0.464	0.1235
NaCl	-0.172	0.011	0.0000	-0.639	0.011	0.0000	-1.002	0.041	0.0000	-1.096	0.063	0.0000	-0.465	0.103	0.0011
	Adjusted r^2 = 0.9958			Adjusted r^2 = 0.999			Adjusted r^2 = 0.9952			Adjusted r^2 = 0.9890			Adjusted r^2 = 0.84900		
	S.E.E.: 0.0193			S.E.E.: 0.020			S.E.E.: 0.0729			S.E.E.: 0.1129			S.E.E.: 0.18545		

Variables	Sampling Interval (Days)								
	9			12			15		
	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level
Intercept	*2.126*	*0.092*	*0.0000*	*2.364*	*0.160*	*0.0000*	*2.141*	*0.1604*	*0.0000*
Glucose	0.213	0.016	0.0000	0.211	0.027	0.0000	0.119	0.0272	0.0014
NH_4Cl	-2.053	0.069	0.0000	-1.710	0.120	0.0000	-1.130	0.1197	0.0000
$CuSO_4$	2.481	0.167	0.0000	1.653	0.290	0.0002	0.578	0.2900	0.0741
Tween 80	3.605	0.282	0.0000	1.502	0.489	0.0118	0.840	0.4894	0.1170
NaCl	-1.231	0.063	0.0000	-1.137	0.109	0.0000	-0.583	0.1088	0.0003
	Adjusted r^2 = 0.9919			Adjusted r^2 = 0.96483			Adjusted r^2 = 0.90251		
	S.E.E.: 0.1129			S.E.E.: 0.19546			S.E.E.: 0.19576		

A spike of 0.375 mM $CuSO_4$ was introduced on day 6.

Main effects (B) = biomass production (g L^{-1}); Std. Err. Of B = standard error of B for uncoded values; *p*-level = 0.05 % and S.E.E = Standard Error of Estimate.

Table 4B: Degree of positive and negative effects of medium components on laccase production according to the Plackett-Burman design.

Variables	Sampling Interval (Days)														
	3			6			9			12			15		
	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level
Intercept	952	146	0.000	2375	30	0.000	1471	26	0.000	540	86	0.000	198	37	*0.000*
Glucose	-57	25	0.044	-124	5	0.000	-97	4	0.000	154	15	0.000	21	6	0.007
NH_4Cl	239	109	0.053	-1310	23	0.000	-946	19	0.000	-1067	64	0.000	-221	28	0.000
$CuSO_4$	-1768	264	0.000	602	55	0.000	2350	46	0.000	1858	155	0.000	1067	67	0.000
Tween 80	-991	446	0.051	3329	92	0.000	1329	78	0.000	1343	262	0.000	873	113	0.000
NaCl	-226	99	0.046	-1284	20	0.000	-457	17	0.000	-449	58	0.000	-302	25	0.000
	Adjusted $r^2 = 0.7997$			Adjusted $r^2 = 0.998$			Adjusted $r^2 = 0.998$			Adjusted $r^2 = 0.9761$			Adjusted $r^2 = 0.9725$		
	S.E.E.: 178.5200			S.E.E.: 36.884			S.E.E.: 31.245			S.E.E.: 104.7401			S.E.E.: 45.1227		

Variables	Sampling Interval (Days)								
	A spike of 0.375 mM $CuSO_4$ was introduced on day 6.	9			12			15	
		B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level
Intercept		507	9.5	0.000	228	29	0.000	143	10	0.000
Glucose		-58	1.6	0.000	-13	5	0.024	-7	2	0.003
NH_4Cl		-146	7.1	0.000	-47	22	0.054	-23	7	0.010
$CuSO_4$		-292	17.1	0.000	-143	53	0.022	-80	18	0.001
Tween 80		-45	28.9	0.151	-329	89	0.004	-153	30	0.000
NaCl		98	6.4	0.000	30	20	0.155	10	7	0.161
		Adjusted $r^2 = 0.9956$			Adjusted $r^2 = 0.66959$			Adjusted $r^2 = 0.82375$		
		S.E.E.: 11.5611			S.E.E.: 35.46043			S.E.E.: 11.88795		

Main effects (B) = laccase production (U L^{-1}); Std. Err. Of B = standard error of B for uncoded values; *p*-level = 0.05 % and S.E.E = Standard Error of Estimate.

Glucose has a positive impact on biomass production throughout. With the exception day 3, $CuSO_4$ and Tween 80 also had a positive impact on biomass however NH_4Cl and NaCl had a large negative impact throughout (Table 4A).

From the $CuSO_4$-spiking experiment in majority of the cases, it was observed that $CuSO_4$-spiking increased the magnitude of impact that the medium components had on biomass production. However, the deviation between the spiked and the unspiked set was not very large.

For laccase production, most of the medium components had a negative impact on day 3. The negative impact of glucose was reversed by day 12. Except for day 3, the impact of NH_4Cl was negative throughout and its maximum negative impact coincided with maximum laccase production on day 6 (Table 4A). When higher amounts of glucose and NH_4Cl were present simultaneously, laccase production was delayed. After a very high negative impact on day 3, the impact of $CuSO_4$ turned positive and remained considerably high for the remaining period. Tween 80 followed a similar trend to $CuSO_4$ but with a higher magnitude of positive impact and its maximum positive impact also coincided with maximum laccase production on day 6. Sodium chloride had a negative impact on laccase production and on day 6 its negative impact increased 6 fold which coincided with maximum laccase production.

The $CuSO_4$ spike on day 6 did not yield the expected spurt in laccase production. In fact, the laccase titer levels had drastically decreased (Table 3B) in such a way that the additional $CuSO_4$ spiking overshadowed the impact of all other medium components. From Table 4B, it is observed that sodium chloride had a negative impact on laccase production but $CuSO_4$ spiking resulted in a reversal of this impact to positive. From Table 3A it is evident that laccase production is at its maximum, 6 days after the addition of $CuSO_4$. For all further experiments, a single addition of 0.375 mM $CuSO_4$ on day 6 was carried out and laccase and biomass production was recorded 6 days after that i.e. on day 12. Since NaCl had a negative impact on both laccase and biomass production and was thus maintained at a constant low level.

Glucose and NH_4Cl as carbon and nitrogen source are essential for biomass accumulation yet both these components if present together at higher concentrations, negatively affected biomass and laccase. The concentration of both components required further optimization. The Central Composite design (CCD) was employed to achieve this.

The experimental values of the CCD are given in Table 5. These values were fitted in a second order polynomial equation. The values of regression coefficients were calculated and the fitted equations (using uncoded values) for predicting biomass (Y_B) and laccase (Y_L) production were;

$$Y_B = 1.575 + (0.5211*A) + (-0.1586*B) + (-0.05917*A*A) + (0.1224* B*B) + (-0.2988*A*B)$$

$$Y_L = 115 + (276*A) + (-364*B) + (10*A*A) + (1358* B*B) + (-634*A*B)$$

Where A, is glucose concentration and B, is NH_4Cl concentration.

Predicted values were generated using the above equation are also presented in Table 5. Irrespective of whether biomass or laccase was concerned, glucose and NH_4Cl interacted antagonistically with each other. Further, for biomass, the linear coefficient of glucose was strongly positive whereas its quadratic coefficient was slightly negative. On the other hand, the interaction of glucose with NH_4Cl was negative. The quadratic coefficient of NH_4Cl for biomass, indicates that biomass accumulation is not very dependant on NH_4Cl concentration and only low concentrations of NH_4Cl appeared favourable for laccase production.

Table 5: Central Composite design matrix for the experimental design with predicted and experimental results for biomass and laccase production.

Trial	Experimental values		Predicted values	
	Biomass ($g\ L^{-1}$)	Laccase ($U\ L^{-1}$)	Biomass ($g\ L^{-1}$)	Laccase ($U\ L^{-1}$)
1	2.0	374	2.01	271
2	1.56	254	1.80	372
3	2.62	917	2.38	690
4	2.24	135	1.81	-2
5	1.93	282	2.09	233
6	1.87	156	1.76	249
7	2.43	153	2.03	281
8	2.26	416	2.38	621
9	1.79	325	1.82	196
10	2.0	271	2.09	233
11	1.9	286	2.09	233
12	1.89	243	2.09	233

A surface plot for both biomass and laccase production elucidating the interaction between glucose and NH_4Cl was plotted using the above equations (Fig. 4A & B). For laccase production (Fig. 4B), glucose and NH_4Cl act antagonistically with each other, hence the inverted bell shaped curve and the low r^2 values.

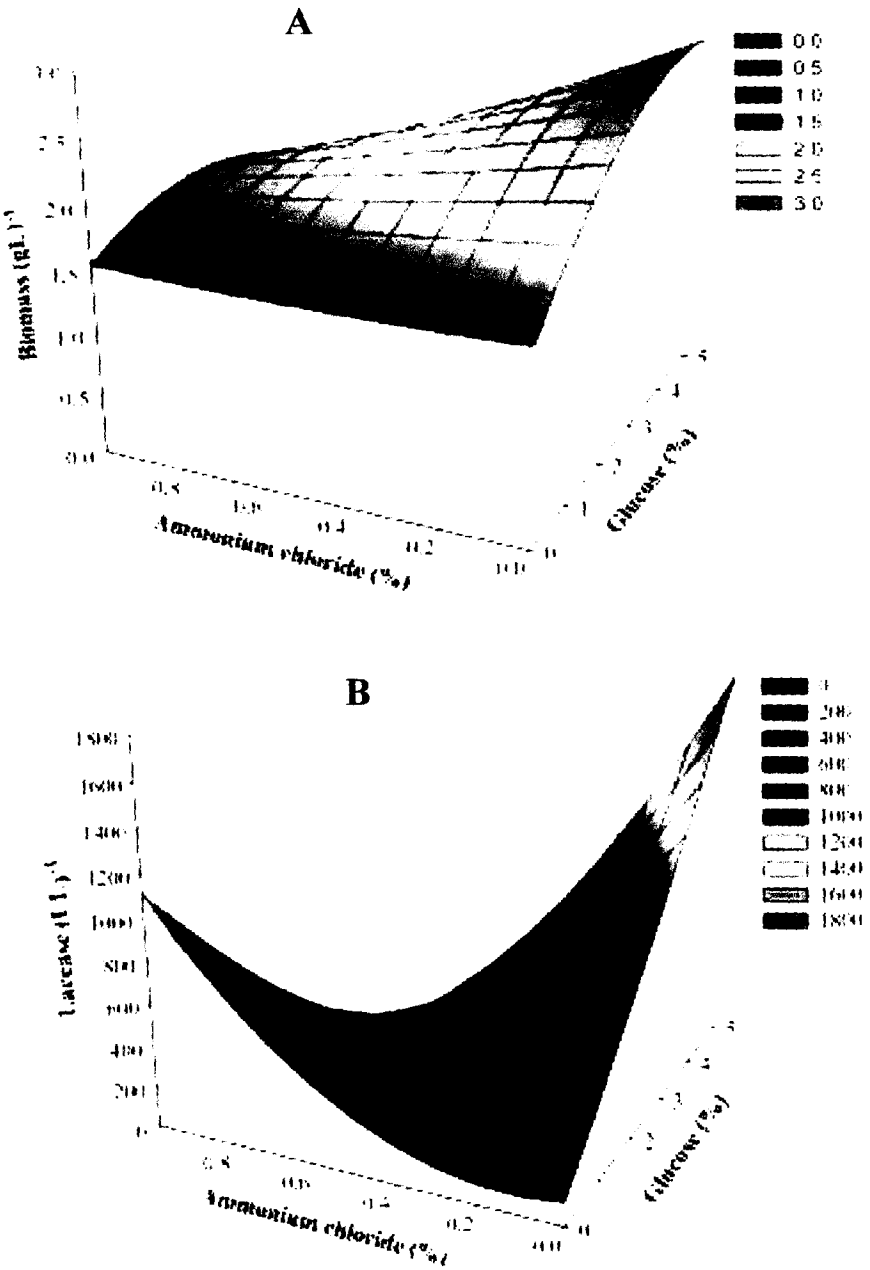

Fig. 4: Surface plots of production of (A) Biomass and (B) Laccase, illustrating the interaction between glucose and ammonium chloride.

A medium containing either a combination of high NH_4Cl and low glucose or a combination of low NH_4Cl but high glucose concentration would support greater laccase production. However only high glucose favours biomass build up which is required for laccase production. Hence a medium containing high glucose and low NH_4Cl concentration would support both biomass and laccase production. From these results, it appears that for laccase production, the concentration of NH_4Cl is more critical than glucose and merits further investigation.

Tween 80 was omitted from the experimental set up in CCD as its concentration was determined independently (Fig. 5).

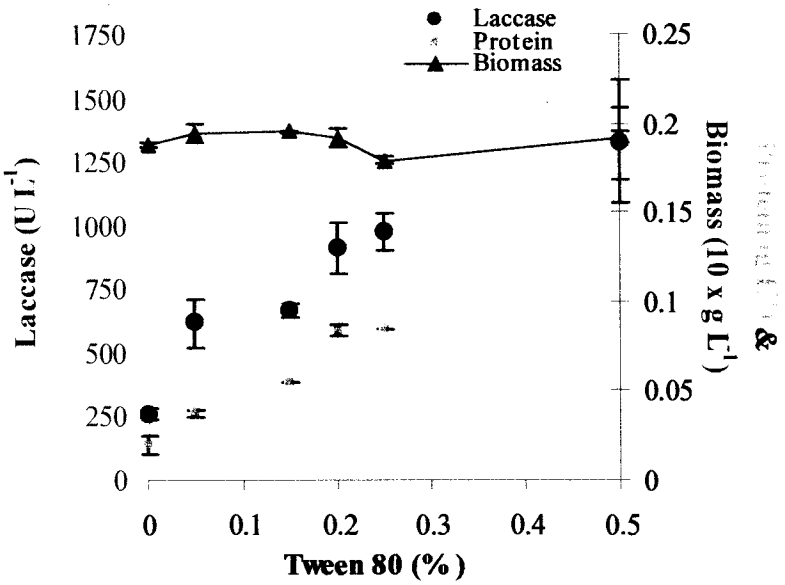

Fig. 5: Effect of varying concentrations of Tween 80 on laccase, protein and biomass production.

Increasing Tween 80 concentrations did not significantly affect biomass production. However, both laccase and protein production were roughly proportional to Tween 80 concentration. Also beyond 0.25 % although laccase concentration increased, its specific activity decreased significantly. Thus 0.25 % Tween 80 was selected for further work.

The effect of surface to volume ratio on biomass, laccase, protein and specific activity are presented in Fig. 6A-D.

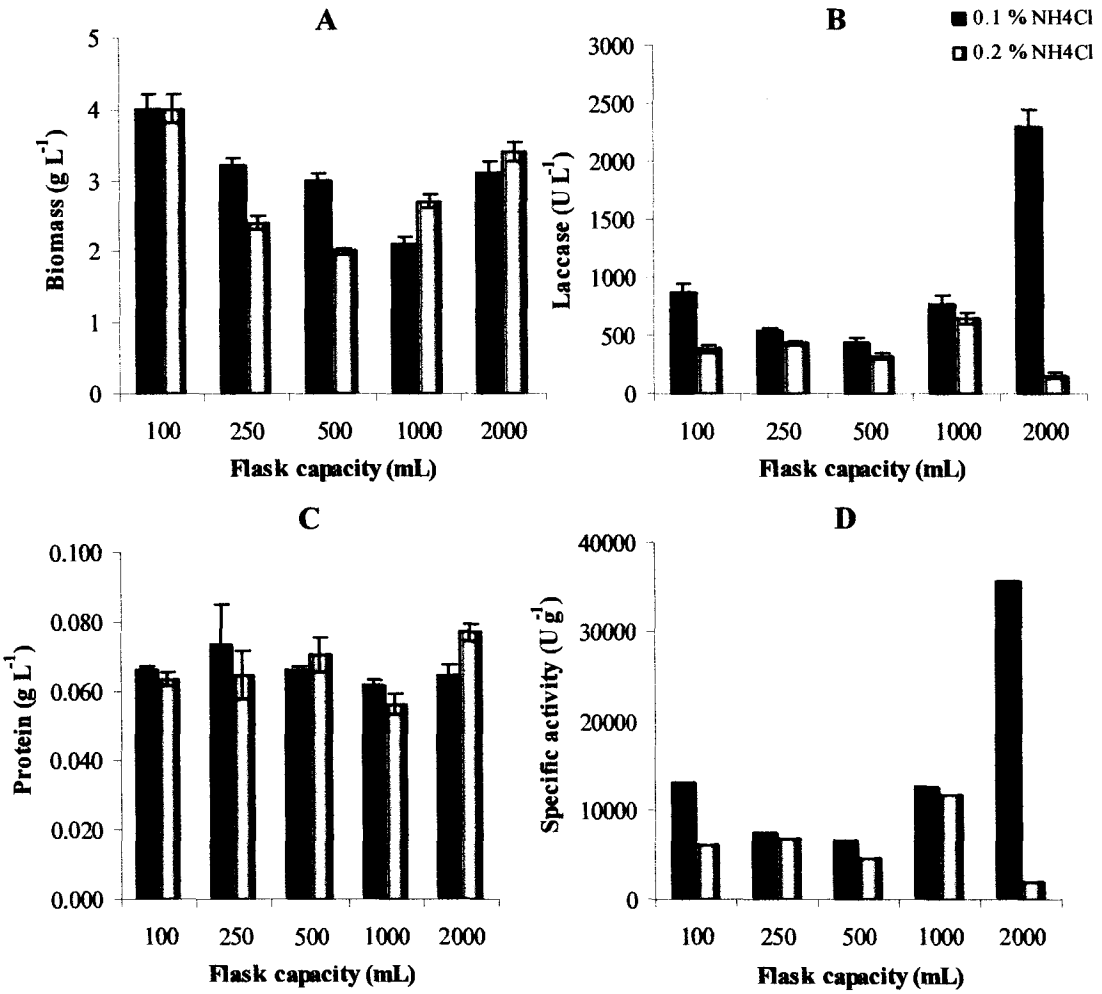

Fig. 6: Effect of surface to volume ratio on (A) Biomass; (B) Laccase; (C) Protein and (D) Specific activity.

The surface to volume ratios of the 100, 250, 500, 1000 and 2000 mL capacity flasks were 1.59, 1.11, 0.87, 0.68 and 0.54 respectively. The surface to volume ratio is roughly inversely proportional to flask capacity. At surface to volume ratios up to 0.87, 0.1 % NH_4Cl supported better biomass production whereas, 0.2 % NH_4Cl supported better biomass production below 0.87-surface to volume ratio (Fig. 6A).

In all the flasks, 0.1 % NH_4Cl supported greater laccase production than 0.2 %. Increasing surface to volume ratios have been reported to favour laccase production however the 1000 and 2000 mL capacity flasks proved to be exceptions. Especially the 2000 mL flask with the least surface to volume ratio, supported maximum laccase activity

at 0.1 % NH_4Cl whereas at 0.2 %, it yielded the minimum (Fig. 6B). With 0.1 % NH_4Cl laccase specific activity increased only below 0.87-surface to volume ratio (Fig. 6D).

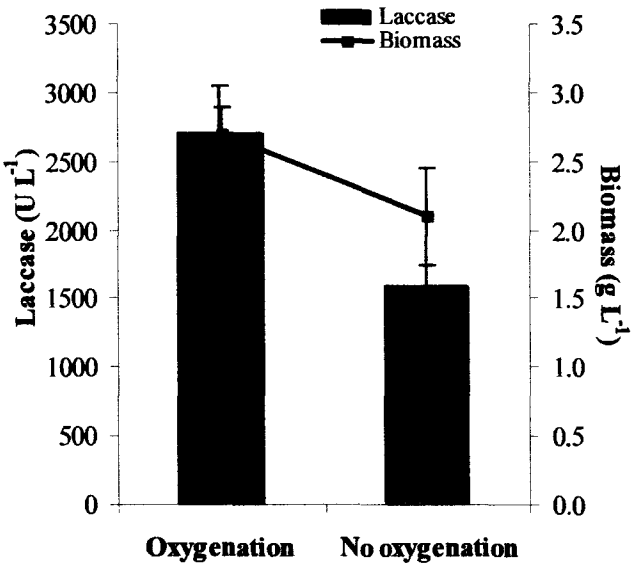

Fig. 7: Effect of oxygenation on laccase and biomass production.

From Fig. 7 it is clear that oxygenation had a positive effect on both laccase and biomass production compared to the unoxygenated culture.

The effect of different known inducers on laccase and laccase specific activity are presented in Fig. 8 A_1 - B_1 and Fig. 8 A_2 - B_2, respectively. Among the aromatic inducers; ferulic acid and guaiacol were the most successful in laccase-induction, ferulic acid also supported high specific activity. Among the various known inducers used, $CuSO_4$ induced the maximum laccase. However the combination of an aromatic inducer such as guaiacol and copper supported consistently better laccase production (Fig. 8 B_1). The effect of industrial effluents on laccase induction and laccase specific activity are presented in Fig. 8 C_1 & C_2, respectively. Among the effluents, 1 % textile effluent B (TeB) induced the highest laccase activity (Fig. 8 C_1) the other effluents stimulated a moderate increase in laccase production.

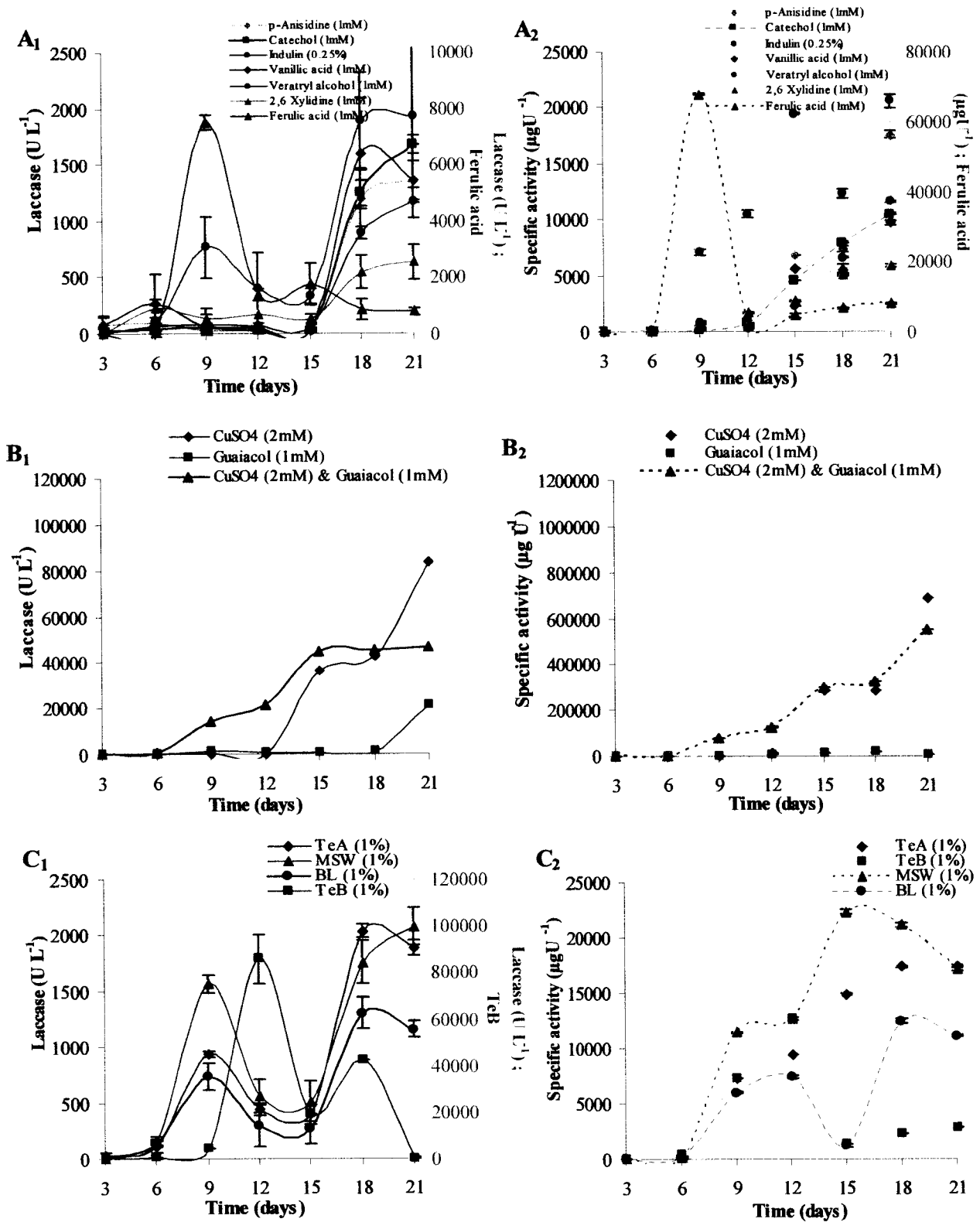

Fig. 8: Effect of different inducers on (A_1-C_1) Laccase production and (A_2-C_2) Specific activity.

The maximum laccase titer induced by either $CuSO_4$ or TeB were similar, however TeB was able to induce the same laccase titer 9 days earlier than $CuSO_4$.

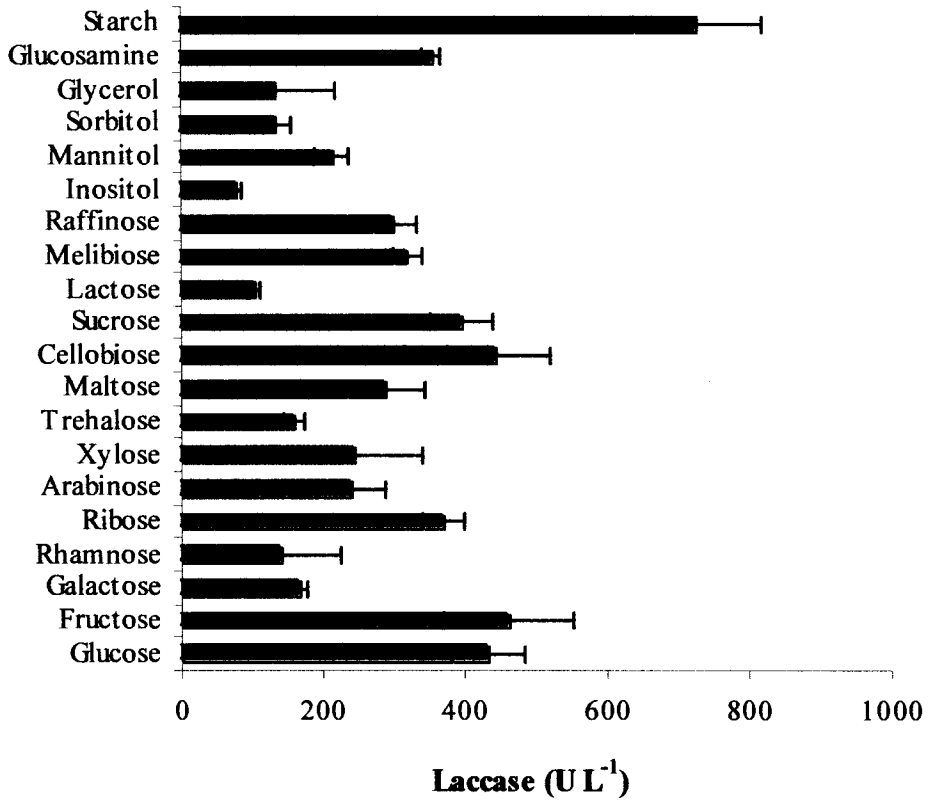

Fig. 9: Effect of different carbon sources on laccase production.

Among the carbon sources tested (Fig. 9), the polysaccharide starch, supported maximum laccase production. Monosaccharide hexoses such as fructose and glucose; disaccharides such as cellobiose and sucrose were also good carbon sources.

4.4 DISCUSSION

The cultures were incubated in the dark at 30°C, since the darkness has been shown to aid in laccase production (Thurston, 1994). Fungi have been cultivated at temperatures between 25° and 30°C for optimal laccase production. Temperatures higher than that have been known to reduce the activity of ligninolytic enzymes (Arora & Gill, 2000; Fåhraeus & Reinhammar, 1967; Lang *et al.*, 2000; Vasconcelos *et al.*, 2000).

As compared to either B&K or MEB, the low nitrogen content of LN most satisfactorily mimicked the nitrogen depletion which is known to trigger laccase production, when the fungus reaches the idiophase (Keyser *et al.*, 1978). Since LN is a completely synthetic medium, the effect of individual components could be estimated accurately. It supported maximum laccase activity as well and hence was chosen to determine the effect of individual medium components for its production.

Thurston, (1994) states that when fungi are grown in a medium at which the pH is optimal for growth (~pH 5) laccase would be produced in excess. Little information is available on the influence of pH on fungal laccase production and most studies indicate that pH levels are set between pH 4.5 and 6 prior to inoculation but not controlled during most cultivations (Arora & Gill, 2000; Fåhraeus & Reinhammar, 1967; Lang *et al.*, 2000; Vasconcelos *et al.*, 2000). In the present work too, the medium was initially set at pH 4.5, using citrate phosphate buffer. The amount of buffer required to set the medium pH to 4.5 contained about 0.2 % citrate whereas glucose was present at 4.5 %. The growth of this fungus was not determined using citrate at 4.5 % as was done with glucose. The effect of citrate was examined whether it serves as an additional carbon source when supplied solely through the buffer. Both served as independent carbon sources for laccase and biomass production. However due to the very low concentration of citrate, whatever biomass production could have been produced, was accumulated by day 3 itself, which itself was minimal. After which, there was no further biomass build-up i.e. the fungus reached its idiophase. When both glucose and citrate were present together, no adverse interaction was observed in fact, the effect was additive on biomass.

When citrate was the sole carbon source, the fungus went into stationary phase by day 3 itself since laccase is known to be produced in this phase, hardly any variation was observed in laccase production and the low laccase titer was probably due

to the low biomass that had been produced. The large variation in laccase titer observed only when glucose was present was probably due to the lack of buffering action that the citrate-phosphate buffer provided. When both glucose and citrate were present together, no adverse interaction was observed. In fact, there was a more consistent production of laccase. However, the percentage of carbon that was contributed individually by either carbon source, to the build up of biomass was not included in this study and merits further investigation. The addition of a basal level of citrate decreased but did not eliminate the temporal variability in laccase titer. Hence, the laccase titer was estimated every 3 days to determine the day at which maximum production was achieved.

The large negative coefficient of ammonium chloride and sodium chloride suggests that higher concentrations of either component would drastically and negatively affect biomass synthesis. However, it appears that inorganic salts affect biomass accumulation. Whereas the level of positive impact of glucose, copper sulphate and Tween 80 on biomass accumulation, reflects their criticality.

Palmieri *et al.* (2000) found that the addition of 0.15 mM copper sulphate to the cultivation media could result in a fifty-fold increase in laccase activity compared to the basal medium. Here, the copper sulphate was added to the medium on the day of inoculation itself. To avoid possible toxic effects of high levels of copper (Baldrian, 2003; Eggert *et al.*, 1996), in the present investigation its maximum concentration used was 0.375 mM. In spite of this, its presence at the time of inoculation resulted in a delay in biomass accumulation however, this impact was later reversed. The same trend was noticed with Tween 80. Prolonged incubation periods often lead to concentration of medium components due to moisture evaporation, this leads to increased concentrations of the other components present in the medium to such an extent that precipitation of critical components could occur or result in adverse interactions between components. The presence of Tween 80 probably decreases the evaporation of moisture, which could account for the very high positive impact it had on the production of biomass.

Overall, however the deviation in biomass between the spiked and the unspiked sets was not very large probably since sufficient growth had already occurred. Therefore, the effect of copper toxicity on biomass was not very evident.

All the medium components including glucose had a negative impact on laccase production on day 3, which got reversed later. This delay in laccase production in

the presence of increased amounts of glucose has also been previously observed by Monteiro and De Carvalho (1998). These findings are in concurrence with the earlier hypothesis that easily assimilable components allow for the constitutive production of laccase, but repress its induction (Bollag & Leonowicz, 1984) This view was later experimentally endorsed when starch, the only polysaccharide from all the carbon sources tested supported maximum laccase production (Fig. 9). The laccase titer in the presence of starch was roughly double than any of the mono- and disaccharides. Also, the presence of both constitutive and inducible laccase isozymes have been previously demonstrated in this fungus when grown in varying nitrogen sources (D'Souza-Ticlo *et al.*, 2006).

The nitrogen sources here included inorganic nitrogen such as potassium nitrate, amino acids such as glycine and glutamic acid and complex organic source such as beef extract and cornsteep liquor. All these nitrogen sources supported the production of a 47 kDa constitutive laccase, whereas glycine, beef extract and cornsteep liquor induced the production of a 55 kDa laccase, in addition to the constitutive laccase. An alternative method to avoid the delay in laccase production would be to use a carbon source that is not very easily assimilable so that instead of a constant but minimal level of constitutive laccase, an inducible laccase at high titer values is produced.

The impact of ammonium chloride an inorganic nitrogen source, was decidedly negative throughout. Its maximum negative impact coincided with maximum laccase production on day 6, indicating that the concentration of nitrogen is especially critical for laccase production.

Higher amounts of glucose and ammonium chloride significantly affected laccase by delaying its production. This may be due to the increased log phase of fungus resulting in a delayed production of laccase which is normally produced in the idiophase of the fungus.

After an initial highly negative impact on laccase production, the impact of both copper sulphate and Tween 80 became highly positive and were the highest among the components tested. Some of compounds affect the metabolism or growth rate while others, trigger laccase production (Froehner & Eriksson, 1974; Lee *et al.*, 1999). It seems that at the concentrations used here, copper sulphate and Tween 80 affected biomass as well as laccase production. Heavy metals especially copper are well known laccase inducers and Faraco *et al.*, (2003) have experimentally proven that the promoter regions

of laccase genes contain various recognition sites that are specific for heavy metals, which induce laccase production. Heavy metals especially copper are also known to dictate the kind of laccases produced, the presence of copper favours the production of inducible laccases over constitutive ones (Robene-Soustrade & Lung-Escarmant, 1997). Apparently, in this medium setup the inducible enzymes are dominant due to the presence of copper. Surprisingly the second addition of copper sulphate led to a dramatic decrease in laccase production.

Sodium chloride, throughout had a negative impact on laccase production and copper sulphate spiking dramatically decreased this impact and turned it to positive. This lowering of the negative impact of sodium chloride after the second addition of copper sulphate was probably due to the drastic decrease in the overall laccase titer. Lower laccase titer values would result in a lowered impact rather than due to a positive interaction between copper sulphate and sodium chloride.

The overall positive impact of Tween 80 on laccase accumulation was probably due to either of the following reasons or a combination thereof ; (1) the surfactant property of Tween 80 which emulsifies the fungal membrane aiding in the release of cell membrane-associated laccases as well as increased secretion of the normal extracellular laccases. (2) The decrease in amount of evaporation of moisture due to the presence of Tween 80, which otherwise would have led to concentration of medium components during prolonged incubation periods and thus increasing adverse interactions between components or the precipitation of critical components.

The initial negative impact of the first addition of copper sulphate on laccase production suggests that either copper has an initial toxic effect on biomass accumulation or merely that the major laccases produced in this medium are not constitutive but of the inducible type and thus the mandatory lag before enzyme production begins. Strangely, an additional spike of copper on day 6 did not yield the expected spurt in laccase production considering that sufficient biomass had accumulated to offset any toxicity resulting from copper addition. In fact, the laccase production had drastically decreased in such a way that the additional copper sulphate spiking overshadowed the effect of all other medium components. Thus, copper sulphate was effective in increasing the laccase titer but when its concentration was increased by 0.375 mM, it had an adverse effect. Thus at 0.375 mM concentration (Trial B), copper sulphate positively affected laccase

production. However, the upper limit of copper sulphate concentration requires careful fine tuning. It is hypothesized that the initial addition of copper sulphate is welcomed by the fungus since it is used in biomass accumulation. Metals are required by fungi for their normal growth but more importantly in laccase synthesis as four copper ions are required per molecule of laccase, for it to be fully functional. However, the second addition of copper contrary to the assumption that sufficient biomass had accumulated to offset copper toxicity, adversely affected laccase production. Whether this toxicity was a universal effect or not cannot be ascertained since sufficient growth had already occurred, however laccase production was negatively affected. Either the synthesis of laccase was affected or merely its activity was affected while assaying due to the inhibiting and prolonged presence of copper. This requires further investigation and would put to rest the questions thrown up by the above observations. Thus, a single addition of copper is preferred. However, since its addition at the time of inoculation results in a delay, if the delay in laccase production was a function of toxicity on growth and not because laccase is generally produced in the idiophase, then a one time of addition of copper sulphate after sufficient growth has occurred would be ideal. This would overcome the toxic effect of copper on biomass yet retain its positive impact on laccase production.

Glucose and ammonium chloride as carbon and nitrogen source are essential and their concentrations critical for biomass and laccase production. The effect of the interactions between these two medium components on biomass and laccase production was interesting.

Irrespective of the whether biomass or laccase was concerned, glucose and ammonium chloride interacted antagonistically with each other. However, the degree of the antagonistic interaction with reference to biomass was negligible as compared to laccase. The linear and quadratic coefficients for biomass indicate that its accumulation was favoured in the presence of increased amounts of glucose but was almost independent of ammonium chloride concentration. Only low concentrations of ammonium chloride appeared to favour laccase production. The inverted bell shaped curve and the low r^2 values of the surface plot for laccase production further emphasizes the antagonistic interaction of glucose and ammonium chloride. These low r^2 values are a result of the antagonistic interaction between these two components and not a reflection

of poor experimental reliability, since for biomass, high r^2 values indicate that there was minimal error in experimental set up.

Since these two components interact antagonistically with respect to laccase production, a medium containing either a combination of high ammonium chloride and low glucose levels or a combination of low ammonium chloride and high glucose levels would be ideal for laccase production. Even though laccase production is known to increase during the stationary phase of growth or in secondary metabolism (Bourbonnais *et al.*, 1995), for laccase production to occur a critical biomass accumulation is required, which is only favoured at higher glucose concentrations. Thus, a combination of high glucose but low ammonium chloride concentration would yield sufficient biomass to produce laccase in an appreciable titer. However, the negative quadratic coefficient of glucose for biomass production and the positive but low value for laccase production, limits the maximum concentration of glucose that can be used, above which biomass and laccase would be detrimentally affected. This has been experimentally proven in laccase-producing fungal strains where excessive concentrations of glucose had an inhibitory effect on laccase production (Eggert *et al.*, 1996). It has also been experimentally proven that laccase production is highly dependent on the conditions of cultivation of the fungus (Heinzkill *et al.*, 1998) and media supporting high biomass does not necessarily support high laccase yields (Xavier *et al.*, 2001). This probably acts as a sort of check on uncontrolled laccase production which would probably have been harmful for the fungus since active growth requires the presence of metabolites and enzymes other than laccase, whose production may have not been possible if the entire cell machinery had been routed to laccase production.

Tween 80 concentrations did not significantly affect biomass production whereas there was approximately a linear correlation between laccase and Tween 80, the same relation was present with total protein. This medium was designed to selectively produce laccase and not the other lignin-degrading enzymes which this fungus is also known to secrete (as observed in Chapter 2). At lower concentrations of Tween 80, laccase was the major protein secreted but beyond 0.25 %, the medium became increasingly frothy due to its surfactant properties and the specific activity decreased greatly.

It is a well known fact that maximum metabolite production occurs under stationary conditions where the fungal mycelium is in maximum contact with the atmospheric surface above the medium and especially under higher oxygen tensions. This was experimentally confirmed since the oxygenated culture produced greater biomass as well as laccase than the unoxygenated culture.

The experimental values of both the concentrations of ammonium chloride were lower than the predicted values since the equations for biomass and laccase were formulated using data obtained with a surface to volume ratio of 2.5, whereas in this case, the surface to volume ratio was only 1.1 less than half, emphasizing the role of surface to volume ratio in fungal metabolite production.

Surface to volume ratios above and equal to 0.87, included the 100, 200 and 500 mL capacity flasks. As the capacity suggests, these flasks were smaller in size than the 1000 and 2000 mL capacity flasks and hence the total biomass produced in these would be limited by their size. Thus, 0.1 % ammonium chloride was more than sufficient for supporting biomass production. Whereas for surface area to volume ratios below 0.87 i.e. the 1000 and 2000 mL capacity flasks, the total surface area was larger and accordingly the total biomass production that could be achieved, for which ammonium chloride at 0.2 % was required to support better biomass production. It seems that biomass production decreases, with decrease in surface to volume ratio up till 0.87. Below 0.87, the biomass production increases with further decrease in surface to volume ratio. It seems that below 0.87, the total surface area provided to the fungus gains more importance than the actual surface to volume ratio as seen in the 1000 and 2000 mL flasks.

The 2000 mL capacity flask with the least surface to volume ratio, supported maximum laccase production. This is probably since the 2000 mL capacity flask had maximum surface area than all the other flasks and thus produced maximum total biomass. The 0.1 % ammonium chloride mimicked the low nitrogen conditions required to induce laccase production. Thus, maximum total biomass and low nitrogen conditions, favoured laccase production, which was the highest among the varying ammonium chloride concentrations and flask capacities.

It has been experimentally proven that an organic nitrogen fared better in inducing laccase production than inorganic nitrogen (Hou *et al.*, 2004). It has also been

found that some fungal strains can be non responsive to varying nitrogen concentrations in terms of ligninolytic activity (Leatham & Kirk, 1983). Heinzkill *et al.* (1998) and Buswell *et al.* (1995) reported a higher yield of laccase in nitrogen-rich media rather than the nitrogen-limited media usually employed for laccase induction although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. Thus, a high carbon to nitrogen ratio cannot be a universal pre-requisite for high laccase production, rather the ratio is strain-dependant as was observed by Buswell *et al.* (1995).

Whether this fungal strain has a strict requirement for low nitrogen concentrations for maximum laccase production to occur or whether this rigid requirement for low concentrations is a result of the inorganic nature of the nitrogen source needs further study. B&K contains forty times more nitrogen that too in the organic form and glucose, less than one fourth present in the LN medium. According to the CCD results, the composition of the B&K medium, with its low carbon to nitrogen ratio should support very low laccase production. However, this medium was also supplemented with several aromatic compounds, an inorganic salt such as copper sulphate and some industrial effluents which also acted as laccase-inducers. Laccase was estimated every 3 days, since the growth conditions were very different from those maintained during the screening and optimization studies and the day at which maximum laccase production occurred may not be the same.

This is the first report wherein high amount of laccase (921 U L^{-1}) is reported to be produced by marine-derived fungus when grown in seawater medium with peptone as a nitrogen source, without any inducer. Biomass production in this medium was rapid in organic medium and could easily sustain the addition of 2 mM copper sulphate even on day 3 which resulted in ~ 100-fold increase in laccase production (83619 U L^{-1}). Besides, addition of aromatic compounds such as *p*-anisidine, catechol, guaiacol, ferulic acid, vanillic acid and veratryl alcohol also induced laccase. This is also the first report where a 1 % textile effluent B (TeB) increased laccase production by ~100-fold. Other effluents such as black liquor from paper and pulp mill and molasses spent wash from alcohol distillery also induced laccase but to a lesser extent. The effluents acted as inducers of laccase were also simultaneously decolourized. These by-products probably further induced laccase. Textile effluent B had an acidic pH of 2.5

whereas textile effluent A had a pH of 8.9 this was probably the reason for the much lower laccase induction and activity.

From the laccase titers obtained, it is apparent that the presence of inducers and nitrogen in the organic form offset the negative effects of the high nitrogen to carbon ratio. A batch culture of this fungus in B&K medium with 1 % textile effluent could be routinely used to obtain laccase activity of ~80 U mL^{-1}. This is much higher than those reported in terrestrial fungi recently (Jordaan & Leukes, 2003; Niku-Paavola et al., 2004; Nyanhongo et al., 2002; Wesenberg et al., 2003).

Although the fungus showed maximum biomass and laccase production in the seawater medium at 25 ppt as seen in Chapter 2, addition of sodium chloride had an inhibitory effect as seen in the screening experiments. Seawater with its other constituents supports growth and apparently unlike sodium chloride. Therefore, for the growth such a marine-derived fungus, sodium chloride may not be able to replace the requirement of seawater. Although sodium chloride at such low concentrations does not affect laccase activity as seen in the previous chapter, it does adversely affect its production. This adverse effect could also be due to the combined stress of the synthetic nature of the medium, which contained only inorganic salts since it is a known fact that fungi have a preference for organic substrates (Hou *et al.*, 2004). The level of tolerance of this fungus to sodium chloride concentrations in organic and inorganic media requires to be investigated further.

Thus, a high carbon to low nitrogen ratio or vice versa may not be the main criteria in maximizing laccase production. Rather the overall conditions such as the kind of form of nitrogen present, time of addition of inducers, presence of seawater rather than pure sodium chloride and especially the presence and concentration of inducers are equally important for laccase production in this fungus.

Chapter 5

Applications of *Cerrena unicolor* MTCC 5159 in Bioremediation

ABSTRACT

Whole culture of *Cerrena unicolor* MTCC 5159 and its lignin-degrading enzymes (LDEs) especially laccases along with its exopolymeric substance (EPS) have been implicated in the decolourization of coloured substances. The fungal mycelia and the EPS decolourize using processes such as active and passive adsorption and absorption whereas the LDEs actively degrade the colourants. Triarylmethane and triphenylmethane dyes are good substrates and also good inducers of these laccases. Diazo dyes act as laccase inducers and are preferentially degraded over monoazo dyes. However, the decolourization of some of the diazo dyes may not be proportional to laccase activity. When applied in biobleaching of sugarcane bagasse, it was found that these laccases are sufficient even at low concentrations. However, the timely addition of the appropriate mediator as soon as repolymerization begins not only reverses repolymerization but also hastens the biobleaching process. *Cerrena unicolor* MTCC 5159 has a great potential in decolourizing dyes and coloured effluents as well as biobleaching of lignocellulosic pulp.

5.1 INTRODUCTION

Industrial effluents especially coloured ones from textile & dye and paper & pulp industries are very difficult to treat. They contain compounds with complex aromatic structures which make them quite difficult to degrade. These dyes are classified as ***anionic*** dyes which include direct, acid and reactive dyes; ***cationic*** dyes which include the basic dyes and ***nonionic*** dyes which include the disperse dyes (Fu & Viraraghavan, 2001). They can also be classified according to the chromophore they contain (Table 1; Fig. 1).

Table 1: Classes of synthetic dyes according to Colour Index (C.I)

(Wesenberg *et al.*, 2003).

Code	Chemical Class	Code	Chemical Class	Code	Chemical Class
10000	Nitroso	42000	Triarylmethane	53000	Sulfur
10300	Nitro	45000	Xanthene	55000	Lactone
11000	Monoazo	46000	Acridine	56000	Aminoketone
20000	Disazo	47000	Quinoline	57000	Hydroxyketone
30000	Trisazo	48000	Methine	58000	Anthraquinone
35000	Polyazo	49000	Thiazole	73000	Indigoid
37000	Azoic	49400	Indamine/Indophenol	74000	Phthalocyanine
40000	Stilbene	50000	Azine	75000	Natural
40800	Carotenoid	51000	Oxazine	76000	Oxidation Base
41000	Diphenylmethane	52000	Thiazine	77000	Inorganic

The chromophores in anionic and nonionic dyes are mostly azo groups or anthroquinone types. The reductive cleavage of azo linkages results in the formation of toxic amines. Sulphonated azo dyes with their high water solubility make their removal difficult (Zhao *et al.*, 2005). Anthroquinone-based dyes are most resistant to degradation due to their fused aromatic structures and effluents containing such structures retain their colour for a longer time. Basic dyes have a high brilliance and intensity and are highly visible even at very low concentrations. The metal-complexed dyes are mostly based on carcinogenic chromium. Among these dyes, the azo dyes account for over 60 % of the total dyes manufactured (Fu & Viraraghavan, 2001).

Table 2: Details of the synthetic dyes used in this study

(http://stainsfile.info/StainsFile/dyes)

Dye [CAS# No.] (Colour Index No.)	Synonyms	Chromophore	λ_{max} (nm)
Brilliant Green [633-03-4] (42040)	Malachite Green G, Emerald Green, Solid Green JO, Diamond Green G, Aniline Green, Benzaldehyde Green, Fast Green J, Basic Green 1, Astradiamant Green GX, Ethyl Green	***Triphenylmethane***	623
Crystal Violet [548-62-9] (42555)	*p*-rosaniline hydrochloride, gentian violet, aniline violet, bismuth violet, hexamethyl violet, methyl violet, methyl violet 10B, paper blue R, oxyozyl, methylrosaniline chloride, adergon, aizen, atmonil, avermin, axuris, badil, brilliant violet 5B, calcozine violet C, genticid, gentioletten	***Triphenylmethane***	589
Aniline Blue [8004-91-9] (42755)	Water Blue, Acid Blue 22, Soluble Blue 3M, Marine Blue V	***Triarylmethane***	585
Methylene Blue [61-73-4] (52015)	Basic Blue 9, Chromosmon	***Phenothiazine***	663
Reactive Orange 16 [12225-83-1] (17757)	Brilliant Orange 3R, Red Label 706, Apricot, Strong Orange LR202, Remazol Brilliant Orange 3R	***Azo***	499
Trypan Blue [72-57-1] (23850)	Diamine Blue, Chloramine Blue, Benzamine Blue, Dianil Blue, Niagara Blue B	***Diazo***	599
Congo Red [573-58-0] (22120)	Direct Red 28, Cotton Red	***Secondary Diazo***	486
Remazol Brilliant Blue R [2580-78-1] (61200)	Reactive Blue 19, Red Label 722, Royal Blue, Intense Blue LR406	***Anthroquinone***	597
Poly-R 478 [68550-77-6] Red poly (vinylamine) sulfonate-anthrapyridone		***Anthrapyridone***	513 ÷ 362

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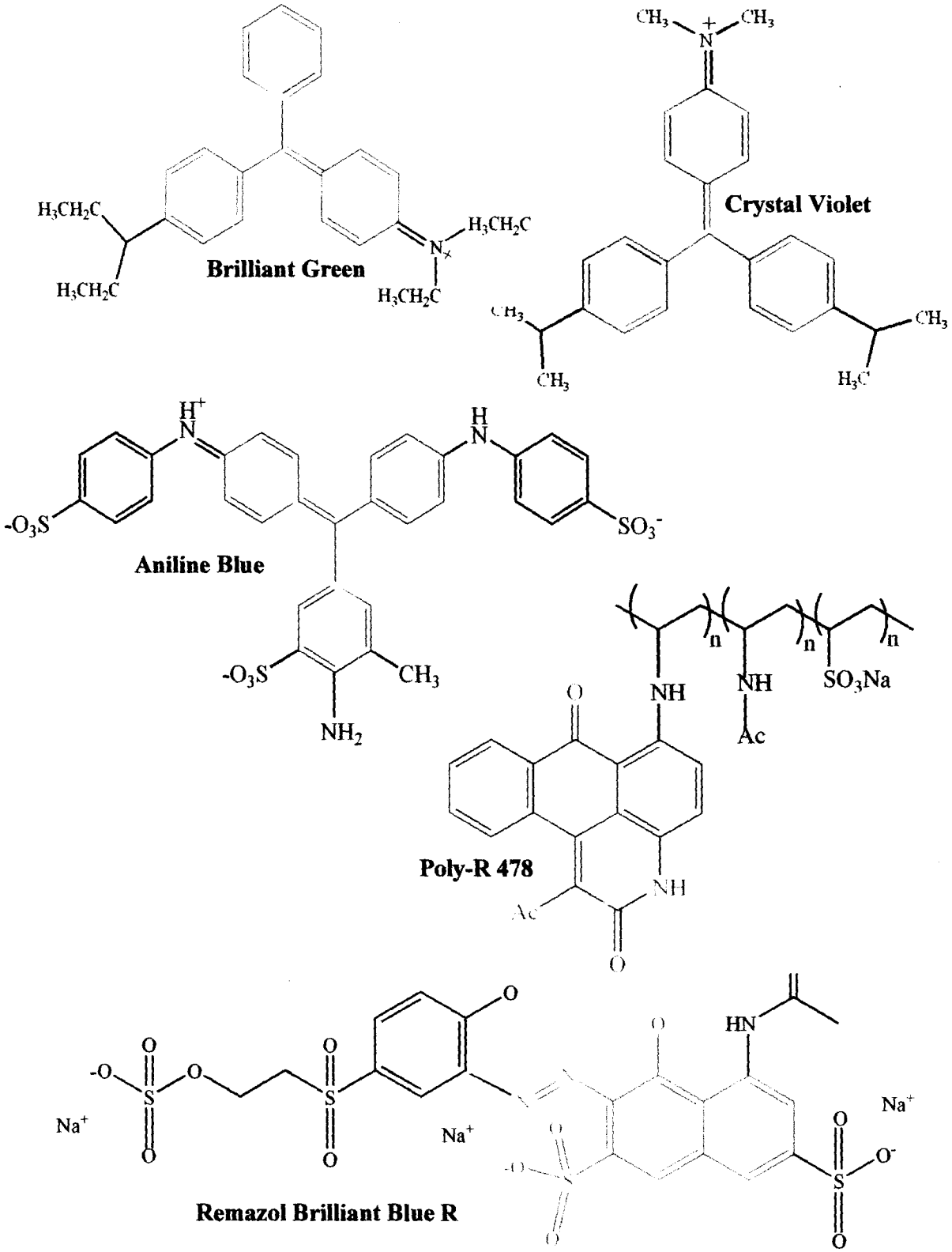


Fig.1: Molecular structure of the synthetic dyes used in this study.

The chromophore is in blue.

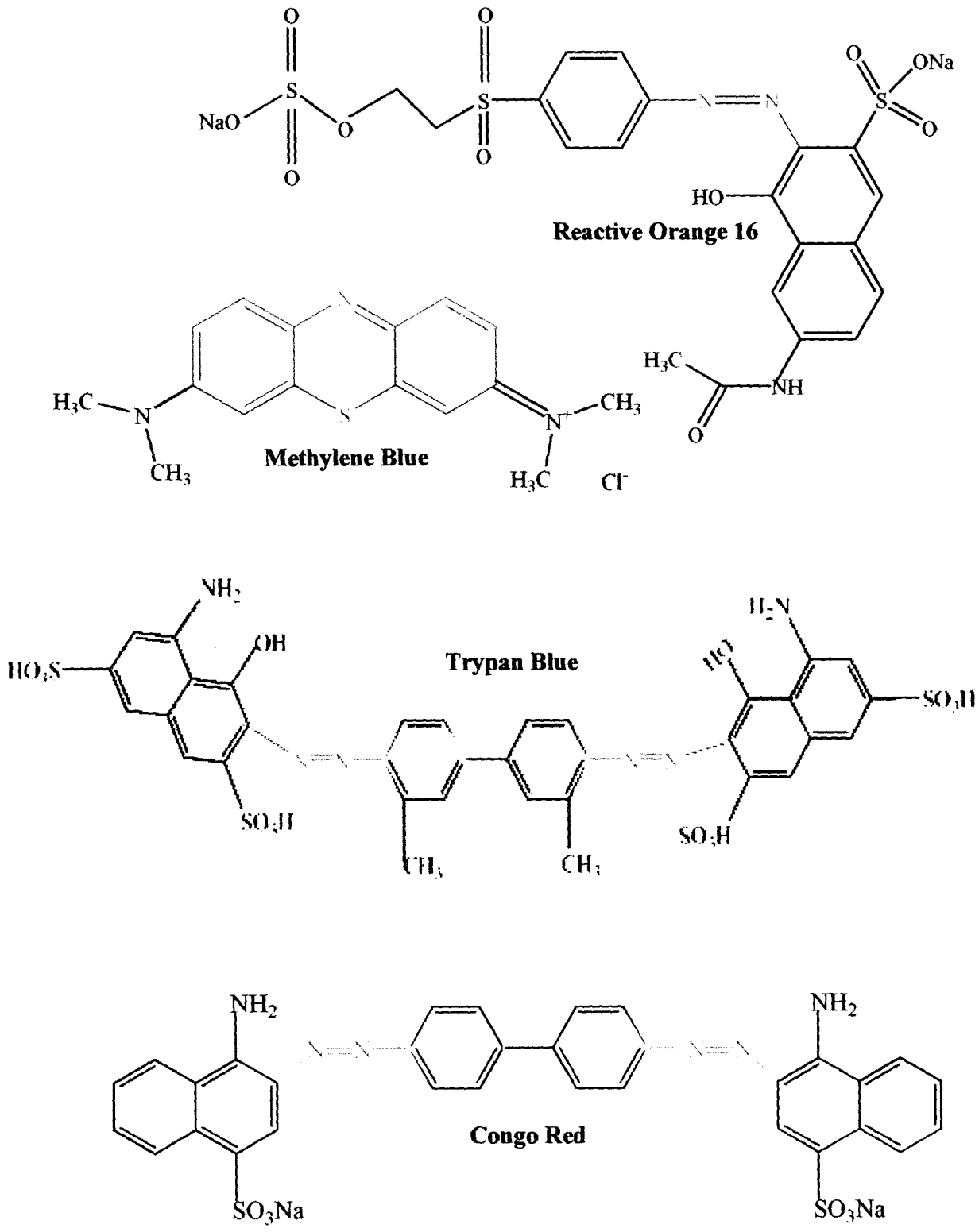


Fig.1 (contd): Molecular structure of the synthetic dyes used in this study.

The chromophore is in blue.

Table 3 : Advantages and disadvantages of the current methods of dye removal from industrial effluents (Robinson *et al.*, 2001b).

Physical/Chemical methods	Advantages	Disadvantages
Fentons reagent	Effective decolouration of soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state, no alteration of volume	Short half-life (20 min)
Photochemical	No sludge production	Formation of by-products
Sodium hypochlorite	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Cucurbituril	Good sorption capacity for various dyes	Expensive
Electrochemical destruction	Break-down compounds are non-hazardous	High electricity consumption
Activated carbon	Good removal of a wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	No adsorbent loss due to regeneration	Not effective for all dyes
Irradiation	Effective oxidation at laboratory scale	Requires high concentrations of dissolved oxygen
Electrokinetic coagulation	Economically feasible	High sludge production

Besides producing an aesthetically unacceptable intense colouring of water and soil bodies, they also result in decreased gas solubility in water bodies as well as block the passage of light to the lower depths of the aquatic system. This results in cessation of photosynthesis, leading to anaerobic conditions, which in turn results in the death of aquatic life causing foul smelling toxic waters. More importantly many dyes have carcinogenic origins (Fu & Viraraghavan, 2001).

Due to the low biodegradability of dyes, conventional effluent treatment systems are inefficient in treating waste water. It is usually treated by physical or chemical treatment processes (Table 3). These include physical or chemical flocculation combined with flotation, electro-flotation, flocculation with Fe^{+2} / $Ca(OH)_2$, membrane-filtration, electro-kinetic-coagulation, electrochemical-destruction, ion-exchange, irradiation, precipitation, ozonation and Katox treatment method involving the use of activated carbon and air mixtures. However besides being inefficient, these technologies are expensive and have low adaptability to diverse types of dye effluents. Adsorption has been observed to be an effective process for colour removal from such effluents. Adsorption on activated charcoal is an effective method but is prohibitively expensive. Low-cost adsorbents have generally low adsorption capacities mediating the need for economical and easily available yet highly effective adsorbents (Fu & Viraraghavan, 2001).

In order to be economically viable, bioremediation methods however require to be safe and less expensive than conventional treatments. Bacteria and fungi along with their products such as enzymes especially the lignin-degrading enzymes (LDEs) and exopolymeric substances (EPS) have been the object of study for quite some time for use in bioremediation (D'Souza *et al.*, 2006). Bioadsorption is a means of bioremediation and includes the use of both living and dead cells of bacteria, fungi, algae, plant biomass or a combination thereof (Robinson *et al.*, 2001b). When only enzymes are involved, maintaining the optimum catalytic activity for a long period under controlled conditions for the treatment of massive volumes of effluents is a rather difficult task (Pritchard *et al.*, 2006). However the use of live cells especially fungal mycelia mediates remediation through several ways. In most cases the dyes adsorb onto the mycelial surface, which is the primary mechanism of decolourization, after which the LDEs come into play. Physical adsorption of the dyes onto the fungal hyphae, and degradation by extracellular

LDEs and / or absorption into the mycelia and subsequent degradation by intracellular LDEs and their associated enzymes are the means of colour removal. The dye-saturated mycelia can be regenerated and used for repeated dye adsorption. This sequential adsorption and degradation of dye molecules makes bioremediation through adsorption a feasible process. Batches of industrial effluents are not identical to each other. They are extremely varied, the adaptability of biological methods to varied conditions gives bioremediation an edge over the physico-chemical methods (Selvam *et al.*, 2003).

The LDEs include the laccases and the peroxidases. These LDEs act on lignin and lignin-analogous compounds since they share common features such as broad substrate specificity, high redox potential and are mostly extracellular in nature. The high redox potential and broad substrate specificity increases the range of pollutants that the enzymes are capable of degrading. These features combined with the fact that LDEs are mostly expressed under nutrient deficient conditions (which is usually the case in the nature) and their ability to oxidize substrates with low solubility have made them the preferred candidates for bioremediation along with the fungi, responsible for their production.

The process of bioremediation can be monitored by measuring any of the following factors: (1) by measuring the redox potential, together with pH, temperature, oxygen content and concentrations of electron acceptor (s) / donor (s) and the breakdown products such as carbon dioxide or (2) by measuring chemical oxygen demand (COD) and biological oxygen demand. Biological oxygen demand (BOD) represents only the organic matter which is capable of being degraded / oxidized by microbes whereas COD represents all the oxidizable matter, including organic matter in any particular effluent. For coloured effluents, bioremediation is measured by estimating the reduction in colour and percentage of detoxification achieved (Marmagne & Coste, 1996).

Industrial effluents also create effluent-specific problems besides the ones mentioned earlier. Mentioned below are problems specific to the effluents that have been used in this study.

Textile effluents are highly coloured effluents released by the textile and dye manufacturing industries that contain large amounts of mixtures of dyes. These dyes are mostly resistant to degradation leading to deleterious effects on aquatic life. Textile

effluents besides containing dyes also contain salts, often at very high ionic strength and have extreme pH values as well. Marine fungi may find application in the decolourization of industrial effluents that contain large amounts of salt and extreme pH values (Raghukumar *et al.*, 2008).

Molasses Spent Wash (MSW) is a by-product of sugar mills and alcohol distilleries, where the starting material is molasses. It contains mostly dark brown coloured recalcitrant compounds formed by the Maillard amino-carbonyl reaction, collectively termed as melanoidin. These compounds are toxic to many microorganisms including those generally involved in wastewater treatment processes. Anaerobic digestion of MSW-containing effluents is one of the treatments followed by distilleries and the resulting dark brown sludge is used as fertilizer. Dark brown colour of these effluents remains a major problem for the distilleries. Laccase and MnP along with glucose oxidase and sorbose oxidase have been used in the decolourization of MSW. The H_2O_2 produced by the oxidases may act as a bleaching agent by itself or assist the peroxidases in the decolourization process (Dehorter & Blondeau, 1993).

Black Liquor (BL) is an intensely coloured effluent released in large volumes by the paper & pulp industry. These effluents contain toxic chlorinated lignin-degradation products which include chlorolignins, chlorophenols and chloroaliphatics (Ali & Sreekrishnan, 2001). Besides, these highly alkaline effluents with a pH ranging from 8 – 11, alter the pH of the soil and water bodies into which they are discharged. Among the LDEs, the peroxidases especially MnP, play a major role in the decolourization of this effluent. Since most of the LDEs have optimum activity between pH 3 – 6 and fungi in general show preferential growth under acidic conditions, BL is usually acidified prior treatment with these fungi. Decolourization of such effluents without the requirement of prior acidification would be useful both economically and environmentally, since acidification results in the water and soils becoming acidified after their discharge (Wu *et al.*, 2005).

It has been reported that effluents from paper and pulp mills and textile dye wastewaters are toxic and mutagenic besides being coloured. In these effluents, laccases have been shown to render phenolics, less toxic via degradation or polymerization

reactions or by cross-coupling of aromatic pollutants with naturally occurring phenols. Besides decolourization, detoxification of wastewaters is an important parameter which also requires monitoring (Raghukumar *et al., in press*).

Paper is manufactured from wood, for this, a pulp of the woody (lignocellulosic) substrate requires to be prepared after which, the required cellulose fibers have to be separated from the hemicellulose and lignin. These cellulose fibers have to be then freed from all traces of lignin so that no traces of colour are present and finally it undergoes finishing producing the final product, paper.

The main processes for paper-pulp production that are currently in use are mechanical and chemical. Using fungi to convert wood chips into paper pulp is termed as ***'biopulping'***. The fungus currently used in biopulping by 'BioPulping International' is *Ceriporiopsis subvermispora* and is reportedly easy to maintain. The wood is treated with steam creating a ventilation system that provides a good environment for the fungus to thrive in. Fungi decay the woody substrate to alter the lignin in the cell walls of the wood, which therefore 'softens' the wood chips. This process is non-polluting since it is chemical-free and since the wood is already softened, the remaining steps of the process require less energy. Biopulping provides a solution to the problems of chemical and mechanical paper production. It also reduces electrical energy needed by an average of 25 - 30 % it also saves about $ 9 - $ 20 per ton of pulp (http://www.biopulping.com/2.html).

The paper-pulp is then subjected to alkali-treatment to free the cellulose fibers from the hemicellulose and lignin. This is then bleached to reduce the colour due to residual lignin still present in the cellulose. Bleaching is done using chlorine, this process however is not environmental friendly. Fungi that live on decaying plant material specially the white-rots, on the other hand produce enzymes which break down hemicellulose and lignin and provide an environmental-friendly alternative to chlorine bleaching and is termed '***biobleaching***'. At present, this has not yet completely replaced chlorine but only augments the bleaching process, thereby decreasing the total residual chlorine load in the effluents. In theory, it should be possible to use the LDEs and laccase-mediator systems (LMS) in the pulping and bleaching phases of paper manufacture resulting in what is described as biopulping and biobleaching. Economical substrate for the fungi would include wood, fiber crops and agricultural residues, due to the variable nature of these substrates the rate of growth of the fungi, the LDE yield and

the type of LDEs produced would vary from batch to batch. Although this would result in decreased repeatability in the biobleaching process, it also means that there is increased adaptability.

Mediators are low molecular weight compounds that aid in lignin-degradation by assisting the LDEs in increasing the effective redox potential. The LMS involves use of these mediators which are oxidized by laccase to stable radicals which in turn oxidize other compounds which could not have been acted upon by laccase directly (Fig. 2). Lack of direct correlation between laccase activity and degradation of xenobiotics further supports the role of LMS. The redox potential of laccase alone is not high enough to break the carbon-hydrogen aliphatic bonds of certain compounds, the presence of a redox mediator allows for the oxidation of such bonds. The enzyme oxidizes the mediator, which can diffuse away from the enzyme and oxidize a substrate. The reduced mediator is then ready for the next cycle.

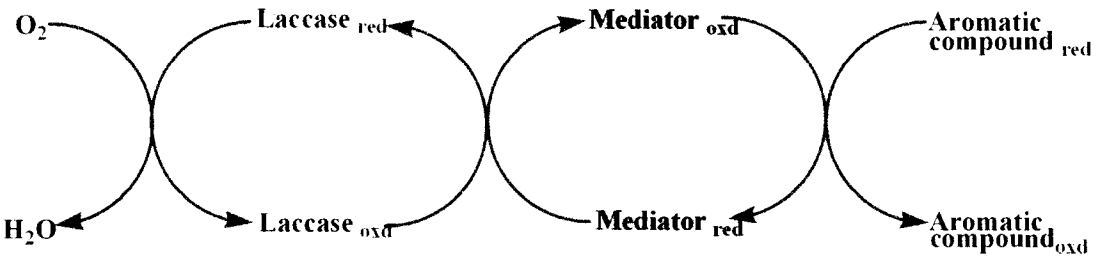


Fig. 2: Mediator-mediated oxidation of aromatic compounds by laccase. The aromatic compounds include lignin, synthetic dyes, effluents and other pollutants.

In nature, these mediators are synthetic compounds or natural compounds produced by fungi or plants. The synthetic compounds include ABTS, 1-hydroxybenzothiazole, violuric acid and N-hydroxyacetanilide (Camarero *et al.*, 2005). Natural mediators include the fungus-produced, 3-hydroxyanthranilic acid and degradation products of lignocellulose such as acetosyringone, *p*-coumaric acid, syringaldehyde and vanillin. Natural mediators have also been extracted from BL of eucalyptus-based kraft pulping. Laccase-mediator system has found applications in paper pulp delignification, degradation of Polycyclic Aromatic Hydrocarbons (PAHs) and industrial dyes (Camarero *et al.*, 2007).

5.2 OBJECTIVE

The objective of this chapter is to determine the bioremediation potential of *Cerrena unicolor* MTCC 5159 in the decolourization of synthetic dyes and industrial effluents and its laccase in the biobleaching of sugarcane bagasse.

5.3 MATERIALS AND METHODS

5.3.1. MICROORGANISM AND CULTURE CONDITIONS

Cerrena unicolor MTCC 5159 was raised as inoculum in the either LNM or B&K medium depending on whether LNM or B&K was used in the proceeding experiment. The 6 day-old culture, was then rinsed with distilled water and homogenized for 5 sec, all under sterile conditions. The mycelial suspension at 5 % v/v was used as inoculum. The day of inoculation in all experiments was considered to be day 0. Cultures were incubated at 30°C in the dark under static conditions.

5.3.2 LACCASE ASSAY

Laccase activity was estimated spectrophotometrically with the ABTS method (Appendix 8.2.2.1.1). Laccase was expressed as either U L^{-1} or U mL^{-1}. An abiotic control without laccase had been used in every experiment and all the assay components had been brought to reaction temperature before assay. Protein concentration was estimated spectrophotometrically using Bradfords reagent (Sigma, St. Louis, Montana, U.S.A), (Appendix 8.2.1.4). All spectrophotometric measurements were carried out using UV-Vis 2450 spectrophotometer (Shimadzu, Japan). All values represent the mean of three measurements of two independent experiments each.

Decolourization of dyes and effluents by whole culture *(in vivo)*

This fungus was tested for its ability to decolourize various dyes and effluents every alternate day after the addition of the dye / effluent over a period of 6 days. Synthetic dyes such as trypan blue, aniline blue and remazol brilliant blue R (RBBR) were added at 0.04 %, methylene blue, crystal violet, brilliant green, Poly-R 478 and congo red at 0.02 % and reactive orange 16 (RO 16) at 0.015 % final concentration. All the four industrial effluents were each added at 10 and 20 % final concentrations, they included textile effluent A (TeA), textile effluent B (TeB), molasses spent wash (MSW) and black liquor (BL).

The dye / effluent was added to a 6 day-old culture grown in both B&K and LNM prepared in 25 ppt seawater. However, the results presented below are only from the B&K medium, since LNM was found to be toxic (Ramsay & Nguyen, 2002). The colour intensity of the dye in the medium at the time of addition was considered as 100 %. The extent of decolourization was recorded as % residual colour. Mean values from triplicate cultures were used for comparing the extent of decolourization of various dyes and effluents.

Decolourization of the dyes and effluents was estimated by monitoring them at their absorbance maxima. Trypan blue, aniline blue, RBBR, methylene blue, crystal violet, brilliant green, congo red and RO 16 were monitored at 599, 585, 597, 663, 589, 623, 486 and 499 nm respectively. Decolourization of Poly-R 478 was determined by the ratio of absorbance at 513 nm versus 362 nm. Industrial effluents such as TeA, TeB, MSW and BL were monitored at 505, 663, 663 and 317 nm respectively. The details of TeA, TeB, MSW and BL are given in Appendix 8.4.1 - 8.4.4 respectively.

Decolourization of effluents by fungus free-culture filtrate *(in vitro)*

Cell-free culture filtrate containing 9 U of laccase was added to effluents such as TeA, TeB, MSW and BL, each at 10, 20 and 50 % final concentrations. These were then incubated at pH 6 and 60°C for 12 h. The percentage of decolourization achieved was calculated with reference to the control samples that were not treated with the enzyme and contained only the effluent at the respective concentration.

LACCASE PRODUCTION MEDIUM FOR BIOBLEACHING

The laccase production medium contained a mineral salts base consisting of 5 % (v/v) basal salts solution (Appendix 8.1.1.1), 10 % (v/v) trace elements solution (Appendix 8.1.1.2) and 1 mL of 0.005 % (w/v) thiamine. To this basal medium, 3 % glucose, 0.1 % glycine, 0.25 % Tween 80 and 0.1 % sodium chloride were added. The pH was adjusted to 4.5 using citrate-phosphate buffer and the final volume was made with distilled water. This is a modified version of the low nitrogen medium by Tien and Kirk (1988) and the medium optimized in the previous chapter. This is referred to as LNM in this chapter, to avoid ambiguity, with various versions of the LN medium.

For large-scale cultivation, 3-L Haffkine flasks with 1 L of LNM were inoculated with 100 mL of mycelial suspension and incubated as described above. On day 6, $CuSO_4$ at 2 mM was added to the cultures aseptically to stimulate laccase production. On day 12 when laccase activity reached its maximum, the cultures were vacuum-filtered to separate the mycelium. The culture filtrate was collected after passing through a series of filter papers namely; Whatman no. 1, GF/C, 0.45 µm and finally 0.22 µm (GV, Millipore, USA). This filtrate was then concentrated using ultrafiltration with positive pressure (Amicon) at 4°C. This concentrate was used as the crude laccase preparation for the biobleaching experiments. This was examined for the presence of cellulase (Appendix 8.2.2.4).

BIOBLEACHING ASSAY

Sugarcane bagasse was the substrate used for the biobleaching experiments. The lignin content of the untreated and treated bagasse samples was estimated using the 'Kappa number' method, given in Appendix 8.2.1.3. Before assay, the enzyme-treated sample was rinsed with distilled water to remove the enzyme, filtered to remove the excess moisture and then immediately placed at 90°C, to inactivate any residual laccase completely dry the sample after which, the lignin content was estimated. The lignin content was calculated in terms of % Klason lignin. Biobleaching is calculated in terms of % biobleaching which is the difference between the lignin content (% Klason lignin) of the untreated or the 0 h sample and enzyme-treated bagasse samples at that sampling interval.

Effect of mediators on biobleaching at 12 and 24 h sampling intervals

Crude laccase containing 1000 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. Mediators such as 1-hydroxybenzotriazole (HBT), veratryl alcohol (VA), 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), syringic acid (SA), hydroquinone (HQ), 3',5'-dimethoxy-4'-hydroxy acetophenone (DMHAP) and 4-hydroxy-2,2,6,6 tetra-methyl piperidine-1-oxyl (HTMP) at 2 mM were added separately to the bagasse. Biobleaching was determined at 12 and 24 h. The control contained only laccase and no mediator.

Effect of ABTS on biobleaching after repolymerization had begun

Crude laccase containing 1000 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. The biobleaching was determined at 12-h time intervals up to 96 h. The mediator, ABTS was added at 2 mM separately when repolymerization just began, to determine how this addition would affect the repolymerization process. The control contained only laccase.

Effect of ABTS on biobleaching at various time intervals.

Crude laccase containing 10, 20 and 30 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse in the presence of 2 mM ABTS and incubated at 60°C using a dry bath. Biobleaching was determined at 12-h time intervals up to 72 h. The control contained 10, 20 and 30 U of crude laccase only.

Effect of mediators on biobleaching at various time intervals

Crude laccase containing 20 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. Mediators such as HBT, VA, ABTS, SA, HQ, DMHAP and HTMP at 2 mM concentration were added separately to the

bagasse at 6 h. The biobleaching was determined at 12-h time intervals up to 72 h. The control contained only laccase.

Impact of time of early addition of mediators on biobleaching.

Crude laccase containing 20 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. Mediators such as HBT, ABTS and HTMP at 2 mM concentration were added separately at 6 h. The biobleaching was determined at 12 and 24 h. The control contained only laccase.

Comparison of two different methods for estimation of biobleaching.

Crude laccase containing 45 effective laccase U prepared in citrate phosphate buffer at either pH 3 or pH 6 on the biobleaching of bagasse (0.1 g) at 60°C was determined at various time intervals. Since the crude laccase was roughly twice as active at pH 3 than at pH 6, the laccase U calculated were 45 effective laccase U at a particular pH, i.e. the actual amount used of crude laccase used when incubated at pH 3 was roughly half that used at pH 6. Here two methods of estimating biobleaching were compared, the kappa number method (Appendix 8.2.1.3) and the brightness index method. For the brightness index method, a mat of the dried bagasse was prepared and placed against a spectrophotometer (Premier Colour Scan, model SS 5100A, India) and the reflectance reading recorded. The control was untreated bagasse for both the estimation methods.

5.4 RESULTS

The B&K medium prepared in 25 ppt seawater had an initial pH of around 7.5, which did not drop below 6 even after the decolourization experiment was completed. Presented below (Fig. 3) are decolourized synthetic dyes prepared in both LNM and B&K medium.

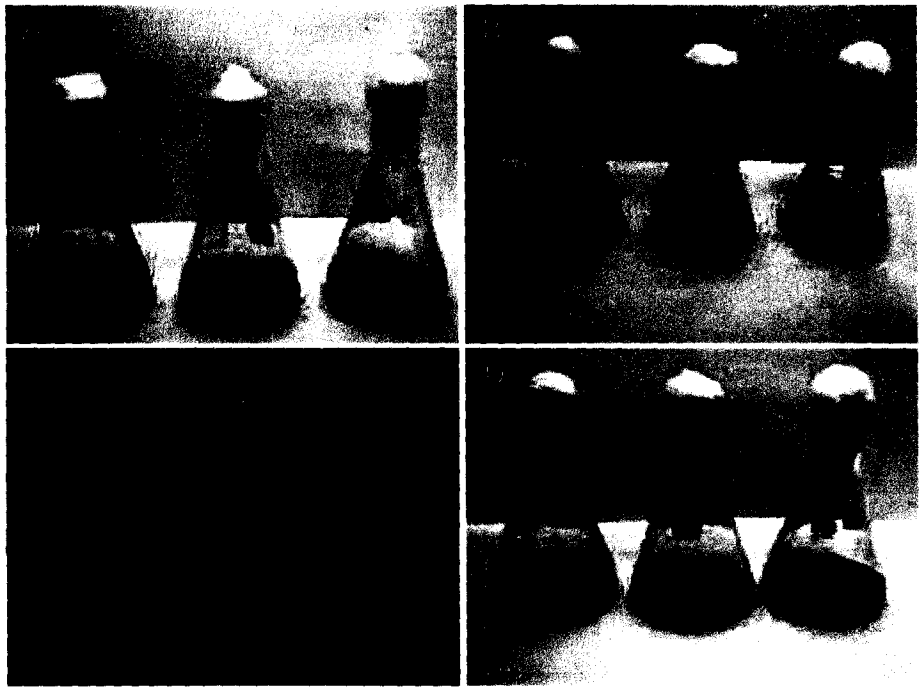


Fig. 3 *Cerrena unicolor* MTCC 5159 after 6 days in the presence of synthetic dyes. (A) Brilliant green (0.02 %) (B) Aniline blue (0.04 %) (C) Congo red (0.02 %) (D) Remazol brilliant blue R (RBBR) (0.04 %). The flask on the left-hand side in each figure contains the culture with dye prepared in B&K medium; in the center, it contains the culture with dye prepared in LNM and on the right-hand side is the control which contains only dye without culture.

Although good amount of decolourization was achieved using LNM, the medium itself is known to be toxic (Ramsay & Nguyen, 2002), defeating the purpose of bioremediation. Henceforth, the results presented or discussed are obtained using only B&K medium prepared in 25 ppt seawater.

The synthetic dye, brilliant green was decolourized to the maximum within 4 days whereas RO 16 was least decolourized (Fig. 4 A_1 - A_2).

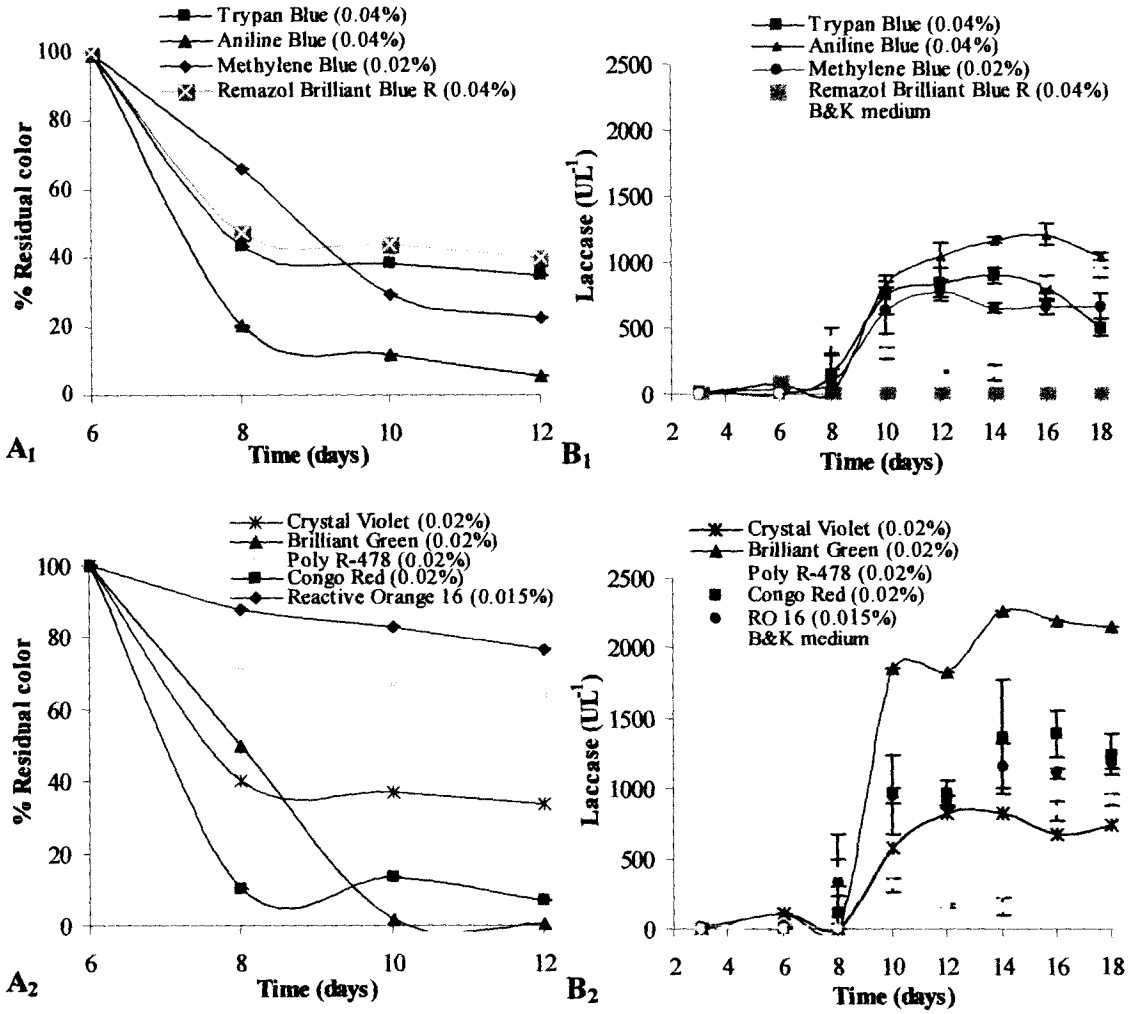


Fig. 4: *Cerrena unicolor* MTCC 5159's (A_{1-2}) Decolourization of synthetic dyes and (B_{1-2}) Induction of laccase in B&K medium with and without dye supplements.

Among the other synthetic blue dyes, aniline blue was decolourized almost completely within 4 days from addition of the dye. Trypan blue, methylene blue and RBBR were decolourized by 60 – 70 % within the same time. However, Poly-R 478 and RO 16 showed low levels of decolourization. The dyes that were decolourized also acted as inducers for laccase (Fig. 4 B_1- B_2) and in turn were further decolourized by the laccase produced. Laccase activity in the presence of Poly-R 478 was lower than equal to the

control and its decolourization was very low as well. Whereas, even though RBBR showed good decolourization, corresponding laccase activity was not observed.

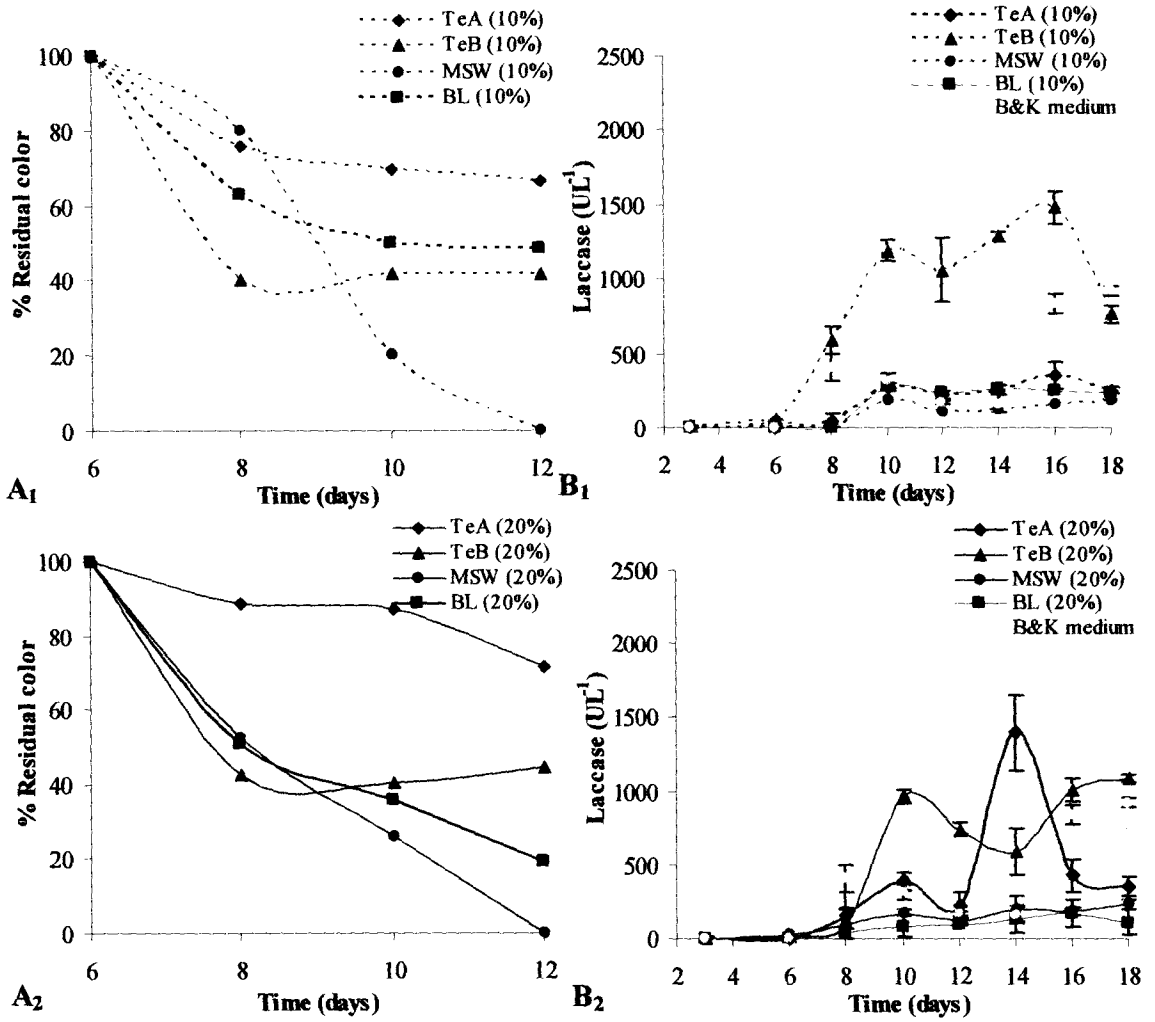


Fig. 5: *Cerrena unicolor* MTCC 5159 (A_{1-2}) Decolourization of industrial effluents and (B_{1-2}) Induction of laccase in B&K medium with and without effluent supplements.

Among the effluents (Fig. 5 A_1 - A_2), ~ 60 % decolourization of BL (10 %) was achieved by day 6 but surprisingly at 20 %, it was decolourized to a greater extent. MSW at 10 and 20 % was completely decolourized within 6 days. Among all the effluents, TeA at both concentrations was least decolourized (Fig. 5 B_1 - B_2). Textile effluent B at both 10 and 20 % was capable of consistently inducing laccase more than the control. However, on day 8 after of addition of dye, TeA at 20 % induced laccase activity comparable to that of TeB at 10 %, which was the maximum among the effluents tested.

The decolourization of synthetic dyes and effluents such as brilliant green, methylene blue, Poly-R 478, TeB (10 %), BL (10 %), aniline blue, trypan blue and crystal violet were proportional to laccase activity with r^2 values of 0.99, 0.99, 0.96, 0.96, 0.95, 0.95, 0.94 and 0.93 respectively. The decolourization of the remaining dyes and effluents such as black liquor (20 %), TeA (10 %) and RO 16, MSW (10 %), TeB (20 %), MSW and TeA at 20 % were not proportional to laccase activity, with r^2 values of 0.79, 0.73, 0.63, 0.59, 0.07, 0.04 and 0.02 respectively. The synthetic dye, congo red also did not show any correlation with laccase activity even though an appreciable laccase titer and significant decolourization was obtained. With RBBR, no laccase activity was observed, yet considerable decolourization occurred.

Two days after the dyes / effluents were added to 6 day-old culture, only TeB at 10 % induced more laccase than control. By the end of the experiment, 6 days after the addition of the dyes / effluents, only MSW at 10 % and 20 % and BL at 20 % still did not induce laccase despite getting completely decolourized. Poly-R 478, TeA at 10 % and 20 % were non-responsive in terms of laccase production, since the laccase titer induced in their presence was less than equal to the control. The remaining dyes and effluents had a positive effect on laccase production, with brilliant green showing maximum influence on laccase production, followed by TeB at 10 %.

Table 3: Effect of crude laccase[#] on effluent-decolourization after 12 h of incubation.

Effluent	Effluent (%)	% Decolourization (SD)
TeA	10	26 (5)
	20	23 (4)
	50	5 (0.5)
TeB	10	43 (6)
	20	73 (8)
	50	18 (2)
MSW	10	58 (5)
	20	50 (5)
	50	0 (0)
BL	10	8 (0.4)
	20	23 (3)
	50	38 (7)

[#] contains 9 U laccase.

The % decolourization of MSW and TeA decreased with increasing concentrations of effluent, whereas the opposite occurred with BL (Table 3).

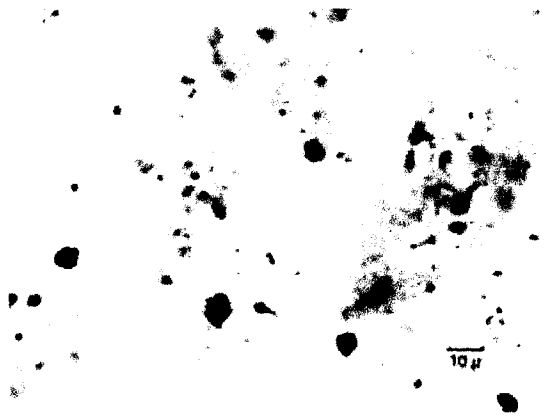


Fig. 6: A 21 day-old culture of *Cerrena unicolor* MTCC 5159 stained with Alcian blue. The hyphae are unstained whereas the surrounding exopolymeric substance stained blue.

A microscopic view of *Cerrena unicolor* MTCC 5159, under 100 X magnification, demonstrating the presence EPS, stained blue with Alcian blue around the unstained mycelium (Fig. 6). The exopolymeric substance (EPS) was very successful in the decolourization of dyes and effluents, even when used alone (D'Souza *et al.*, 2006).

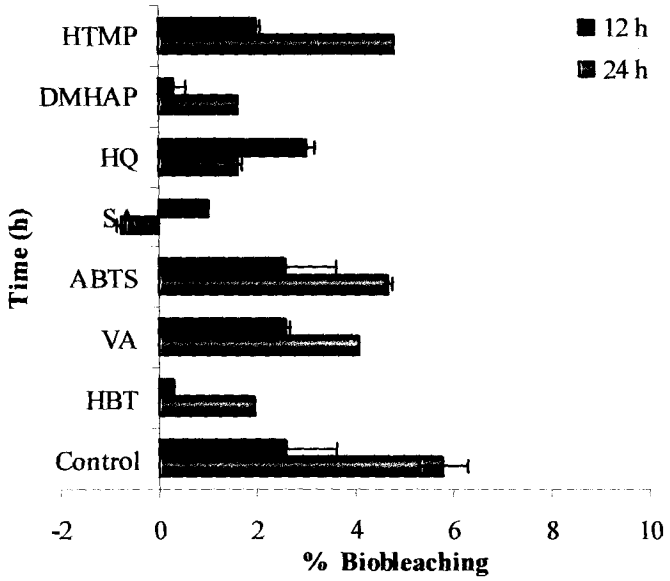


Fig. 7: Biobleaching (%) of sugarcane bagasse using 1000 U of crude laccase in the presence of the mediators was determined after 12 and 24 h, at pH 3 and 60°C. The control contained only crude laccase.

The medium employed did not induce the production of celullases. The HQ-supplemented bagasse showed maximal biobleaching after 12 h (Fig. 7), however at 24 h a high degree of repolymerization was observed. From the mediators; HQ and SA-supplemented bagasse showed repolymerization whereas although the other mediators showed continued depolymerization, they were not efficient enough as compared to the control containing only laccase, showed a stable trend of delignification up to 24 h which was more than any of the mediator supplemented samples, with the maximum amount of biobleaching. However, the mediators VA & ABTS showed roughly similar, albeit lower biobleaching than the control.

It was observed that the biobleaching continued steadily up to 24 h followed by mild repolymerization till 48 h, and then continued depolymerization which stabilized at 60 h and remained so till 96 h (Fig. 8).

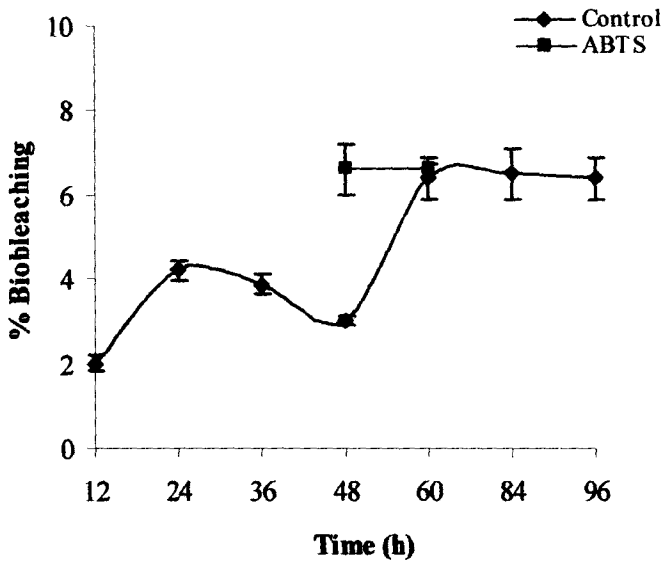


Fig. 8: Biobleaching (%) of sugarcane bagasse using 1000 U of crude laccase determined at pH 3 and 60°C at various time intervals. The mediator, ABTS was added at 36 h, after the repolymerization reaction began after 24 h.

Since repolymerization was first observed at 36 h, ABTS was immediately added as a mediator supplement to curb the repolymerization process. It was chosen among the mediators due to its maximal and consistent aid in depolymerization. Addition of ABTS helped to curb repolymerization and decreased the time required to achieve maximal

biobleaching by 12 h. Laccase at 10, 20 and 30 U with and without ABTS supplementation were screened for their ability to delignify bagasse, at 12 h intervals (Fig. 9). The ABTS-supplemented 10 laccase U showed maximal biobleaching in half the time as compared to that of the control (only 10 laccase U, no ABTS).

Where 20 laccase U were used, the control (only 20 laccase U) was able to biobleach the bagasse to a greater extent than the ABTS-supplemented 20 laccase U. After which, slight repolymerization was experienced which got stabilized by 60 h. The control and the ABTS-supplemented laccase observed the same trend for biobleaching, however the control was more efficient than the ABTS-supplemented laccase.

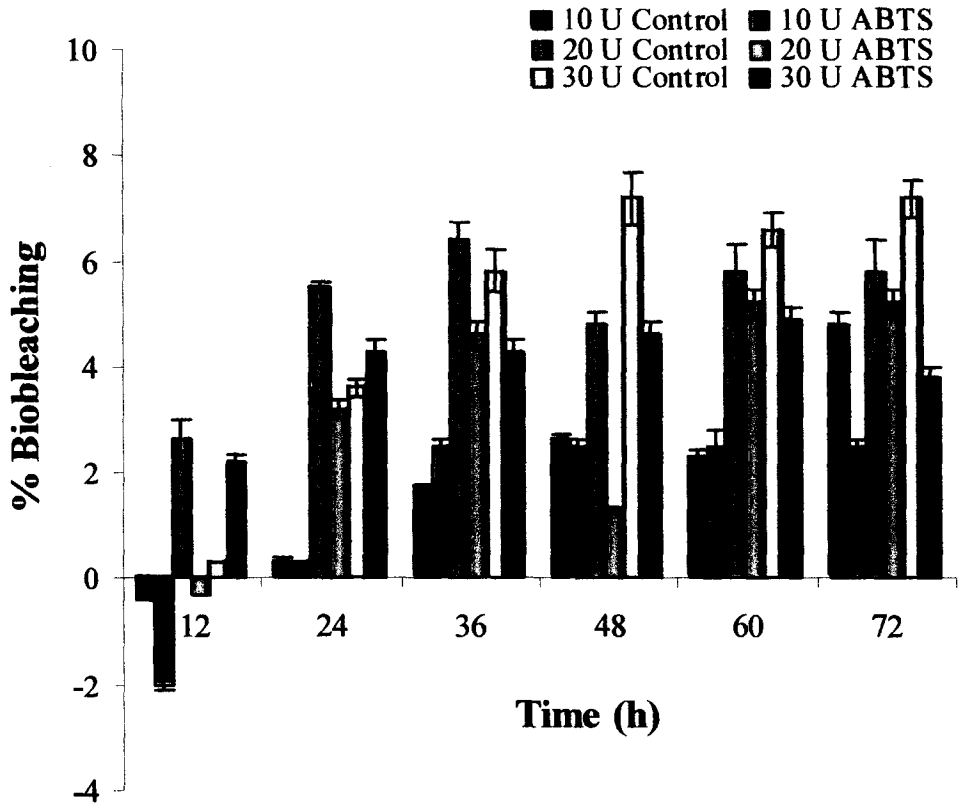


Fig. 9: Biobleaching (%) of sugarcane bagasse determined at various time intervals using 10, 20 and 30 U of crude laccase with and without the mediator ABTS, at pH 3 and 60°C.

When 30 laccase U were used, the control (only 30 laccase U, no ABTS) and the ABTS-supplemented 30 laccase U behaved in similar fashion to the 20 laccase U and its ABTS-supplemented 20 laccase U. Here however, the difference between the control and the ABTS-supplemented 30 laccase U was more pronounced.

HTMP-, HBT- and ABTS-supplemented 20 laccase U initially supported minimal biobleaching which later got reversed (Fig. 10). In general, the mediators that were capable of bringing effective biobleaching were HBT and DMHAP. Among these, HBT showed repolymerization after 12 h followed by an increase in biobleaching.

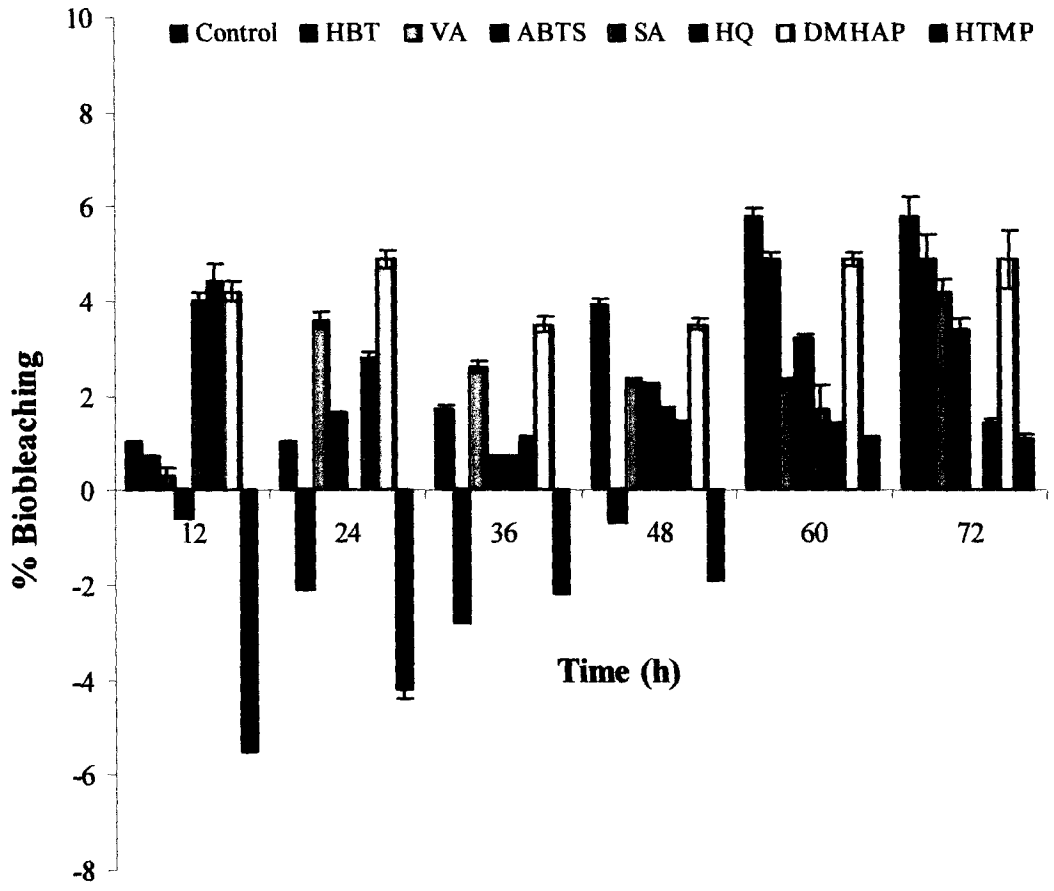


Fig. 10: Biobleaching (%) of sugarcane bagasse determined at various time intervals using 20 U of crude laccase in the presence of mediators added at 0 h, at pH 3 and 60°C. The control contained only crude laccase.

Mediators such as HTMP, HBT and ABTS when added at 0 h, showed a high initial repolymerization, which decreased over a period of time (Fig. 10). The same mediators

when used with the same amount of crude laccase (20 U), did not show any repolymerization when added after 6 h of biobleaching had taken place with the enzyme alone (Fig. 11). This proved to be successful since the repolymerization was avoided.

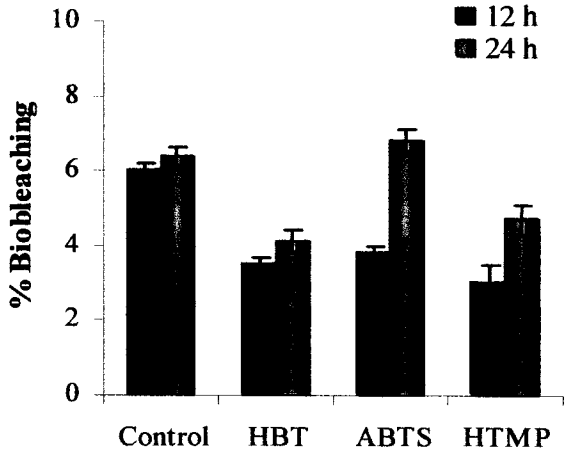


Fig. 11: Biobleaching (%) of sugarcane bagasse determined at 12 and 24 h at pH 3 and 60°C, using 20 U of crude laccase in the presence of mediators; HBT, ABTS and HTMP, added at 6 h. The control contained only crude laccase.

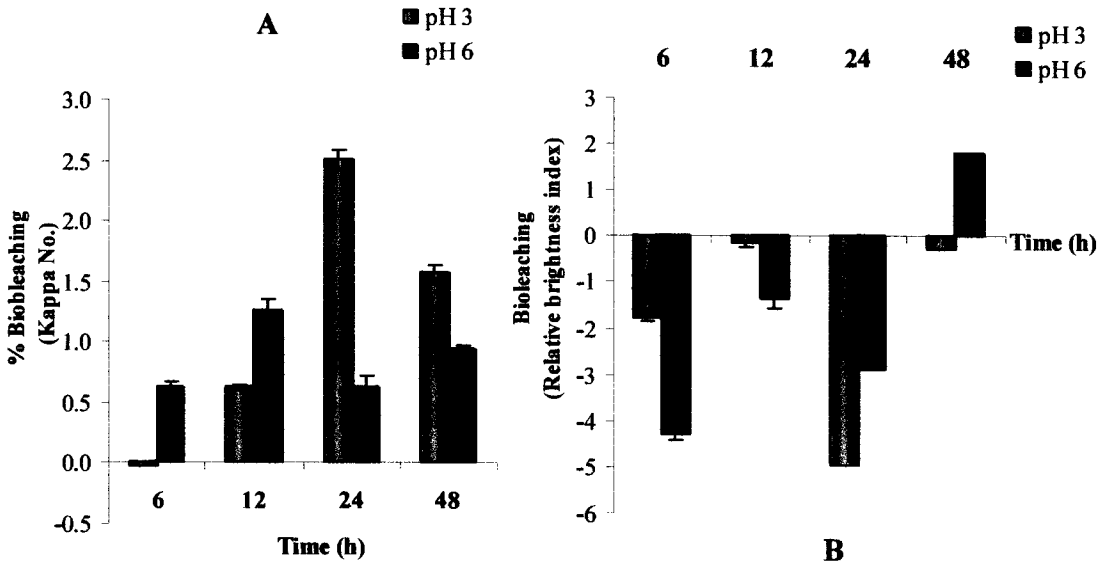


Fig. 12: The effect of 45 effective U of crude laccase on the biobleaching of sugarcane bagasse determined at various time intervals at 60°C at pH 3 and 6 and by (A) Kappa number and (B) Brightness index method. The control was untreated dry bagasse.

The two methods for biobleaching estimation, the kappa number method and the brightness-reflectance method gave opposing results (Fig. 12) with the exception of 48 h reading, although they reflected the same trend.

5.5 DISCUSSION

In the dye decolourization studies done by Ramsay and Nguyen (2002), the authors found that LNM was more toxic than the dye itself. An organic medium such as B&K yielded a very high titer of laccase even in the presence of 25 ppt seawater, which was not achieved in an inorganic and economically expensive medium such as LNM (as seen in Chapter 4). The inclusion of seawater was imperative since industrial effluents often contain large amounts of salts. To be able to function under such conditions and still produce appreciable decolourization was the objective of using a marine-derived fungus in these studies.

Even after active growth and decolourization of the dyes and effluents, the pH of the B&K medium which was initially 7.5 did not fall below 6, due to the buffering action of the seawater. This also indicated that the decolourization observed was not due to acidification of the medium, which is often the cause of many dyes to be converted into their leuco-form (they lose colour), which can readily be reconverted upon readjusting the pH of the medium. Several of the dyes tested for decolourization in fact induced laccase production.

Most of the dyes and effluents added to the culture medium were decolourized and simultaneously acted as inducers. Among these, brilliant green induced highest laccase production and its decolourization was proportional to laccase activity. This was followed by 10 % TeB which contained 8 reactive dyes induced maximum laccase production (among the effluents), it showed a high correlation between laccase activity and decolourization. The larger amount of TeB decolourization in comparison to TeA can be accounted for due to its reactive blue base and pH of 2.5. Reactive blue dyes include anthroquinone and diazo dyes, both of which have shown good decolourization by these laccases, its pH is also around the optimum pH for activity of these laccases

which is 3 whereas TeA was mainly composed of Azo dye-20 and had a pH of 8.9. From the results, it was observed that mono-azo dyes showed minimal decolourization and the alkaline pH was almost equal to the pH at which MTCC 5159 laccase was most stable (pH 9) with no activity. Here TeA did not induce laccase activity greater than the control even after 6 days, neither did a significant co-relation exist between its decolourization and laccase production. Any decolourization that occurred may be due the presence of enzymes working either alone or in conjunction with laccase. Even though TeA, induced considerable laccase activity on day 8 after effluent addition, it was unlikely that it would have made a difference in its decolourization, since this effluent was mainly composed of an azo dye which has shown to be more or less resistant to these laccases.

When TeB was used at 1 % (Chapter 4), laccase production was increased by ~100-fold, far greater than even brilliant green. Textile effluent B is mainly composed of reactive blue dyes which include anthroquinone and diazo dyes, both these dyes although get decolourized but anthroquinone dyes do not seem to induce laccase production whereas diazo dyes are good laccase inducers (Bhatt *et al.*, 2005). Hence, its acidic pH and the presence of diazo reactive blue dyes probably make TeB a good laccase inducer. What component of this mixture of dyes was actually involved in inducing such high laccase titers merits further investigation since the composition of effluents do not remain constant. The complete decolourization of MSW to some extent may be accounted for by its acidic pH of 4.3. High production of ligninolytic enzymes during treatment of paper mill effluents was reported in *Trametes versicolor* (Manzanares *et al.*, 1995).

The mechanism of laccase-catalyzed dye decolourization can differ depending upon dye structure. The white-rot fungus *Trametes versicolor* was shown to use anthroquinone dyes as direct substrates for its laccase. In this study however, although the anthroquinone dye, remazol brilliant blue R, got decolourized by 50 % within 6 days yet no laccase activity was detected, not even the basal amount induced by the medium itself. Obviously, its decolourization was due to enzymes other than laccase or by some other mechanism. Similar results have been obtained by (Vyas & Molitoris, 1995) who shown that a oxygenase called 'RBBR oxygenase' was implicated in the decolourization of RBBR which is distinct from MnP, LiP and laccase.

Azo and indigo dyes have been shown to be indirect substrates (non-substrate) i.e. their decolourization may be dependant upon small molecular weight

metabolites being secreted into the culture medium (Wong & Yu, 1999). These metabolites mediate interactions between laccase and dyes, which under normal conditions would not have been degraded by laccase. The decolourization rate of the non-substrate dyes was shown to be limited by the concentrations of mediating compounds rather than laccase activity in the culture filtrate (Wong & Yu, 1999). In the present study, among the synthetic dyes, it was found that brilliant green and aniline blue induced the highest laccase production and were decolourized to a greater extent than other dyes. Crystal violet on the other hand induced moderate amounts of laccase which was sufficient to bring about its decolourization which also showed good correlation to laccase activity. Poly-R 478 has been used for screening for the presence of peroxidases since it generally is decolourized by MnP and LiP whereas laccase only slightly modifies this dye (Wesenberg *et al.*, 2003) This fungus preferentially produces laccases which explains why Poly-R 478 was unable to induce laccase and whatever laccase was produced was probably due to the medium alone.

Although congo red, a secondary diazodye did not show any correlation with laccase activity, it still showed appreciable laccase induction. There are two explanations for this occurrence;

1) An enzyme other than laccase is induced by congo red and is responsible for its decolourization.

2) Although laccase may still be largely responsible for the decolourization of congo red, congo red might not be a direct substrate of laccase and its degradation may involve the production of a metabolite which is the substrate of laccase and effects the decolourization of congo red.

Similar results with congo red were obtained earlier (Wong & Yu, 1999). In the present study however, trypan blue, a diazo dye showed appreciable laccase induction and correlation with laccase activity. On the other hand, even though RO 16, a monoazo dye induced moderate laccase activity, its decolourization was negligible at ~ 20 %. (Svobodová *et al.*, 2007) have shown that laccase in combination with MnP was very successful in decolourization of RO 16. From these results, it is obvious that great variation exists within a single group of dyes such as the azo class in eliciting laccase

production and effecting its own decolourization. Apparently, the diazo group is far more successful in inducing laccase and mediating its own decolourization compared to the monoazo group. It has also been shown that batch decolourization processes of azo dyes using laccase cannot be attained. The application of laccases alone in bioremediation has been limited due to the occurrence of polymerization reactions. These authors have shown that laccase modifies azo dye structures by destroying their chromophoric structure, observed visually as a reduction in colour. However, over long periods of oxidation, the products obtained during the degradation reaction can undergo further reactions leading to couplings between themselves or with the unreacted dye, producing a large amount of coupled products leading to a darkening of the solution. This could explain the occurrence of repolymerization, within 4 days in congo red, a diazo dye and TeB (10 & 20 %), a textile effluent containing a mixtures of several dyes. Immobilization of the enzyme has been proposed to avoid such coupling reactions (Zille *et al.*, 2005).

Triarylmethane dyes are the parent structures of triphenyl methane dyes. In this study, the triaryl methane and the triphenyl methane dyes showed good laccase production and correlation between laccase titer and decolourization as well, indicating that these dyes are not only good inducers of laccases of MTCC 5159 but are good substrates as well. Similar results were seen with methylene blue which is a phenothiazine dye.

The cell-free culture filtrate was successful in decolourizing MSW and TeA, the amount of decolourization decreased with increasing concentrations of effluent. However for BL, increasing concentrations resulted in increased decolourization, this may be due to the increased presence of phenolics at higher concentrations of BL which acts as laccase inducers (Ali & Sreekrishnan, 2001). At lower concentrations of BL, the concentration of phenolics may not be sufficient to induce laccase production.

Presence of LiP and MnP, the other major lignin-degrading enzymes play an important role in dye decolourization. In *Cerrena unicolor* MTCC 5159, laccase was the major LDE produced. Similarly laccase as the major LDE was reported with *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996b) *Phlebia tremmellosa* (Robinson *et al.*, 2001a) and *Pleurotus sojarcaju* (Chagas & Durrant, 2001). The white-rot fungus *Clitocybula dusenii* producing laccase as its major LDE, also showed decolourization of industrial textile dye effluents (Wesenberg *et al.*, 2002).

D'Souza *et al.*, (2006) have showed that decolourization of several dyes and effluents could be achieved within 6 - 12 h by incubating with the laccase-containing mycelia-free culture supernatant. However, on incubation of the culture supernatant with TeA and TeB, reduction in colour was seen within the initial 2 h after which there was no further reduction in colour. Addition of redox mediators may improve this situation as was reported in the case of pure laccase from a commercial formulation (Soares *et al.*, 2001). The EPS produced by white-rot fungi is reported to play an active role in lignin degradation (Bes *et al.*, 1987). In concurrence in majority of the cases, the EPS produced by this fungus was more efficient in decolourization than the culture supernatant. Even RO 16 and Poly-R 478 that were not decolourized when added to the growing culture were totally decolourized by the EPS. This might be due to the combined effect of adsorption and enzymatic decolourization, since EPS is known to trap extracellular enzymes within its matrix (Wingender *et al.*, 1999).

The medium had been optimized to almost exclusively support laccase production (Chapter 4), cellulase was not induced under the present medium conditions, which makes this especially suitable for use in the biopluping process, where the lignin is selectively removed without weakening the cellulose fiber (Bernier *et al.*, 1992).

Biobleaching is an environmentally friendly alternative to harsh chemical treatments such as chlorination of paper pulp. Xylanases are one of the most prominent enzymes involved in this process and reduces the total requirement for chlorine (Raghukumar *et al.*, 2004). Laccase is also an enzyme that achieves this goal however, the economics of laccase production and stability need to be considered before applying this technology on a large scale. Mediators have been shown to increase the effectiveness of the enzymes. However, the problem encountered when dealing with laccase is that besides achieving biobleaching of pulp via depolymerization, it can also catalyze the reverse action, i.e. repolymerization, which is counter productive to the biobleaching process. While utilizing it for biobleaching, this fact requires to be taken into consideration and the entire process merits careful observation to determine the minimum yet effective amount of laccase required in bringing about large amount of biobleaching within the shortest possible period. This also includes the time at which repolymerization occurs and steps required to be taken to avoid it.

From the biobleaching experiments it was observed that if laccase was supplied in excess, the need of mediators to enhance biobleaching could be done away with. The economics of whether low amounts of laccase supplemented with effective low-cost mediators are better than larger amounts of laccase alone, needs to be investigated. If the former option is more economical then, the type and concentration of mediator and the time of its addition requires to be standardized along with laccase concentration prior to large-scale biobleaching.

From Fig. 7, it is clear that when crude laccase was used at 1000 U, the requirement of mediators became superfluous. None of the mediators could match the biobleaching brought by the enzyme alone, although ABTS and HTMP came comparatively closer. At such high amounts of laccase, mediators were either antagonistic or at the least, non-responsive.

The previous experiments were carried out using laccase at a 1000 U, which is not commercially viable whereas laccase at 10 to 30 U is. Thus, laccase at 10, 20 and 30 U with and without ABTS supplementation were screened for their ability to delignify bagasse. Here the ABTS-supplemented 10 laccase U and its respective control, on an average achieved similar levels of biobleaching. Even though at this concentration of laccase, the effect of mediators is clearly visible, the total biobleaching achieved was not sufficient. When 20 laccase U were used, the control was able to bleach the bagasse to a greater extent than the corresponding ABTS-supplemented laccase within the same period although both followed the same trend. This indicated a slight excess of enzyme. The difference between the control and the ABTS-supplemented 30 laccase U was more pronounced than with the 20 laccases U, indicating a definite excess of enzyme. Laccase at 20 U was the minimum concentration required to achieve sufficient biobleaching without becoming too superfluous.

Mediator-supplemented 20 laccase U such as HTMP, HBT and ABTS showed minimal biobleaching initially but this got reversed with time (Fig. 11). The mediators which resulted in effective biobleaching in majority of the cases, were HBT and DMHAP. Among these, HBT showed repolymerization after 12 h followed by a steady increase in biobleaching. To determine whether this repolymerization could be halted by manipulation of the time of addition of the mediators; HTMP-, HBT- and ABTS which showed high initial repolymerization were added after 6 h of biobleaching

had taken place instead of at 0 h. This change in time of addition of the mediators eliminated the problem of repolymerization. However, in most of the above experiments, regardless of the amount of laccase used, the presence of mediators seemed to be superfluous. Crude laccase preformed well and for most of the time was better than if supplemented with mediators. Only when repolymerization began, the addition of mediator seemed to help in maintaining biobleaching by reversing the repolymerization action. Thus, we can infer that not only does the concentration of laccase determine at what time and to what extent biobleaching occurs but also the time of addition of the mediator is critical to avoid repolymerization and this too, is dependant of the type of mediator used.

In Fig. 12, although the effective laccase U were equal in pH 3 and 6, since at pH 3 the activity was double that at 6, the amount of enzyme used was roughly half (calculated by precise spectrophotometric measurements at both the temperatures). After the initial delay in removal of lignin, the greater efficiency of the enzyme at pH 3 is obvious in the latter half of the experiment (Fig. 12 A). However, this breakdown of lignin due to the presence of laccase does not translate into increased brightness of the pulp as observed in Fig. 12 B. This may be since the experiment was carried out for only 48 h and success in increasing the brightness was observed only at 48 h. Brightening of the pulp may have been achieved, if incubated for longer periods with the enzyme.

However, it is to be noticed that the pH that supported maximal biobleaching or removal of lignin at any given time (Fig. 12 A), supported minimum brightness (Fig. 12 B). Brightness and residual lignin in terms of kappa number are both assays for biobleaching and should in theory, if biobleaching had occurred, give at least a similar trend. However, this was not the case in the above experiment. These contradicting results are probably due to the fact that although lignin has been degraded by laccase, reflected in lower kappa number the degradation products which are also coloured aromatics have not been completely removed before 48 h which prevent the brightness of the pulp from increasing. Sometimes these products may repolymerize to give more intensely coloured products than the original lignin, thus decreasing brightness (Zille *et al.*, 2005). This repolymerization can be avoided by using laccase in conjunction with MnP (Svobodová *et al.*, 2007).

Biobleaching cannot be determined by the traditional 'kappa number' method alone, since even though this method is precise in monitoring the percent lignin in the sample and detects polymerization and depolymerization as well, it may not be a true reflection of the actual biobleaching that takes place. This is because some of the lignin molecules which may have been altered by the enzyme and hence not estimated by this method, through coupling reactions result in a darkening of the sample, this may or may not be recorded by the kappa number method. It would be better to confirm the action of biobleaching with the 'brightness index' test, which is a spectrophotometeric reflectance reading.

Laccase is the major extracellular enzyme of *Cerrena unicolor* MTCC 5159 responsible for decolourizing majority of the dyes and effluents containing dyes of varying structures. Some dyes such as triarylmethanes and the triphenylmethanes are excellent substrates of laccase and they induce laccase production, as well. Many dyes are not the direct substrate of laccase such as some diazo dyes and their degradation depends on the presence of some small molecule metabolites or its own degradation products which bring about further decolourization. The production of the small molecule metabolites by MTCC 5159 may not be proportional to the release of laccase, the decolourization of such dyes cannot be correlated with laccase activity.

Industrial effluents contain a consortium of dyes, whose composition varies on a regular basis. These effluents may contain dyes which are good substrates or non-substrates or indirect substrates of laccase, however in such a consortium, the substrate dyes in industrial effluents may mediate the interaction between laccase and other non-substrate dyes effecting the decolourization of both, substrate as well as non-substrate dyes and also act as inducers for more laccase. The decolourization rates of non-substrate dyes, which otherwise would never have been decolourized could now be significantly improved by this mechanism. Further investigation should be conducted on the relationship between specific laccases which have commercial viability and dyes structures, since the preferences for substrates vary with the type of laccase. Also the potential of dyes to act as mediators and the kinetics of the mediator-involved dye degradation of non-substrate dyes and laccase also merit intense investigation (Wong & Yu, 1999).

In conclusion, the marine-derived *Cerrena unicolor* MTCC 5159 decolourized several synthetic dyes and effluents in the presence of sea salts. Cell-free

culture supernatant containing laccase or EPS precipitated from the culture medium were also very successful in the decolourization process. It was also able to bring about biobleaching without the need of large amounts of mediators. This fungus appears to be a good candidate for industrial application in bioremediation of coloured waste waters in the presence of chlorides and sulphates as well as in biobleaching.

Chapter 6

Summary

The oceans are the last frontier on Earth that has yet to be fully explored. Marine life holds great promise for the isolation of novel and useful compounds and metabolites. Marine sponges have been touted as the single most important source of novel marine metabolites. Obligately marine and marine-derived fungi too hold great potential as a source of such compounds or at least similar metabolites but with novel properties that give them a commercial edge, their enzymes which are salt tolerant especially hold great promise.

The object of the present study was to isolate and identify obligate marine or marine-derived fungi that were hyper-laccase producers. The laccase from the selected marine-derived fungus was to be characterized and its application in bioremediation and biobleaching, studied. These objectives were fulfilled in the thesis.

Seventy-five fungi were isolated from decaying lignocellulosics from the mangrove swamps of Chorað Island in Goa, using techniques such as particle-plating, hyphal and single spore isolation methods. Among these, fifteen isolates were found to be laccase producers. Out of these, three anamorphic fungi proved to be the most efficient dye decolourizers and laccase producers and were designated NIOCC #2a, NIOCC #7a and NIOCC #Xa. Among these, NIOCC #2a was the laccase hyper-producing strain selected for this study, since it was superior in terms of both laccase production and dye decolourization. It was deposited at the Microbial Type Culture Collection (MTCC) under accession no. 5159 and identified using rDNA homology to be a *Cerrena unicolor* strain. The fungus NIOCC #2a is therefore referred to as *Cerrena unicolor* MTCC 5159. Its marine origin was confirmed by achieving maximum growth in full strength (34 ppt) seawater and maximum laccase production in 25 ppt seawater.

Crude laccase, contained a consortium of laccase isozymes with differing surface charges and molecular masses. It collectively showed optimum activity at pH 3 and 60°C. Out of these isozymes, an isozyme, 'Lac IId' was purified to homogeneity with a final yield of 17 %. Its UV-visible spectrum showed a shoulder at 330 nm and peak at 610 nm which is characteristic of blue laccases. The N-terminal and an internal peptide sequence, both deposited under the accession no. P85430 in the 'Uniprot' database confirms Lac IId to be a blue laccase. Lac IId has a molecular mass of 59 kDa and a pI of 5.3 with 17 % *N*-linked glycosylation. This glycosylation besides playing a structural role

also confers upon Lac IId, resistance to proteases, which are also produced by *Cerrena unicolor* MTCC 5159 in copious amounts (data not shown).

Lac IId showed optimum activity at pH 3 and 70°C and a half life of 90 min at 70°C. It retained more than 60 % activity up to 180 min at 60°C and pH 9. Even after 1 year of storage at -20°C, no loss in activity was observed. This coupled with its extremely low energy of activation (2.5 kJ mol^{-1}) at pH 3 recorded between 60° to 70°C, attests to its long shelf life and thermostability. Lac IId could catalyze the oxidation of a wide range of substrates and its substrate range increased in the presence of certain mediators. Of all the inhibitors tested, only azide was able to effectively inhibit it up to 95 % of its original activity. Pure sodium chloride up to 0.3 M, did not inhibit it above which, the laccase was reversibly inhibited. From the wide range of metal ions tested, Lac IId was maximally inhibited by 56 % with chromium whereas the other metals showed lesser inhibition.

After having confirmed the commercial potential of laccases from this fungus, *Cerrena unicolor* MTCC 5159 was subjected to medium optimization to maximize laccase production. It was found that carbon and nitrogen act antagonistically with each other with respect to laccase production, when nitrogen is used in an inorganic form. A combination of low inorganic nitrogen and high carbon concentrations favour good biomass and laccase production. When nitrogen was supplied in the inorganic form but in low concentrations, laccase titer was maximal. However, organic nitrogen at much higher concentrations also supported good laccase production. Buffering action of citrate-phosphate buffer was required to decrease the temporal variation in laccase titer. Inducible laccases are produced in the idiophase only after addition of an appropriate inducer. Known inducers like $CuSO_4$ induced maximum laccase activity (~84,000 U L^{-1} by 21 days of growth). However comparable activity had been obtained with raw textile effluent B when used at 1 % (~86,000 U L^{-1} by 12 days of growth). This is economically lucrative. Only a single addition of inducer effectively aided in inducing laccase, it is especially effective if added after sufficient growth had occurred. It was observed that pure sodium chloride cannot replace seawater for growth of the fungus and that oxygenation enhances laccase production. Addition of a surfactant such as Tween 80 at 0.25 % after sufficient growth had already occurred positively impacted both biomass and laccase production. At higher concentrations however, the specific activity of laccase

decreased. The medium is designed to almost exclusively promote and maximize laccase production with a high specific activity. The surface to volume ratio of culturing flasks is also a critical factor for biomass and laccase production and requires to be taken into consideration.

The medium had been optimized to almost exclusively support laccase production, cellulase was not induced under the present medium conditions, which makes this especially suitable for use in the biopulping processes. When applied in biobleaching of sugarcane bagasse, it was found that these laccases are quite self sufficient even at low concentrations and do not require the presence of mediators. However, the timely addition of the right kind of mediators, as soon as repolymerization begins not only reverses this process but also reduces the time required to attain the same level of biobleaching.

The whole culture of *Cerrena unicolor* MTCC 5159 and its lignin-degrading enzymes especially laccases along with the exopolymeric substance (EPS) work together in the decolourization processes. The fungal mycelia and the EPS decolourize using processes such as active and passive adsorption and absorption whereas the lignin-degrading enzymes actively degrade the colourants.

Triarylmethane and triphenylmethane dyes are not only good substrates of these laccases but also good inducers of them. Diazo dyes are preferentially degraded over monoazo dyes and also act as laccase inducers. However, the decolourization of some of the diazo dyes may not be proportional to the laccase activity. The simple diazo dyes such as trypan blue are good laccase inducers and their decolourization is proportional to laccase activity. Whereas although congo red, a secondary diazo dye gets decolourized to a similar extent as trypan blue, its decolourization is not proportional to the laccase activity it induces. An anthroquinone dye such as remazol brilliant blue R gets decolourized by 50 % within 6 days, no laccase activity is detected in its presence. Its decolourization is possibly due to the presence of enzymes other than laccase.

Cerrena unicolor MTCC 5159 has a great potential in biobleaching processes as well as decolourizing dyes and industrial effluents and possesses at least one laccase which is of commercial importance.

Chapter 7

Future Prospects

Production of metabolites and enzymes in fungi can be increased by several methods. The most common and easiest way is to vary the nutritional and physicochemical parameters which influence product synthesis (Mundra *et al.*, 2007; Soni *et al.*, 2007). Directed evolution is a technique wherein a gene that codes for a particular product is expressed in another host which has a high doubling rate and capacity for successfully translating the inserted gene as seen in the expression of a fully functional laccase from *Myceliophthora thermophila* in *Saccharomyces cerevisiae* (Bulter *et al.*, 2003).

Genetic transformation to increase the output of the original producer organism and make it a hyper producer has also been achieved. In *Aspergillus terreus*, the production of itaconic acid was elevated by the introduction of a gene from *Vitreoscilla stereocoraria* that codes for *Vitreoscilla* hemoglobin (Vhb). The Vhb aided in scavenging oxygen in periods of lowered oxygen tension thus maintaining the production level of itaconic acid, even under oxygen limitation (Lin *et al.*, 2004).

The present study emphasizes the industrial applications of *Cerrena unicolor* MTCC 5159 and its enzymes such as a high quality laccase. This fungus has maximum homology with marine sponge-derived basidiomycetes, suggesting that it has probably been associated with the marine environment long enough for secondary adaptations to occur which are reflected in the ITS rDNA region, making these adaptations stable and capable of being vertically transferred. Fungi which have undergone such adaptations in the marine environment are lucrative for identifying novel marine metabolites and marine-sponge-derived fungi are one of the most important sources for discovering novel marine metabolites (Jensen & Fenical, 2002). This fungus also produces large amounts of exopolymeric substance which is capable of extensive dye decolourization (D'Souza *et al.*, 2006) which merits further investigation. There is an immense scope for further study with regards to this fungus and marine fungi in general.

To determine the type of laccases produced by *Cerrena unicolor* MTCC 5159, the following work was carried out. Degenerate primers which amplify a 200 bps region between the conserved copper binding regions I and II of the laccase gene from white rot and brown rot terrestrial fungi (D'Souza *et al.*, 1996) were used on MTCC 5159. The sequenced region was found to be different from terrestrial laccases (data not included in the thesis). Since this region corresponds, to roughly only 50 AAs, less than

$1/10^{th}$ the actual size of laccase. Finding the laccase from the plethora of isozymes produced by this fungus to which this region corresponds, is a daunting task, especially keeping in mind the low expression level of several of the isozymes.

To first identify a laccase which has desirable properties such as thermotolerance, salt and metal tolerance and then sequence it is a more practical approach. One such a laccase isozyme 'Lac IId' from *Cerrena unicolor* MTCC 5159 has been successfully identified and characterized. Two peptide sequences namely the N-terminal peptide and an internal peptide which is situated near the C-terminal end, have been identified. Using the sequences of these two peptides, primers can be designed which scan the entire length of the gene by various modifications of the PCR technique.

Another alternative is by using the two sets of DNA primers I have designed (data not included in the thesis) which target laccases from terrestrial and marine basidiomyceteous fungi. One set of primers contain a forward and reverse primer which target the conserved copper binding region I and IV and the other set targets the conserved copper binding region II and IV. Since the conserved copper binding region, IV is at the distal end of laccase, more than $4/5^{th}$ the laccase gene can be sequenced using the traditional PCR technique. This region will also include introns, which can be spliced *in-silico*. Properties such as thermostability and substrate range can be tested for *in-silico* and the most favourable sequence can then be isolated from the laccase gene pool of *Cerrena unicolor* MTCC 5159 by designing specific primers.

Once the sequence is obtained, the entire laccase gene can be transformed into a suitable host which has a short replication time and suitable post translational machinery required for glycosylation of laccase. Fully functional laccase can then be produced on a large scale for industrial and bioremediation purposes.

Transformation of the laccase gene to another host does not utilize the potential of *Cerrena unicolor* MTCC 5159. This fungus also produces lignin-degrading enzymes other than laccase such as MnP and LiP. Their titer was low in the medium used since the entire machinery of the fungus was routed to produce laccase via medium modification. The titer of these enzymes (MnP and LiP) may be enhanced by simply including manganese and veratryl alcohol in the medium and reducing the level of copper to a bare minimum. Medium modification with the intention to increase the MnP or LiP titer requires further investigation. Besides laccase, MnP and LiP, it is worth while to

screen for the production of other commercially important enzymes and metabolites from this fungus.

The decolourization and delignification capability of MTCC 5159 has been well documented in this thesis (Chapter 5). However, the toxicity and other effects of the resulting degradation products requires further investigation before the fungus can be used for bioremediation purposes.

Oxygenation has been known to enhance production of many secondary metabolites (Zhu *et al.*, 2006) in fungi. It has been observed that oxygenation greatly increases laccase production, an enzyme generally produced in the idiophase. Oxygenation of the culture is an expensive and laborious process. If the culture itself had the innate capability to scavenge available oxygen, external oxygenation could be avoided or reduced. This can be accomplished by transforming *Cerrena unicolor* MTCC 5159 with an oxygen scavenging gene such as *Vitreoscilla* hemoglobin gene (Vgb) to make it self sufficient. The effects of *Vitreoscilla* hemoglobin (Vhb) have been well documented. In the wild, *Vitreoscilla stereocoraria* produces Vhb under oxygen limiting conditions (Boerman & Webster, 1982) which captures oxygen and feeds it to the terminal oxidase (Wakabayashi *et al.*, 1986).

It has been successfully transformed into filamentous fungi (Lin *et al.*, 2004) as well as in basidiomyceteous yeast to increase cell density and to enhance metabolite and enzyme production by enhancing oxygen uptake (Zhu *et al.*, 2006). However, *Vitreoscilla stereocoraria* the source of the gene is a prokaryote if this prokaryotic gene is introduced into eukaryotic *Cerrena unicolor* MTCC 5159, it may experience a codon bias. Codon bias is the unequal use of synonymous codons for encoding amino acids and has been found across kingdoms, this bias varies among organisms and even within the genes of the same organism. It is thought that optimal codons help to achieve faster translation rates and high accuracy. As a result of these factors, translational selection is often stronger in highly expressed genes. Whereas in organisms that do not show high growing rates or genes that are minimially expressed, codon usage optimization is normally absent. Codon usage differs greatly between prokaryotes and eukaryotes, this difference is more pronounced between a basidiomycete, which use GC rich codons instead of the AT rich codons used by prokaryotes (Codon Usage Database;

http://www.kazusa.or.jp/codon). This could result in low Vgb translation which may not meet the critical concentration required for effective oxygen scavenging.

To overcome this, *Vitreoscilla stereocoraria* strain C1 was used as the source of Vhb with superior oxygen scavenging capabilities. The Vgb sequence of this organism was corrected for codon bias such that this gene should be optimally expressed in basidiomycetes, more specifically with *Phanerochaete chrysosporium* (most well documented basidiomycete). Once transformed, growth and secondary metabolite production of the basidiomycete should be enhanced. This gene has already been synthesized by recursive PCR and deposited at National Center for Biotechnology Information (NCBI) under accession number DQ787436. It has been transformed into p416 vector containing a fungal glyceraldehyde-3-phosphate dehydrogenase, (GPD) expression promoter. This promoter-gene cassette requires to be tagged with a reporter gene for easy identification of clones with the fully functional Vhb. After which it can be transformed into *Cerrena unicolor* MTCC 5159. This should result in enhanced growth or normal growth under lowered oxygen tensions as well as up-regulation of several secondary metabolite / enzyme-coding genes.

It would be worth while to complete these studies to obtained high quality and greater yields of metabolites and enzymes from this fungus.

Appendix

8.1 MEDIA

8.1.1 Low nitrogen (LN) (Tien & Kirk, 1988) **modified medium:** Glucose and glycine at 3.75 % and 0.5 % concentration respectively were added to a mineral salts base which contained the following components; 0.0001 %, 7 % & 10 % final concentration of thiamine, trace elements solution and basal salts solution respectively. The pH was adjusted to 4.5 using citrate phosphate buffer. The volume was brought up to 100 mL using either DW or SW, as per the requirement.

8.1.1.1 Basal salts solution: KH_2PO_4, $MgSO_4$ and $CaCl_2$ at 2 %, 5 % & 0.1% respectively, the final volume was made with DW.

8.1.1.2 Trace elements solution: 1.5 g nitrilic triacetate was dissolved in 800 mL distilled water and the pH was adjusted to 6.5 using 1 N KOH to which, the following components were added:

$MgSO_4$	3 g
$MnSO_4$	0.5 g
NaCl	1 g
$FeSO_4.7H_2O$	0.1 g
$CaCl_2.6H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g
$CdCl_2$	0.08 g
$AlK(SO_4)_2.12H_2O$	0.01 g
H_3BO_3	0.01 g
$Na_2MoO_4.2H_2O$	0.01 g

The solution was made up to 1 L with DW and stored in the dark.

8.1.2 Boyd and Kohlmeyer (B&K) medium (Kohlmeyer & Kohlmeyer, 1979): contained 1 % glucose, 0.2 % peptone and 0.1 %yeast extract. The volume was made up with either DW or SW or half strength (50 %) SW, as per the requirement. In case of solid medium, 1.8 % agar was used.

8.1.3 Malt extract broth (MEB): 2 % (w/v) of ready made malt extract broth powder (HiMedia, Mumbai) dissolved in either DW or SW or half strength (50 %) SW, as per the requirement. In case of solid medium, malt extract agar (MEA), 1.8 % agar was used.

8.1.4 Carrot juice modified agar (Hasan *et al.*, 1995): 10 % (v/v) filtered carrot juice and 0.5 % (w/v) yeast extract was added to LN medium base to which 1.8 % agar was added.

Ready-made media were from Hi-media (Mumbai, India) and other chemicals were from either Sigma or Merck and were of molecular biology or analytical grade as per the requirement.

8.2.1 ESTIMATION METHODS

8.2.1.1 Dissolved Oxygen Estimation (Wrinklers Method) (Eaton *et al.*, 2005)

A 300-mL glass Biological Oxygen Demand (BOD) stoppered bottle was placed below the water surface and filled up to the brim. Immediately without the introduction of air bubbles, 2 mL of $MnSO_4$ followed by 2 mL of KI-azide reagent was added. The bottle was then stoppered with care to ensure no air was introduced. The sample was mixed by inversion several times. A brownish-orange cloud of precipitate indicated the presence of dissolved oxygen. It was allowed to precipitate after mixing several times by inversion. Just above the surface, 2 mL of concentrated H_2SO_4 was added and the bottle was then stoppered and inverted several times to allow the precipitate to get dissolved by the acid. An aliquot of this treated sample (201 mL) was titrated with $Na_2S_2O_3$ using starch as indicator. The concentration of dissolved oxygen (1 mg L^{-1} dissolved oxygen) in the sample is equivalent to the amount of titerant (mL) used.

8.2.1.2 Chloride Content Estimation (Argentometric Method) (Eaton *et al.*, 2005)

The chloride content of the water sample was estimated via titration with $AgNO_3$. The $AgNO_3$ precipitates out the chlorides in the water sample due to the reaction of Ag^+ with the Cl^- present in the water sample to form a white precipitate of AgCl. Once all the Cl^- ions are used up, the Ag^+ then reacts with the CrO_4^{-2} of the indicator to form Ag_2CrO_4 which is a reddish brown precipitate. The end-point of the reaction is reached when a stable reddish brown precipitate is obtained.

To 5 mL of water sample, 5 mL of (0.35 %) K_2CrO_4 indicator solution was added. This was titrated against 0.2184 M $AgNO_3$ with constant stirring until a presence of a persistent orange colour occurred. The volume of $AgNO_3$ required for the colour change to occur was recorded.

CALCULATIONS:

0.2184 M $AgNO_3$ * vol of $AgNO_3$ (L) = 'x' mol $AgNO_3$ = 'x' mol Ag^+ = 'x' mol Cl^-

('x')*(35.45g Cl^- ÷ 1 mol Cl^-) = mass Cl^- = 'y' g of Cl^-

('y' ÷ mass of sample[†])*(1000) = ***z chlorinity (ppt)***

[†]*mass of sample* = volume of sample (mL) * density of sample[#]

[#] when the sample is sea water, the density of sea water is 1.05 g mL^{-1}

NOTE: 1.80655 Cl (ppt) = ***Salinity (ppt)***

8.2.1.3 Lignin Estimation (Kappa Number Method) (Anonymous, 1988)

STANDARDIZATION OF POTASSIUM PERMANGANATE ($KMnO_4$) SOLUTION

The burette was filled with $KMnO_4$ whose normality had to be determined. 20 mL of 0.1 N ferrous ammonium sulphate containing 0.2 % H_2SO_4 was taken in a conical flask to which, 10 - 15 mL of 1 M H_2SO_4 was added. This solution of ferrous ammonium sulphate was titrated by slowly adding small amounts of $KMnO_4$ from the burette with continuous mixing. The pink colour obtained upon addition of $KMnO_4$ solution disappears on mixing. The titration is continued until a permanent pale pink colour appears.

STANDARDIZATION OF SODIUM THIOSULPHATE ($Na_2S_2O_3$) SOLUTION

10 mL of 0.1N $K_2Cr_2O_7$ solution was taken in a conical flask to which, 20 mL of 1N HCl was added. To this mixture, 2 g of KI was added and mixed well. The mixture was kept in the dark for 10 min after which, it was titrated against $Na_2S_2O_3$ solution present in the burette. After adding about 3 - 4 mL of $Na_2S_2O_3$ or till the solution in the conical flask turned pale yellow, 1 mL of starch was added. The titration was continued till the end-point, which was bluish green.

PROCEDURE

To 0.1g of moisture-free sugarcane bagasse, 79.5 mL distilled water was added and mixed until the bagasse was completely wet. To this, a mixture of 10 mL (0.1N) $KMnO_4$ & 10 mL (4N) H_2SO_4 was added and mixed well. The solution was gently mixed for 10 min and the temperature of the solution was recorded mid way of the reaction. The reaction was stopped with 2 mL of (1 N) KI. This too was well mixed and then the free I_2 released as a result was titrated against (0.2 N) $Na_2S_2O_3$ using starch (0.2 %) as an indicator. The volume of $Na_2S_2O_3$ was recorded. The blank determination was carried out using exactly the same method but omitting the pulp.

CALCULATIONS:

$$\mathbf{p} = [(b - a) * N] \div 0.1$$

Where; p = amount of $KMnO_4$ (0.1N) actually consumed by the test (mL).

b = amount of $Na_2S_2O_3$ consumed by the blank (mL).
a = amount of $Na_2S_2O_3$ solution consumed by the test (mL).
N = normality of $Na_2S_2O_3$.

Using the value of '**p**', the value of '**f**' is determined from the table below:

Factor 'f' correction for different percentages of permanganate used

f	0	1	2	3	4	5	6	7	8	9
30	0.958	0.960	0.962	0.964	0.966	0.968	0.970	0.973	0.975	0.977
40	0.979	0.984	0.983	0.985	0.987	0.989	0.991	0.994	0.996	0.998
50	1.000	1.002	1.004	1.006	1.009	1.011	1.013	1.015	1.017	1.019
60	1.002	1.024	1.026	1.028	1.030	1.033	1.035	1.037	1.039	1.042
70	1.044									

When,

When a constant temperature bath was not available, the temperature was determined after 5 min of reaction period. This was assumed the average reaction temperature throughout the test. If the temperature was not higher than 30°C or lower than 20°C, the correction for the kappa number is as follows:

$$\mathbf{K} = [p * f * (1+0.013\ (25-t))] \div w$$

Where;

K = kappa number
f = correction factor to a 50 % $KMnO_4$ consumption, dependent on the value of '**p**'
w = weight of the moisture free bagasse (g)
p = amount of 0.1N $KMnO_4$ actually consumed by the test (mL).
t = temperature in °C of the reaction mixture midway during incubation period.

Relationship with lignin:

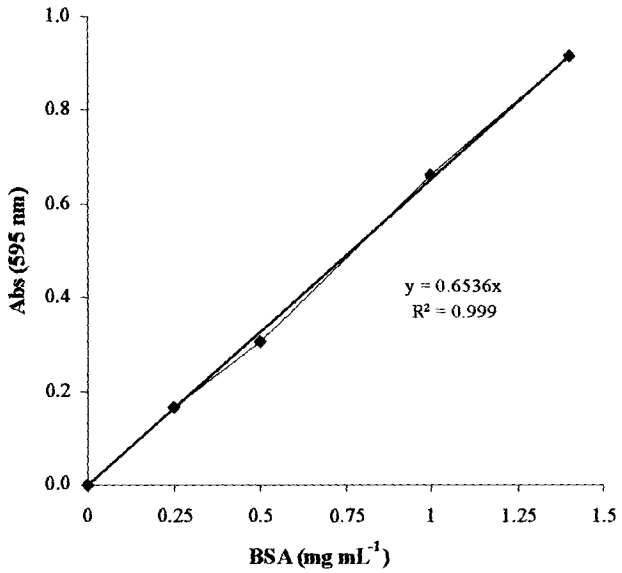
The kappa number gives essentially a straight line relationship with both Klason lignin and chlorine number for pulp below 70 % total pulp yield.

Klason lignin (%) ≈ K x 0.1

8.2.1.4 Protein Estimation (Bradfords Method) (Bradford, 1976; Stoscheck, 1990)

Bovine serum albumin (BSA); 2 g was dissolved in 1 mL distilled water and used as stock for the preparation of protein standards for protein estimation by Bradfords method. The various dilutions were prepared in distilled water.

To 30 µL of each protein dilution, 900 µL of Bradfords reagent was added and incubated at RT for 30 min. The absorbance was recorded spectrophotometrically at 595 nm and a standard graph for the same was plotted.



CALCULATIONS:

$$\text{Protein (mg mL}^{-1}) = [A_{595} * 0.6536 * \text{dilution factor}]$$

Where;

A_{595} = Absorbance at 595 nm

Protein was expressed as **mg mL^{-1}** or **g L^{-1}**.

8.2.1.5 Biomass Estimation

The fungal biomass was estimated as its dry weight. The culture broth was filtered though pre-weighed Whatman No.1 filter paper. The paper was dried at 60°C, until a constant weight was achieved. This was then conditioned at room temperature and then reweighed. The difference in weight was considered as the dry weight and expressed as **g L^{-1}** of culture medium.

8.2.2 ENZYME ESTIMATION METHODS

8.2.2.1 Laccase Assay

8.2.2.1.1 Using ABTS (Childs & Bardsley, 1975; Niku-Paavola *et al.*, 1988)

Laccase activity in the sample was spectrophotometrically determined by monitoring the rate of product (dark green colour) formation due to the enzymatic oxidation of ABTS.

In a 1 mL of cuvette, the following components were added;

0.5 mL of 2 mM ABTS prepared in buffer of desired pH range + 0.05 to 0.5 mL of enzyme (to be tested) was added, the same buffer in which the ABTS was prepared in, was used to make up the total volume to 1 mL

The kinetic reaction was spectrophotometrically measured at 405 nm for 1 min at the desired temperature, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

$$\text{Laccase (U L}^{-1}) = [\Delta A_{405} * \text{total vol} * \text{dilution factor} * 10^6] \div [\epsilon_{ABTS} * \text{sample vol}]$$

Where;

ΔA_{405} = rate of reaction i.e. final abs - initial abs ÷ time (min)
ϵ_{ABTS} = molar extinction coefficient of the radical-cation ABTS (35000)
Total vol = total volume of reaction mixture (mL)
Sample vol = volume of enzyme used (mL)

Laccase was expressed as enzyme units per liter i.e. **U L^{-1}** or **(µmol min^{-1}) L^{-1}**, where one enzyme unit is expressed as µmol of the product formed per minute.

8.2.2.1.2 Using Syringaldazine (Harkin & Obst, 1973)

Laccase activity in the sample was spectrophotometrically determined by monitoring the rate of product (dark pink colour) formation.

In a 1 mL of cuvette, the following components were added;

0.1 mL of 10 mM syringaldazine prepared in ethanol + 0.4 mL of buffer of desired pH range + 0.05 to 0.5 mL of enzyme (to be tested) was added, buffer, was used to make up the total volume to 1 mL

The kinetic reaction was spectrophotometrically measured at 525 nm for 1 min at 30°C, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

$$\text{Laccase (U L}^{-1}) = [\Delta A_{525} * \text{total vol} * \text{dilution factor} * 10^6] \div [\epsilon_{\text{Syringaldazine}} * \text{sample vol}]$$

Where;
ΔA_{525} = rate of reaction i.e. final abs - initial abs ÷ time (min)
$\epsilon_{\text{Syringaldazine}}$ = molar extinction coefficient of the product (65000)
Total vol = total volume of reaction mixture in (mL)
Sample vol = volume of enzyme used in (mL)

Laccase was expressed as enzyme units per liter i.e. **U L^{-1}** or **(µmol min^{-1}) L^{-1}**, where one enzyme unit is expressed as µmol of the product formed per minute.

 # NOTE: Both ABTS and syringaldazine also act as substrates for peroxidases in the presence of hydrogen peroxide. To exclude the inclusion of peroxidase in laccase estimation, endogenous peroxide is required to be removed by pre-incubating the enzyme sample with catalase (10 µg mL^{-1}) with stirring prior to assay for laccase activity.

***8.2.2.1.3 Using Guaiacol* (modified)** (Setti *et al.*, 1998; Xiao *et al.*, 2001)

Laccase activity in the sample was spectrophotometrically determined by monitoring the rate of product (dark brown colour) formation.

In a 1 mL of cuvette, the following components are added;

0.1 mL of 10 mM guaiacol prepared in ethanol + 0.4 mL of buffer of desired pH range + 0.05 to 0.5 mL of enzyme (to be tested) was added, buffer, is used to make up the total volume to 1 mL.

The kinetic reaction was spectrophotometrically recorded at 460 nm for 3 min after 1 min delay at 30°C, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

Laccase (U L^{-1}) = [ΔA_{460}*total vol*dilution factor*10^6] ÷ [$\epsilon_{Guaiacol}$*sample vol]

Where;

ΔA_{460} = rate of reaction i.e. final abs - initial abs ÷ time (min)
$\epsilon_{Guaiacol}$ = molar extinction coefficient of the product (27.75)
Total vol = total volume of reaction mixture in (mL)
Sample vol = volume of enzyme used in (mL)

Laccase was expressed as enzyme units per liter i.e. **U L^{-1}** or **(µmol min^{-1}) L^{-1}**, where one enzyme unit is expressed as µmol of the product formed per minute.

8.2.2.2 Lignin Peroxidase (LiP) Assay (Paszczynski *et al.*, 1988)

LiP catalyses the oxidation of veratryl alcohol to veratraldehyde by H_2O_2. Only the aldehyde shows absorbance at 310 nm.

In a 1 mL cuvette, the following components are added;
0.5 mL of reaction mixture #
+ 0.5 mL of enzyme solution to be tested

Reaction mixture (25 mL) = 20 mL of 125 mM d-tartaric acid buffer (pH 2.5) + 2.5 mL of 40 mM veratryl alcohol + 2.5 mL of 8 mM H_2O_2.

The kinetic reaction was spectrophotometrically measured at 310 nm for 1 min at the desired temperature. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme.

CALCULATIONS:

$$\text{LiP (U L}^{-1}) = [\Delta A_{310} * \text{total vol} * \text{dilution factor} * 10^6] \div [\epsilon_{\text{Veratrylaldehyde}} * \text{sample vol}]$$

Where;
ΔA_{310} = rate of reaction i.e. final abs - initial abs ÷ time (min)
$\epsilon_{\text{Veratrylaldehyde}}$ = molar extinction coefficient of veratrylaldehyde (9300)
Total vol = total volume of reaction mixture (mL)
Sample vol = volume of enzyme used (mL)

Lignin peroxidase was expressed as enzyme units per liter i.e. **U L^{-1}** or **(µmol min^{-1}) L^{-1}**, where one enzyme unit is expressed as µmol of the product formed per minute.

8.2.2.3 Manganese-dependant Peroxidase (MnP) Assay (Paszczynski *et al.*, 1988)

MnP catalyses the oxidation of Mn^{+2} to Mn^{+3} by H_2O_2 the product Mn^{+3}, forms a transient stable complex with tartaric acid, showing a characteristic absorbance at 238 nm.

In a 1 mL cuvette, the following components are added;
0.8875 mL reaction mixture [#] + 0.0125 mL of 8 mM H_2O_2
+ 0.1 mL enzyme solution to be tested

[#] **Reaction mixture** (17.75 mL) = 4 mL of 500 mM sodium tartarate buffer (pH 5) + 0.2 mL of 10 mM $MnSO_4$ + 13.55 mL DW.

The kinetic reaction was spectrophotometrically measured at 238 nm for 1 min at the desired temperature, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

$$MnP\ (U\ L^{-1}) = [\Delta A_{238} * \text{total vol} * \text{dilution factor} * 10^6] \div [\epsilon_{Mn}^{+3} * \text{sample vol}]$$

Where;
ΔA_{238} = rate of reaction i.e. final abs - initial abs ÷ time (min)
ϵ_{Mn}^{+3} = molar extinction coefficient of Mn^{+3}–tartarate complex (6500)
Total vol = total volume of reaction mixture (mL)
Sample vol = volume of enzyme used (mL)

Manganese dependant peroxidase was expressed as enzyme units per liter i.e. **U L^{-1}** or **(µmol min^{-1}) L^{-1}**, where one enzyme unit is expressed as µmol of the product formed per minute.

8.2.2.4 Estimation of Cellulase (modified) (Miller, 1959; Trigiano *et al.*, 2003)

Paper is the source of cellulose, the reducing sugars released as a result of cellulase action on cellulose is estimated by the dinitro salicyclic acid (DNSA) method using D-glucose as the sugar standard.

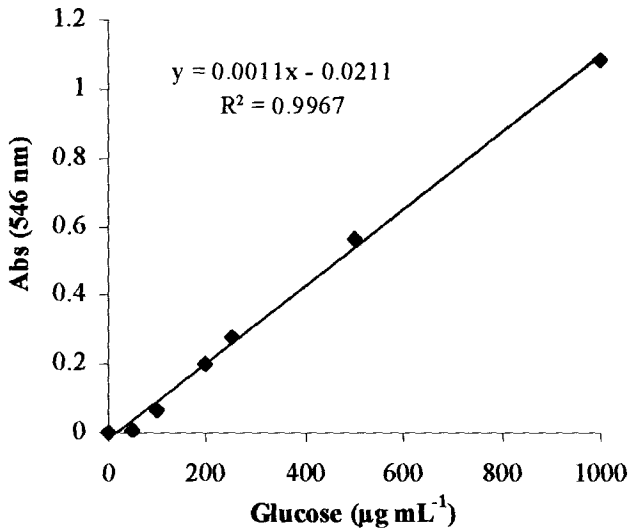
Finely cut-pieces of Whatman No. 1 paper, 0.01 g was incubated with the enzyme sample dissolved in 0.2 M Na-acetate buffer, pH 4.5 at 30°C for 1 h. One mL of this sample (without the paper) + 1 mL of DNSA reagent[#] were incubated in a boiling water bath for 10 min. It was then cooled to RT, the total volume was brought to 10 mL with DW and the absorbance was spectrophotometrically recorded at 546 nm. The blank was enzyme free.

[#]**DNSA Reagent** (to be prepared without heating)**:**

Solution A – Sodium potassium tartarate (Rochelle salt), 150 g dissolved in 100 mL of distilled water was added to DNSA, 5 g dissolved in 20 mL of 10 % NaOH. The total volume was made to 250 mL using distilled water.

Solution B – Sodium bisulphate, 0.25 g was dissolved in a solution containing 1 g of phenol prepared in 30 mL of 10 % NaOH. The total volume was made to 250 mL using distilled water.

Solution A & B are mixed and stored in the dark at 4°C.



CALCULATIONS:

AMOUNT OF REDUCING SUGAR

The concentration of reducing sugar was determined as mg mL^{-1}, from the following equation obtained from the above Std. graph;

$$Y = 0.0011X - 0.0211 * \text{dilution factor}$$

Where Y = 'A' mg reducing sugar mL^{-1}
X = Absorbance of sample at 546 nm

Total Reducing sugar:

A* total vol of reaction mixture (mL) = **'B' mg total reducing sugars**

Amount of mg moles of D-glucose:

Molecular weight of D-glucose = 180.2 g i.e. 180.2 g $mole^{-1}$
Converting g to mg,
$180.2 * 10^3 = 180200$ mg D-glucose $mole^{-1}$---------- (1)

AMOUNT OF PROTEIN

Using the Bradfords method protein concentration was determined, 'C' mg mL^{-1}.

Total Protein:

Total protein = C * vol of sample used in assay (mL) = **'E' mg total protein**

Since the sample was incubated for 1 h,

= (B mg total reducing sugars) ÷ (E mg total protein) ÷ 60
= 'F' (mg reducing sugar mg^{-1} total protein) min^{-1} ---------- (2)

AMOUNT OF CELLULASE

Dividing equation (2) by (1),

F ÷ 180200 = 'G' (moles of reducing sugar mg^{-1} total protein) min^{-1}
= G* 10^6 = 'H' (μmoles of reducing sugar mg^{-1} total protein) min^{-1}

Cellulase activity = 'H' **(μmoles of reducing sugar mg^{-1} total protein) min^{-1}**
One unit of cellulase was defined as the amount of enzyme which produced 1 μmole glucose equivalent per minute under the assay conditions and expressed as **(μmoles mg^{-1} total protein) min^{-1}**.

8.2.3 LACCASE CHARACTERIZATION

8.2.3.1 Molecular mass of laccase using size exclusion chromatography (Superdex 75 column)

K_{av} = (Elution vol – Void vol) ÷ (Total vol – Void vol)

Where Total vol = 120.637 mL ;

Void vol = 39.810001 mL

This value is substituted in the following equation which was previously determined in the laboratory using the std proteins (BSA, chicken egg albumin, carbonic anhydrase, α-lacto albumin) for Superdex 75 column at the same flow rate (1 mL min^{-1}) which was used to separate the protein. K_{av} = -0.2287 * ln (Mol wt) + 2.6367.

By solving the equation, the molecular mass is obtained in **Daltons (Da)**.

8.2.3.2 LACCASE KINETIC PARAMETERS (Nelson & Cox, 2004)

8.2.3.2.1 V_{max} (maximum velocity)**:** A reciprocal plot of velocity of reaction (y-axis) and substrate concentration (x-axis) [Line Weaver burke Plot] is plotted. Upon extrapolating the line, the reciprocal of the intercept on the positive Y-axis is the **V_{max} (µmol min^{-1})**.

8.2.3.2.2 K_m (Michaelis constant)**:** From the above plot, the reciprocal of the intercept on the negative X-axis is the **K_m (µM)** [the –ve sign of the value is ignored].

8.2.3.2.3 K_{cat} (Catalytic constant)**:** $K_{cat} = V_{max} \div E_t$ (µmol). Where E_t = Total amount of enzyme (in terms of total protein in µg) ÷ Mol mass of laccase (kDa). The unit for K_{cat} is either **min^{-1}** or **sec^{-1}**.

8.2.3.2.4 Specificity Constant = $K_{cat} \div K_m$.

The unit for specificity constant is **µM min^{-1}**.

8.2.3.2.5 Q_{10} (temperature coefficient) http://www.csupomona.edu/~seskandari /physiology/physiological_calculators/Q10.html: This represents the factor by which the rate (R) of a reaction increases for every 10 degree rise in the temperature (T) in degrees Celsius or Kelvin (K).

$$Q_{10} = [R_2 \div R_1]^{[10 \div (T_2 - T_1)]}.$$

Q_{10} is a unitless quantity.

8.2.3.2.6 E_{act} (Energy of activation) (http://www.rod.beavon.clara.net/Q10.html): This has been determined in terms of Q10 value.

$$E_{act} = [R^{*}\ln (k_2 \div k_1)] \div [(1 \div T_1) - (1 \div T_2)]$$

Where ; k_1 and k_2 are the rate constants of the reaction at temperatures T_1 and T_2 in Kelvin (K).

R (gas constant) = 8.314472 J K^{-1} mol^{-1} or 1.987 C K^{-1} mol^{-1}.

The units of E_{act} is either **J mol^{-1}** or **Cal mol^{-1}** depending of the units of 'R'. It is converted in k J mol^{-1} or Cal mol^{-1} by multiplying the result with 10^{-3}.

8.2.3.3 PURIFICATION TABLE (Nelson & Cox, 2004)

8.2.3.3.1 Total Specific Activity:

= Total laccase (U) in step *x* ÷ Total protein (mg) in step *x*.

The unit for total specific activity is U mg^{-1}.

8.2.3.3.2 Yield:

= Total laccase (U) in step *x* ÷ Total original laccase (U).

The unit for yield is % relative considering original as 100%.

8.2.3.3.3 Purification Fold:

= Specific activity in step *x* ÷ Original specific activity

8.2.3.4 Specific Activity (U mg^{-1}): = Laccase (U mL^{-1}) ÷ protein (mg mL^{-1}).

The unit for specific activity is U mg^{-1}.

8.3 PROCEDURES & PROTOCOLS

8.3.1 Fungal DNA Extraction (van Burik *et al.*, 1998)

The fresh fungal biomass was rinsed in sterile DW twice, lyophilized and then ground with a mortar and pestle. This was re-suspended in extraction buffer and 1 % β-mercaptoethanol preheated to 65°C. The mixture was then subjected to a deproteination step which consisted of extraction in a mixture of Tris-saturated phenol (pH 8), chloroform and isoamyl alcohol in the ratio of 25:24:1, after vigorous mixing, the solution was subjected to centrifugation (13,000 *g* / 15 min / 4°C). The supernatant was subjected to RNase (10 % of 2 mg mL^{-1} stock) treatment at 37°C and immediately followed by an extraction in a mixture of chloroform and isoamyl alcohol in the ratio of 24:1. The mixture was subjected to centrifugation (13,000 *g* / 15 min / 4°C), 2.5 vols of isopropanol was added to the supernatant and stored at -20°C for at least 1 h. This caused the DNA to precipitate which was then rinsed in 70 % ethanol, air-dried and then resuspended in high salt Tris EDTA (TE) buffer.

The quality of the DNA was estimated via agarose gel electrophoresis at ~ 60 V using 1X Tris-Borate-EDTA (TBE) running buffer. The gel contained 0.8 % (w/v) agarose in 1X TBE buffer, to which ethidium bromide was added. After electrophoresis, the gel was visualized under UV light, DNA appears as fluorescent orange bands due to the intercalation of ethidium bromide with the DNA double helix. The image was then documented using a gel documentation system (Alpha Imager, Alpha Innotech, C.A, USA).

Extraction buffer: 100 mM Tris-HCl, 20 mM Na-EDTA, 2 % CTAB, 1.4 M NaCl &1 % poly vinyl pyrolidine in DW

High Salt TE Buffer: 10 mM Tris-HCl, 1 mM Na-EDTA & 1 M NaCl in DW

10X TBE Buffer: 900 mM Tris-base, 0.5 M Na-EDTA & 900 mM boric acid in DW

DNA Loading Dye: 0.25 % bromophenol blue, 0.25 % xylene cyanol & 0.25 % ficoll in DW.

8.3.2.1 SDS PAGE Composition & Reagents (Sambrook & Russell, 2001)

COMPONENTS	12 % Resolving gel (mL)	5 % Stacking gel (mL)
DW autoclaved	3.4	5.7
Monomer solution	4.0	1.7
Gel buffer	2.5[†]	2.5[#]
SDS (10 %)	0.1	0.1
APS (10 %) freshly prepared	0.05	0.05
TEMED	0.02	0.01

[†]**Resolving buffer:** 1.5 M Tris-HCl, pH 8.8 adjusted with 6 N HCl.

[#]**Stacking buffer:** 0.5 M Tris-HCl, pH 6.8 adjusted with 6 N HCl.

Monomer solution (acrylamide-bisacrylamide solution, 30 %): 29.2 g of acrylamide and 0.8 g of N'N'-bis-methylene-acrylamide was made to a final volume of 100 mL using DW. The stock solution was filter sterilized though 0.22 µm filter and stored at 4°C in dark.

SDS-PAGE sample solubilizing buffer (6×): contained 300 mM Tris-HCl (pH 6.8), 7.5 % β-mercaptoethanol, 10 % SDS, 60 % Glycerol, 0.6 % bromophenol blue prepared in autoclaved DW.

SDS-PAGE running buffer (pH 8.3, 10×): 30.2 g Tris base, 144 g glycine & 100 mL of 10 % SDS, the final volume is made to 1 L using DW.

8.3.2.2 Electro-Blotting (http://www.oardc.ohio-state.edu/stockingerlab/Protocols/ProteinElectroblotting.pdf)

After SDS PAGE was over, the gel was washed in milli Q water to remove the SDS and then soaked in 1X CAPS buffer for 5 min. The packing (Whatman sheets) were also similarly soaked. The PVDF membrane was soaked in 100% methanol for 5 min. The gel was then placed onto the membrane and the air

bubbles, if any were removed. On either side, CAPS buffer-soaked Whatman sheets were placed. Together they were placed in the electro blotting chamber with the gel facing the negative electrode and the PVDF membrane, the positive electrode. Electrophoresis was carried out overnight in the cold.

The PVDF membrane was stained in Coomassie stain for 2 - 3 min and then destained using 50 % methanol with 2 - 3 intermittent changes. After the background was removed the membrane was dried between clean dry Whatman sheets.

10X CAPS Buffer: 5.535 g CAPS was dissolved in 200 mL milli Q water. The pH was adjusted to 11 with 2 N NaOH. The total volume was made up to 250 mL with milli Q water.

1X CAPS Buffer: 100 mL of 10 X CAPS Buffer was added to 800 mL milli Q water and then chilled. 100 mL methanol was added just before use to the chilled mixture.

8.3.2.3 Polyacrylamide gel / PVDF Membrane Staining Procedures

8.3.2.3.1 ACTIVITY STAINING FOR LACCASE (Xiao *et al.*, 2004)

After electrophoresis was over, the polyacrylamide gel was placed in a citrate phosphate buffer (pH 6) solution containing 10 % guaiacol till staining occurs. Protein bands corresponding to laccase oxidize guaiacol to a deep brown colour and these bands retain the colour. The excess guaiacol was removed with repeated rinses in distilled water. The gel was placed in fixative (same as used in silver staining of proteins). This removes the background and previously invisible laccase bands begin to appear. The gel was then placed in water for complete rehydration and the image was then documented using the gel documentation system.

NOTE: All incubation steps are carried out on a gel rocker.

8.3.2.3.2 PROTEIN STAINING

8.3.2.3.2.1 Silver Staining (Heukeshoven & Dernick, 1985)**:** After electrophoresis was over, the gel was placed in fixative for at least 1 h. After which it was transferred to methanol (for dehydration) and kept for at least 20 min. This was repeated at least 2 - 3 times using fresh methanol each time. The gel was then rehydrated in distilled water till complete rehydration occurred. It was then placed in 0.02 % $Na_2S_2O_3$ for 2-3 min and then subjected to 30 min incubation in 0.2 % $AgNO_3$ in the dark. The $AgNO_3$ was rinsed off with distilled water and then the gel was placed in developer and shaken very gently till protein bands appeared. The reaction was stopped by placing the gel in fixative for 5 - 10 min. The gel was then placed in water for complete rehydration and the image was then documented using the gel documentation system.

Fixative: 250 mL methanol, 62.5 mL acetic acid & 500 µL formalin in 187 mL DW.

Developer: 6 g Na_2CO_3, 40 µL of 10 % $Na_2S_2O_3$ & 75 µL formalin in 100 mL DW.

8.3.2.3.2.2 Coomassie Blue Staining (http://www.ohsu.edu/proteomics/sampleprep/coomassie.pdf)**:** After electrophoresis was over, the gel was placed in Coomassie Blue stain for 2 h and then destained overnight using Coomassie Blue destain solution with several intermittent changes, until the background was cleared and only the protein bands stained blue.

Coomassie Blue staining solution: 40 % methanol, 10 % acetic acid, 0.25 % Coomassie brilliant blue R-250 prepared in milli Q water.

Coomassie Blue destaining solution: 40 % methanol, 10 % acetic acid, prepared in milli Q water.

8.3.2.3.3 GLYCOPROTEIN STAINING (Schiffs Method) (Zacharius *et al.*, 1969)**:** After electrophoresis was over, the polyacrylamide gel was placed in fixative for at least 30 min. This step was repeated at least 4 times using fresh fixative solution each time. The fixation overnight was continued in fresh fixative and then the gel was subjected to another two washes for 30 min each in fresh fixative. The glycoprotein bands were oxidized by placing them in 1 % periodic acid – 3 % acetic acid mixture for 60 min. The oxidized gel was washed with water at least 10 times, with 10 min incubation each time, to remove traces of periodic acid. The gel was then incubated in Schiffs reagent (Sigma, USA) for 60 min in the dark. Red-purple glycoprotein bands began to appear within the first 20 min itself. To remove the background (for image capture), the gel was washed at least thrice, with a 10 min incubation each time in 0.58 % potassium metabisulphite - 3 % acetic acid prepared in water. Washing with this solution at 30 min intervals using fresh solution each time, was continued till all the background was eliminated.

NOTE: For all staining procedures, the incubation steps were carried out on a gel rocker.

8.3.3 Exopolymeric Substance (EPS) Staining (Long & Azam, 1996)

A 21 day-old fungal culture grown in liquid medium was placed on a slide and teased to separate the fungal strands. A drop of 0.22 µm filtered Alcian blue stain was placed onto the preparation, over which a cover slip was placed. The Alcian blue stains the EPS blue whereas the fungal hyphae remain transparent.

Alcian blue stain: 0.02 % Alcian blue in 0.06 % acetic acid (pH 3.3)

8.4 DETAILS OF THE INDUSTRIAL EFFLUENTS

8.4.1 Textile Effluent A (TeA) was obtained from Atul Ltd., Gujarat, India and contained mainly Azo dye - 20 with 0.34 % carbonate. The effluent was deep purple and had a pH of 8.9.

8.4.2 Textile Effluent B (TeB) was obtained from from Atul Ltd., Gujarat, India and contained a mixture of dyes with 21700 colour units, total solids 4.2 %, Na^{+1} 2440 ppm, Ca^{+2} 31 ppm, SO_4^{-2} 0.6 %, Cl^{-1} 3.2 % and PO_4^{-3} 0.5 %. The dyes in this mixture were reactive blue 140 base, reactive blue 140, reactive blue 160 base, reactive blue 163, reactive red 11, reactive yellow 145, reactive green 19 and reactive blue 4. The effluent was dark green in colour and had a pH of 2.5.

8.4.3 Molasses Spent Wash (MSW) in the raw untreated form was obtained from Rheaa Distilleries Ltd., Goa, India. It had a BOD of 42,000 mg L^{-1} and COD of 80,000 mg L^{-1}. It was dark brown in colour and had a pH of 4.3.

8.4.4 Black Liquor (BL) was obtained from Seshasayee Paper Mills, a bagasse and wood chip-based newsprint manufacturing unit in Tamil Nadu, India. The effluent was black in colour and had a COD of 416 mg L^{-1} and BOD of 190 mg L^{-1}.

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Publications

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2. Raghukumar, C., **D'Souza-Ticlo, D.** and Verma, A. K., (2008) Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. *Critical Reviews in Microbiology*, **34**, 189 - 206.

3. **D'Souza-Ticlo, D.**, Verma, A. K., Mathew, M. and Raghukumar, C. (2006) Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by a fungus isolated from mangrove wood. *Indian Journal of Marine Science*, **35**: 364 - 372.

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Patent

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Presentations at Symposia & Conferences

1. **D'Souza-Ticlo. D** and Raghukumar, C. (2009) Bioremediation of high saline colored waste waters with a marine-derived fungus *Cerrena unicolor* MTCC 5159. *FEMS 2009 – 3rd Congress of European Microbiologists-Microbes and Man Interdependence and Future Challenges*, Sweden, 166 (Poster presentation).

2. **D'Souza-Ticlo, D.**, and Raghukumar, C. (2007) Characteristics and applications of laccase from an estuarine fungus NIOCC #2a. *New Trends in Biotechnology*, India, 13 (oral presentation).

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A NOVEL PROCESS FOR DECOLORIZATION OF COLORED EFFLUENTS

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Title: A NOVEL PROCESS FOR DECOLORIZATION OF COLORED EFFLUENTS

Abstract: The present invention relates to a novel process for decolorization of colored effluents. More particularly it relates to a process for decolorization of colored effluents of textile mills, dye-making industries, paper and pulp industries and molasses spent wash from alcohol distilleries using an unidentified marine fungus NIOCC #2a isolated from mangrove wood and deposited in the microbial type culture collection (MTCC) of the Institute of Microbial Technology, Chandigarh, India, under the accession number MTCC 5159. Further, this invention relates to decolorization of these effluents using the fungus directly, its cell-free culture supernatant or immobilized fungus or extracellular polymeric substances produced by the fungus. Furthermore, the decolorization of effluents can be carried out from 30oC to 60oC and at pH 3 to 6. The decolorization of various colored effluents occurs in the presence of sea water with 25 parts per thousand salinity. Besides, several synthetic dyes are also decolorized under similar conditions of temperature and pH by using free mycelia or immobilized fungus or extracellular culture fluids or extracellular polymeric substances.

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Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes

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Abstract

Paper and pulp mills, textile and dye-making industries and alcohol distilleries release highly colored effluents that are relatively difficult to decolorize by chemical and physical treatments. White-rot basidiomycetous fungi that produce lignin-degrading enzymes are reported to be the most efficient in decolorizing such effluents. We report here decolorization of all the three effluents by a marine fungal isolate, NIOCC # 2a cultured from decaying mangrove wood. The fungus also decolorized several synthetic dyes. Laccase was the most dominant lignin-degrading enzyme produced by this fungus with very low activities of manganese-dependent peroxidase and no lignin peroxidase activity. The growth and production of laccase was best in a medium prepared with seawater having salinity in the range of 25–30 ppt. The pH optimum for the laccase activity was 3.0 and 6.0 and the temperature optimum was 60 °C. Laccase production was increased in the presence of phenolic and non-phenolic inducers. A several fold enhancement in laccase production was found during treatment of colored effluents from textile, paper and pulp mill and distillery waste. Industrial effluents and synthetic dyes added to the growing culture of this fungus were decolorized to a great extent. The culture supernatant without the fungal biomass was also effective in decolorization of these effluents to various degrees within 6 h of incubation. Extracellular polymeric substances (EPS) produced by this fungus were also useful in decolorization of these effluents. Thus, efficiency of this fungus in decolorization of various effluents with laccase that is active at pH 3.0 and 6.0 and 60 °C in the presence of seawater has great potential in bioremediation of industrial effluents. Enhanced laccase production in the presence of industrial effluents in this fungus is an added advantage during bioremediation of effluents.

Keywords: Laccase; Inducers; Marine fungus; Mangroves; Colored effluents; Decolorization

1. Introduction

Lignin peroxidase, manganese-dependent peroxidase and laccase are the three major lignin-degrading enzymes with great potential in industrial applications. Laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) is a multi-copper blue oxidase capable of oxidizing *ortho-* and *para*-diphenols and aromatic amines by removing an electron and proton from a hydroxyl group to form a free radical. These enzymes lack substrate specificity and are thus capable of degrading a wide range of xenobiotics including industrial colored wastewaters. The most efficient microorganisms to break down colored pollutants so far reported are white-rot fungi. These comprise mostly basidiomycetous fungi capable of extensive aerobic lignin degradation and mineralization. This is possible through several extracellular lignin-degrading enzymes synthesized by these fungi [1].

Paper and pulp mills, textiles and dye-making industries, alcohol distilleries and leather industries are some of the industries that discharge highly colored effluents. The paper and pulp industry release large volumes of intensely colored black liquors containing toxic chlorinated lignin degradation products, including chlorolignins, chlorophenols, chloroguiacols and chloroaliphatics [2]. Chlorinated organic compounds are acute or chronically toxic besides being mutagenic and carcinogenic.

Dye-making and textiles industries release industrial dyes into the environment. About 10–15% of the total dye finds its way into the waste waters [3]. Several of these dyes

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resist microbial attack and are thus recalcitrant. These can be transformed to carcinogenic compounds under anaerobic conditions [4]. The fast colored dyes such as azo dyes are a major source of concern to environmentalists since such pollutants, besides causing aesthetic damage to sites are also toxic and carcinogenic [5].

Distilleries producing beverage alcohol by fermentation use sugarcane molasses as the raw material. The effluents from such distilleries contain large amounts of dark brown pigments called molasses or melanoidin pigments [6]. Melanoidin pigments (MP) are the products of the "Maillard reaction" between sugars and amino compounds produced on heating [6]. Pollution of aquatic ecosystems by molasses spent wash (MSW) is due to its intense brown color that cuts off light, prevents photosynthesis and causes anaerobic conditions. When MSW is dispersed in soil, it acidifies the soil and thereby affects agricultural crops [7].

Besides color, the various effluents from the above mentioned industries also contain various inorganic chemicals such as sulfides, sulfates, chlorides, carbonates, sodium hydroxide, peroxides and chlorine bleach compounds. The pH varies between a range of 7–12. These chemicals may also add taste and odors [8]. Obligate and facultative marine fungi occurring in coastal marine environments can be an important source for use in bioremediation of saline soil and wastewaters. We reported decolorization of some of these colored effluents by a lignin-degrading fungus isolated from sea grass detritus [9,10]. Search for better and more efficient fungi from newer sources for application in bioremediation of such colored effluents, especially in the presence of high salt contents of the effluents continues.

We report here decolorization of black liquor, textile dye wastewaters and molasses spent wash by a marine fungal isolate NIOCC # 2a, producing laccase as the major lignin-degrading enzyme. Although laccase is a constitutive enzyme, its production can be stimulated by the presence of several inducing substrates, nitrogen and carbon levels [11]. We further discuss enhanced laccase production by this fungus in the presence of colored effluents and other synthetic dyes.

2. Materials and methods

2.1. Organism and culture conditions

Decaying wood pieces from mangrove swamps from Chorao island in Goa, India (73°55′E and 15°30′N) were collected in sterile plastic bags and processed within 3 h. They were washed free of attached soil particles and other extraneous matter using sterile seawater. The wood pieces were then incubated in sterile bags lined with moist filter paper for a fortnight. As soon as fungal mycelia were observed to colonize the wood pieces, as little mycelia as possible were picked up using a sterile glass needle and transferred onto Boyd and Kohlmeyer (B&K) agar [12] fortified with 10% antibiotic solution to prevent bacterial growth. The stock solution of antibiotics contained 400,000 units of procaine penicillin and 1 g of streptomycin sulphate in 100 ml of sterile distilled water. B&K agar contained 10 g glucose, 2 g peptone, 1 g yeast extract and 18 g agar in 1 l of 50% seawater. The same medium was used for maintenance of the cultures. The culture was routinely checked for purity by light microscopy.

2.2. Qualitative assay for laccase

The isolates were screened for laccase production by growing them on plates of B&K medium containing 4 mM guaiacol (S.D. Fine-Chemicals Ltd., Mumbai) or 2 mM ABTS, i.e. 2,2′-azino-bis-(3-ethyl benzothiazoline-6-sulphonic acid) (Sigma Chemical, USA). The production of an intense brown color under and around the fungal colony in the case of guaiacol-supplemented agar and a deep green color in the case of ABTS-supplemented plates was considered as a positive reaction for the presence of laccase activity.

2.3. Quantitative estimation of lignin-degrading enzymes

The fungal culture grown in B&K broth (20 ml in 100 ml Erlenmeyer flasks) for 7 days was blended in sterile seawater with sterilized glass beads and was used at 10% (v/v) concentration for growing stationary cultures. The culture filtrate was obtained by filtering the culture through Whatman GF/C filter paper and centrifuged at 5000 × g for 10 min at 5 °C. Laccase activity was assayed using ABTS substrate [13]. The enzyme units were expressed as micromole of substrate transformed per minute per liter of culture filtrate i.e. as enzyme units per liter of culture filtrate ($U l^{-1}$). In the absence of the enzyme activity, no increase in the rate of absorbance was observed.

Lignin peroxidase (LiP) activity was determined by measuring the rate of oxidation of veratryl alcohol to veratraldehyde [14]. Manganese-dependant peroxidase (MnP) activity was determined by measuring the rate of oxidation of veratryl alcohol to veratraldehyde in the presence of Mn [15].

2.4. Partial purification and optimization of laccase activity in the culture supernatant

About 500 ml of culture supernatant was concentrated to 10 ml by ultrafiltration using Amicon YM10 membrane (Millipore, USA) at 4 °C. An aliquot of the concentrate was passed through an anion exchange column "Resource Q" (Amersham Biosciences, Upssala, Sweden). It was eluted using a NaCl gradient (0.1–1.0 M) at a flow rate of 0.5ml/ min. Fractions of 2 ml were collected during the elution. Fractions showing laccase activity were pooled and concentrated further using nanosep 10K Omega tubes (Paul Corporation, USA). This partially purified enzyme was used for determining the optimum temperature and pH.

The laccase activity was measured at temperatures of 5–90 °C in an electrically controlled cell holder of a spectrophotometer (Shimadzu, UV-2450, Japan). Laccase activity was estimated at pHs 3–11. To obtain pH 3, glycine–HCl buffer (0.2 M), for pH 4 and 5 acetate buffer at 0.2 M concentration, for pH 5–7 phosphate buffer (0.2 M), for pH 8 and 9 glycine–NaOH buffer (0.2 M) and carbonate–bicarbonate buffer (0.2 M) for pH 11 were used.

2.5. Effect of salinity on growth and enzyme production

The effect of salinity on growth of the marine fungal isolate was tested by preparing the B&K medium with seawater to give final concentration of 10, 15, 20, 25, 30 and 34 parts per thousand (ppt) salinity. For determination of mycelial dry weight, duplicate cultures were vacuum-filtered through tarred Whatman GF/C filter papers, rinsed with 100 ml of distilled water, dried to a constant weight, and the cell mass was calculated by difference. Laccase, LiP and MnP activities were measured in the culture supernatants of the fungus grown under different salinities. The average values of duplicate cultures were plotted.

2.6. Enhancement of laccase production by inducers

In order to enhance the laccase production, various inducers like *p*-anisidine, catechol, guaiacol, ferulic acid, vanillic acid, veratryl alcohol and 2,5 dimethyl aniline were used at a final concentration of 1 mM. Cupric sulphate at a concentration of 2 mM, indulin at 0.25 mg ml^{-1} and a combination of 2 mM cupric sulphate and 1 mM guiacol were added on day 6 to shallow stationary cultures grown in B&K medium at 28–30 °C. Dyes like trypan blue, aniline blue and remazol brilliant blue R at a final concentration of 0.04%, methylene blue, crystal violet, brilliant green and congo red at 0.02% and reactive orange 176 at 0.015% final concentration were tested for their laccase induction properties. Textile effluents as well as molasses spent wash (MSW) from an alcohol distillery and black liquor from a paper mill industry were also screened for their laccase inducing properties, each at the final concentrations of 1, 10 and 20%. The values from triplicates were used for comparing the effect of various inducers.

2.7. Decolorization of various effluents and dyes in the culture medium

The fungus was tested for its ability to decolorize various dyes and effluents by monitoring the change in the respective specific absorbance maxima every alternate day after the addition of the dye/effluent over a period of 6 days. The dye was added to a 6 day old culture and the time of addition was considered to be the 0 day. The final concentration of the dye in the medium on day 0 was considered to be 100%. The extent of decolorization was recorded as residual color (in percentage). Mean values from triplicate cultures were used for comparing the extent of decolorization of various dyes and effluents. Trypan blue, aniline blue, remazol brilliant blue R, methylene blue, crystal violet, brilliant green, congo red and reactive orange 176 (RO 176) were monitored at their absorbance maxima at 599, 585, 597, 663, 589, 623, 486 and 499 nm, respectively. Decolorization of Poly-R 478 was monitored by determining the ratio of absorbance at 513 nm versus 362 nm. (All the dyes were from Sigma Chemicals, USA; RO 176 was a gift from Dr. C. Novotny, Czech Republic). Effluents like textile effluent A, B, MSW and black liquor were also monitored at their absorbance maxima at 505, 663, 663 and 317 nm, respectively. Textile effluent A (from Atul Ltd., Gujarat, India) contained mainly azo dye-20 and had a pH of 8.9 with 0.34% carbonate. The textile effluent B from the same source contained a mixture of dyes and had a pH of 2.5 with 21,700 color units, total solids 4.2%, Na^+ 2440 ppm, Ca^+ 31 ppm, SO_4 0.6%, Cl 3.2% and PO_4 0.5%. The dyes in this mixture were reactive blue 140 base, reactive blue 140, reactive blue 160 base, reactive blue 163, reactive red 11, reactive yellow 145, reactive green 19 and reactive blue 4. The black liquor obtained from Seshasayee Paper Mills, Tamil Nadu was from bagasse and wood chip-based newsprint manufacturing unit. As per the data provided by the paper mill, the effluent had COD of 416 mg l^{-1} and BOD of 190 mg l^{-1}. Raw untreated molasses spent wash used in this study (obtained from Rheaa Distilleries Ltd., Goa, India) was dark brown in color. According to the data provided by the unit, the raw MSW had a pH of 4.3, a BOD of 42,000 mg and COD of 80,000 mg l^{-1}.

2.8. Decolorization of effluents and dyes by using the fungus free culture supernatant

The culture filtrate with maximum laccase activity was used as a source of enzyme to test its efficiency in decolorization of various effluents and dyes. This in brief was carried out by incubating the enzyme with effluents and dyes buffered at pH 6 for 6–12 h at 60 °C in duplicates. The percentage of decolorization achieved was calculated with reference to the control samples that were not treated with the enzyme.

2.9. Decolorization of effluents and dyes by using the exopolymeric substances (EPS) produced by the fungus

The exopolymeric substance (EPS) of the fungus was prepared as follows: 1 l of frozen culture filtrate was allowed to thaw. The EPS precipitated on thawing was removed by decantation. Methanol at a final concentration of 70% was added to the supernatant to further precipitate the remaining EPS from the culture supernatant. The precipitated EPS was collected by centrifugation and lyophilized and the dry weight determined. Carbon, nitrogen and sulfur ratio was analyzed in CNS analyzer (Model NCS 2500/S. no.980813, Thermoquest Italia, S.P.A.) using MAG-1 and Sulphan standards. Various dyes and effluents were added to 10 mg of EPS in phosphate buffer at pH 6 and incubated at 60 °C in

duplicates. The decolorization was monitored at 6 and 12 h at the absorbance maxima specific to that particular dye/ effluent. Dyes and effluents without addition of EPS were used as control.

3. Results

Out of 40 fungi isolated from decaying mangrove wood, 3 fungi showed positive reaction for laccase activity when grown in the presence of guaiacol and ABTS. One isolate designated NIOCC # 2a used for the present work was deposited in the Microbial Type Culture Collection (MTCC, Chandigarh, India) under the accession number MTCC 5159 under the Budapest treaty for patent culture deposition [16]. Based on partial large subunit rRNA (D2) gene (~350 bp) alignment with GenBank database, it was shown to have 99% homology to an unidentified basidiomycete species AY187277 (MIDI LABS Inc, Newark, USA). As the fungus does not produce spores, its further identity is not possible. Using the ITS sequencing also it showed homology to an unidentified basidiomycete. The sequencing data of ITS and D2 region are deposited in the GenBank under the accession nos. AY939879 and AY939878, respectively.

The salinity in the mangrove environment fluctuates from 5–35 parts per thousand (ppt). Therefore the effect of various salinities on growth of the fungus and production of lignin-degrading enzymes LiP, MnP and laccase were determined. The results showed that the maximum biomass of the fungus was produced at 34 ppt salinity, whereas laccase production was best at 25 ppt (Fig. 1a and b). The production of MnP and LiP by this fungus was negligible and this was further inhibited by seawater at all the salinities.

Among the various inducers used, $CuSO_4$ and guaiacol induced the maximum laccase production, individually as well as in combination (Table 1). Among the various dyes, brilliant green induced good laccase activity (Table 2). Trypan blue, methylene blue, remazol brilliant blue R, Poly R and crystal violet did not induce laccase activity as the other dyes (Table 2). Among the effluents used, textile effluent B at 1% concentration induced the highest laccase activity (Table 1), the other effluents stimulated moderate increase in laccase production. The partially purified laccase showed highest activity at pH 3 and 6 and at 60 °C.

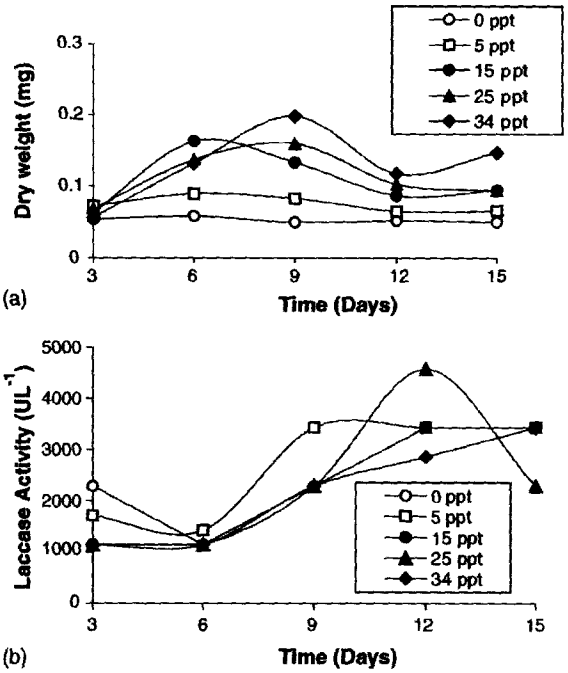

Fig. 1. (a) Fungal biomass (dry weight in mg) in 20 ml of B&K medium at various salinities. The values are mean of two replicates. (b) Laccase activity in the culture supernatant of the fungus grown in B&K medium of different salinities. The values are mean of two replicates.

Table 1
Enhancement of laccase production by various inducers and effluents

Inducers	Laccase activity (UI^{-1})						
	3 days	6 days	9 days	12 days	15 days	18 days	21 days
B&K medium (control)	0	0	404 ± 95.5	166 ± 21.7	158 ± 58.6	921 ± 166.7	838 ± 286
p-Anisidine (1mM)	73 ± 8.3	92 ± 60	32 ± 13.3	35 ± 23.6	24 ± 10.9	1200 ± 250.8	1351 ± 323.2
Catechol (1mM)	21 ± 6.4	36 ± 2	53 ± 27.3	27 ± 25.3	27 ± 1.1	1246 ± 109	1675 ± 78.5
Copper sulphate (2mM)	16 ± 2.5	197 ± 67.2	185 ± 104.8	119 ± 60.2	36571 ± 30.7	42933 ± 76.8	83619 ± 134
Guaiacol (1mM)	0	85 ± 41.3	979 ± 132	384 ± 51.6	344 ± 196.3	1258 ± 904.7	21112 ± 961.8
Copper sulphate (2mM)& Guaiacol (1mM)	36 ± 6.5	485 ± 88.3	14078 ± 970.9	21347 ± 447.8	44457 ± 273	45257 ± 30.3	46781 ± 64.9
Ferulic Acid (1mM)	9 ± 4.9	70 ± 48.5	7502 ± 255.8	1407 ± 1280.1	1755 ± 721	873 ± 377	780 ± 142
Indulin (0.25%)	0	63 ± 29.9	79 ± 9.6	66 ± 32	62 ± 29	894 ± 50.6	1174 ± 10.6
Vanillic acid (1mM)	75 ± 8.6	265 ± 47	68 ± 30.5	48 ± 18.8	32 ± 14.9	1599 ± 493.4	1359 ± 172.8
Veratryl alcohol (1mM)	14 ± 5.1	112 ± 14.9	768 ± 273.6	395 ± 33.2	332 ± 61.4	1889 ± 420.6	1931 ± 636.3
2,5 Dimethyl aniline (1mM)	0	232 ± 30.4	133 ± 95.3	177 ± 11.8	128 ± 43.6	548 ± 143.2	633 ± 153.2
Textile effluent A (1%)	25 ± 2.2	113 ± 59.7	937 ± 323.9	440 ± 230	404 ± 70.9	2036 ± 97.8	1886 ± 171.5
Textile effluent B (1%)	7 ± 2.6	358 ± 66.3	4545 ± 503.2	85829 ± 71.5	19086 ± 255.7	42248 ± 116.1	266 ± 59.1
Molasses spent wash (1%)	0	159 ± 38	1566 ± 83.8	563 ± 87.9	505 ± 97.2	1756 ± 298.1	2075 ± 167.3
Black liquor (1%)	2 ± 3.6	139 ± 27.1	738 ± 123.1	296 ± 38.9	268 ± 134.1	1299 ± 242.1	1156 ± 79.8

Laccase activity was measured in the culture supernatant using ABTS substrate at pH 3.0.

Table 2
Enhanced laccase production by various dyes

Inducer	Laccase activity ($U l^{-1}$)							
	3 days	6 days	8 days	10 days	12 days	14 days	16 days	18 days
Trypan blue (0.04%)	21 ± 4.5	1 ± 2.5	149 ± 15.2	747 ± 103.7	844 ± 155.8	897 ± 189.5	812 ± 125.8	505 ± 156.9
Aniline blue (0.04%)	12 ± 4.5	67 ± 13.5	21 ± 2.1	825 ± 67.9	1051 ± 118.5	1166 ± 178.2	1211 ± 101	1048 ± 97.8
Methylene blue (0.02%)	0	51 ± 15.9	93 ± 16.7	634 ± 205.7	783 ± 200.3	651 ± 189.5	656 ± 109.4	666 ± 98.9
RBBR (0.04%)	3 ± 2.7	43 ± 7.9	0	0	0	0	0	0
Crystal violet (0.02%)	4 ± 2.5	104 ± 19.9	0	581 ± 106.8	825 ± 189.5	819 ± 111.5	671 ± 105.8	745 ± 98.6
Brilliant green (0.02%)	13 ± 8.8	53 ± 11.8	0	1852 ± 79.6	1821 ± 157.9	2271 ± 259.6	2191 ± 205.2	2156 ± 189.3
Poly-R 478 (0.02%)	17 ± 2.5	36 ± 7.9	0	187 ± 15.9	248 ± 78.5	241 ± 67.9	266 ± 56.9	325 ± 47.6
Congo red (0.02%)	5 ± 3.3	5 ± 2.2	115 ± 12.9	955 ± 12.9	966 ± 89.9	1363 ± 126.9	1389 ± 187.6	1241 ± 198.4
RO 176 (0.015%)	21 ± 10.2	8 ± 3.3	335 ± 57.9	949 ± 112.3	904 ± 89.9	1158 ± 89.9	1108 ± 125.9	1180 ± 118.6

Laccase activity was measured in the culture supernatant using ABTS substrate at pH 3.0.

The dyes that acted as inducers of laccase production in the culture medium were in turn decolorized by the enzyme produced. Brilliant green was decolorized to the maximum by day 4 whereas RO 176 was least decolorized (Fig. 2a). Among the synthetic blue dyes, aniline blue was decolorized almost totally by day 4 (Fig. 2b). Poly R and crystal violet did not induce high laccase activity and their decolorization was also very low (Fig. 2a). On the other hand trypan blue, methylene blue and RBBR were decolorized by 60–70% but they did not induce laccase activity (Table 2). About 60% decolorization of black liquor (used at 10% concentration) from paper and pulp mills was achieved by day 6 (Fig. 2c). Molasses spent wash from distillery waste when used at 10% concentration was totally decolorized by day 6 (Fig. 2c). Textile effluent B at this concentration was decolorized by 60% on day 2 and no further reduction in color was observed (Fig. 2c).

The fungus free-culture supernatant with laccase activity ($18 U ml^{-1}$) when incubated with various dyes at pH 6 and 60 °C showed about 79% decolorization of brilliant green dye within 12 h of incubation (Table 3) and about 71% of the color was removed from 10% solution of black liquor within 6 h (Table 3).

The fungus produced 2.3 g exopolymeric substance (EPS) l^{-1} of the culture medium. Analysis of EPS in cultures grown with 1% glucose showed CNS ratio of 4.5:0.76:10, respectively, and therefore appeared to be a sulphated polysaccharide. We determined the efficacy of this in decolorization of various dyes and effluents. Almost total decolorization of some of the dyes and effluents was noticed within 24 h of incubation with 10 mg of EPS (Table 3).

4. Discussion

This is the first report wherein high amount of laccase ($921 U l^{-1}$) is reported to be produced by a marine fungus

Table 3
Decolorization of dyes and effluents using fungus free- culture supernatant and EPS produced by the fungus

	Decolorization by the culture supernatant (%)		Decolorization by the EPS of the fungus (%)	
	6 h	12 h	12 h	24 h
Dye				
Trypan blue (0.04%)	22	25	20	79
Aniline blue (0.04%)	55	40	46	75
Methylene blue (0.02%)	3	5	4	6
RBBR (0.04%)	67	46	19	100
Crystal violet (0.02%)	44	54	45	80
Brilliant green (0.02%)	72	79	2	90
Poly-R 478 (0.02%)	21	43	33	90
Congo red (0.02%)	54	47	18	29
RO 176 (0.015%)	ND	ND	35	100
Effluents				
Textile effluent A (10%)	9	11	11	100
Textile effluent B (10%)	14	22	35	100
Molasses spent wash (10%)	34	33	12	100
Black liquor (10%)	71	59	41	100

Five hundred microlitres of culture supernatant having $18 U ml^{-1}$ laccase activity was incubated with 500 μl of dye solution at pH 6.0 and 60 °C. The absorbance was measured at appropriate wavelengths to calculate the percentage of decolorization after 6 and 12 h. Ten milligrams of freeze-dried EPS of the fungus was incubated with dye solutions prepared in phosphate buffer pH 6.0 at 60 °C. Decolorization was measured at 6 and 12 h at the absorbance maxima specific to the dye and effluents. The percentage decolorization was calculated based on the initial readings. All the values are mean of two replicates.

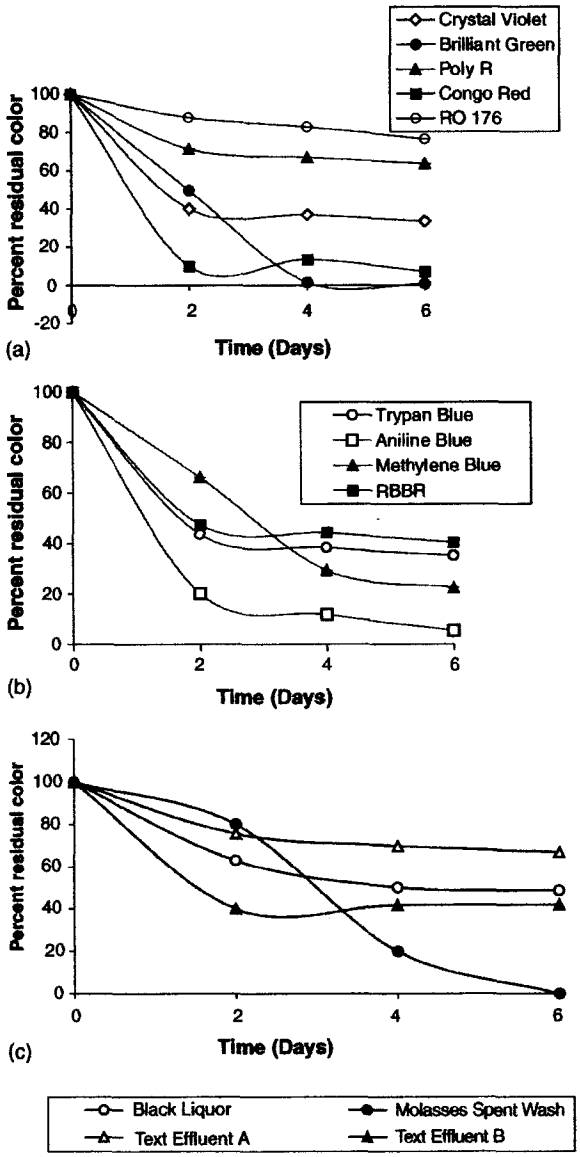

Fig. 2. (a) Decolorization of synthetic dyes measured as percentage of residual color with reference to untreated dyes. The results are shown as percent residual color with reference to the color measured immediately after addition of the dyes to the fungal culture (day zero). (b) Decolorization of some more synthetic dyes by the fungus measured as percentage of residual color with reference to untreated dyes. The values represent mean of three replicates. The S.D. values were below 5% in all the cases. (c) Decolorization of paper mill black liquor, molasses spent wash from alcohol distillery and textile mill effluent A and B used at 10% concentration. The values represent mean of three replicates. The S.D. values were below 5% in all the cases.

NIOCC # 2a when grown in seawater medium with peptone as a nitrogen source. On addition of 2 mM copper sulphate to this medium, ~100-fold increase in laccase production (83619 $U l^{-1}$) was achieved. Besides, addition of aromatic compounds such as *p*-anisidine, catechol, guaiacol, ferulic acid, vanillic acid and veratryl alcohol also induced laccase production. Several of the dyes tested for decolorization in fact induced laccase production. This is also the first report where textile effluent at low concentration (1%) increases laccase production by ~100-fold. Other effluents such as black liquor from paper and pulp mill and molasses spent wash from alcohol distillery also induced laccase production when used at a concentration of 1% (v/v). Using batch culture of the fungus in B&K medium with peptone and glucose as nitrogen and carbon source and 1% textile effluent, we could routinely obtain laccase activity of ~80 U ml^{-1}. This is much higher than those reported in terrestrial fungi recently [17–20].

The optimum pH for laccase activity reported so far in majority of the fungi is between 3 and 5 [11,18–20]. Partially purified laccase of NIOCC # 2 showed biphasic pH curve having maximum activity at pH 3.0 and 6.0. The enzyme peak at pH 3.0 cannot be due to MnP activity as the enzyme assay was done without addition of Mn and H_2O_2 [18]. Optimum temperature of 60 °C is reported in terrestrial fungi *Botrytis cinerea*, *Fomes fomentarius* and *Chaetomium thermophile* and a mesophilic white-rot fungus ([18] and references therein). Laccase active at high temperature may find potential application in treatment of heated industrial effluents.

Most of the dyes and effluents added to the culture medium were simultaneously decolorized and also acted as inducers. Among these, the textile effluent B containing eight reactive dyes induced maximum laccase production. Its acidic nature (pH of 2.5) might induce more laccase activity as the laccase activity of this fungus showed peak at pH 3.0. The textile effluent A on the other hand with its alkaline pH of 8.9 induced much lower laccase activity and was decolorized to a much lesser extent (Table 1 and Fig. 2c). Similar high production of ligninolytic enzymes during treatment of paper mill effluents was reported in *Trametes versicolor* [21].

The mechanism of laccase-catalysed dye decolorization can differ depending upon dye structure. The white-rot fungus *T. versicolor* was shown to use anthroquinone dyes as enzyme substrates that were oxidized by its laccase whereas decolorization of azo and indigo dyes depended upon metabolites having small molecular weights secreted in the culture medium [22]. These small molecular weight metabolites mediated the interactions between the dyes and the enzyme. Thus, the decolorization rate of the non-substrate dyes was limited by the concentrations of mediating compounds than laccase activity in the culture filtrate [22]. In our studies we found that the synthetic dyes, brilliant green and aniline blue induced the highest laccase production and were also decolorized much more than other dyes (see Table 2 and Fig. 2a and b). Poly R and crystal violet induced lowest laccase activity and their decolorization was also lower than all the other dyes (see Table 2 and Fig. 2a). On the other hand, reactive orange (RO 176), an azo dye induced laccase activity moderately but its decolorization was much lower than the other dyes (compare Table 2 and Fig. 2a).

Presence of LiP and MnP, the other lignin-degrading enzymes play an important role in decolorization of dyes. In

NIOCC # 2a, laccase was the major lignin-degrading enzyme as is reported in *Pycnoporus cinnabarinus* [23] *Phlebia tremmellosa* [24] and *Pleurotus sojarcaju* [25]. Poly R and crystal violet are generally decolorized by white-rot fungi producing MnP and LiP [19]. Decolorization of textile dye industrial effluents by white-rot fungus producing laccase as the major lignin-degrading enzyme as seen in our culture was reported in the white-rot fungus *Clitocybula dusenii* [26].

Decolorization of several dyes and effluents was achieved within 6–12 h by incubating the culture supernatant (without the fungal biomass) containing laccase enzyme (Table 3). However, on incubation of the culture supernatant with textile effluent A and B, reduction in color was seen in the initial 2 h after which there was no further reduction in color. Addition of redox mediators may improve this situation as was reported in the case of pure laccase from a commercial formulation [27]. The exopolymeric substance produced by the fungus was more efficient in decolorization of most of the dyes and the effluents than the culture supernatant (Table 3). Even RO 176, Poly R 478 that were not decolorized when added to the culture medium were totally decolorized by the EPS. This might be due to the combined effect of adsorption and enzymatic decolorization. The EPS produced by white-rot fungi is reported to play an active role in lignin degradation [28].

In conclusion, the unidentified marine basidiomycetous fungus NIOCC # 2a decolorized several synthetic dyes when added to the growing fungal culture medium or cell-free culture supernatant containing laccase or EPS precipitated from the culture medium. It decolorized textile effluents, black liquor from paper and pulp mill and molasses spent wash from alcohol distillery in the presence of sea salts. The fungus grew and produced laccase in seawater medium of 25 parts per thousand containing 1% textile effluent B. The synthetic dyes and textile effluent acted as laccase inducers when added to the growing culture of the fungus. The fungus with biphasic pH optima and temperature optimum of 60 °C thus appears to be a good candidate organism for industrial application in bioremediation of colored wastewaters in the presence of chlorides and sulphates.

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Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by the fungus NIOCC #2a, isolated from mangrove wood

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Carbon and nitrogen sources in the growth medium play an important role in the production of lignin-degrading enzymes in the white-rot basidiomyceteous fungi. The role of nutrient nitrogen sources in growth media on production of lignin-degrading enzymes namely laccase, lignin peroxidase and manganese peroxidase as well as on the decolorization of industrial effluents like black liquor, molasses spent wash and textile mill effluents was studied using the basidiomycetous fungus NIOCC #2a isolated from mangrove wood. The results indicated that the type of nitrogen source used, not only influences the amount and type of lignin-degrading enzymes produced but also has an effect on the decolorization of these effluents. The amount of extracellular peroxidases increased by several fold in the presence of effluents whereas in their absence they were of negligible quantity. Some of the effluents had an inhibitory effect on laccase production. The effect of nitrogen sources in the absence as well as presence of the effluents, on the expression of laccase isoenzymes was studied by non-denaturing SDS-PAGE. It was noticed that a few new isozymes of laccase were induced in the presence of industrial effluents. Decolorization of these effluents by the concentrated culture filtrate obtained from media containing different nitrogen sources further proved the importance of the type of nitrogen source in decolorization of colored industrial effluents.

[**Key words**: Laccase, isoenzymes, lignin-degrading marine fungus, nutrients, fungi, effluents]

1. Introduction

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is a copper oxidase capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water. It is predominantly present in fungi and higher plants. Laccase is an important enzyme in the lignin-degrading enzyme complex in ligninolytic fungi, the other being lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Laccase has a wide range of substrate specificity and is thus used in the degradation of several of xenobiotics including synthetic dyes and industrial effluents[1]. Ligninolytic enzymes of white-rot basidiomycetous fungi have been extensively studied for the degradation of recalcitrant compounds and colored effluents from textile and dye-making industries[2]. Laccases have gained importance recently due to a number of diverse applications such as delignification of lignocellulosics and cross-linking of polysaccharides, food technological uses, personal and medical care applications, biosensors and analytical applications[3].

Effective decolorization of bleach plant effluent from paper mill, molasses spent wash from alcohol distillery and synthetic dyes by the basidiomycetous fungus *Flavodon flavus* isolated from decaying seagrass in a lagoon of the Lakshadweep island Kavaratti was reported[4]. This fungus produced all the three lignin-degrading enzymes namely LiP, MnP and laccase, the highest titre being that of MnP[5]. Another white-rot basidiomycetous fungus, NIOCC # 2a, isolated from mangrove wood produced laccase as the major lignin-degrading enzyme and effectively decolorized the colored effluents from paper mill, alcohol distillery and textile mill[6].

The secretion of extracellular lignin-degrading enzymes by white-rot fungi depends on nutrient source, either carbon or nitrogen[7,8]. The lignin-degrading enzymes are produced during secondary metabolism under conditions of limited nitrogen[9,10]. Industrial effluents varying in their nitrogen content and source may sometimes inhibit the activity of fungal growth or their enzymes. Thus for effective

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bioremediation of such colored effluents by white-rot fungi, it is imperative to study these interactions. Differential regulation of ligninolytic enzyme-encoding genes in response to culture conditions has been documented[11]. Effect of inducers of specific laccase isozymes has been demonstrated in some of the basidiomycetes[12,13].

Therefore, the major objective of this study was to investigate the effects of various nitrogen sources incorporated in the growth medium on laccase production and decolorization of industrial effluents by the basidiomycetous fungus NIOCC # 2a. Decolorization of these effluents by the culture filtrate (*in vitro*) containing lignin-degrading enzymes obtained from media with different N sources were also compared. Further, we describe the effect of various nitrogen sources and effluents incorporated in the growth medium on the production of laccase isozymes.

2. Material and Methods

The fungal culture NIOCC #2a used in this study was isolated from decaying mangrove wood from Chorao island in Goa, India (73°55'E and 15°30'N). The fungus was maintained on malt extract agar slants prepared with half-strength seawater. The culture has been deposited at the Microbial Type Culture Collection (MTCC) Chandigarh, India, under the accession number MTCC 5159 under the Budapest treaty for patent culture deposition[14]. Based on partial large subunit rRNA (D2) gene (~350 bps) alignment with GenBank database, it was shown to have 99% homology to an unidentified basidiomycete species AY187277 (MIDI LABS Inc, Newark, USA). Since the telomorphic stage of the fungus has not yet been identified, its further identification is presently not possible. From the ITS sequence, it also showed homology to an unidentified basidiomycete. The sequencing data of ITS and D2 region are deposited in the GenBank under the accession No. AY 939879 and AY 939878 respectively.

2.1 Quantitative estimation of lignin-degrading enzymes

A seven day-old fungal culture grown in malt extract broth was washed with sterile water, mechanically homogenized using glass beads under sterile conditions and the resulting mycelial suspension was used at 10% (v/v) concentration for inoculating low nitrogen medium containing different nitrogen sources at a final concentration of 0.1% nitrogen. The low nitrogen (LN) medium contained a final concentration of 2.5% fructose (1% carbon), 0.01% thiamine, 7% trace elements, 5% basal salts in a total volume of one liter with distilled water. The *p*H was adjusted to 7 with citrate phosphate buffer. Trace elements contained 1.5 g nitrile triacetate in 500 ml distilled water, the *p*H was adjusted to 6.5 with 1 N KOH, to which the following components were added; 3 g $MgSO_4$. 0.5 g $MnSO_4$, 0.08 g $CdCl_2.6H_2O$, 0.1 g $FeSO_4.7H_2O$, 0.1 g $ZnSO_4.7H_2O$, 0.01 g $AlK(SO_4)_2.12H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4.2H_2O$ in a total volume of one liter. Basal salts contained 40 g KH_2PO_4, 10 g $MgSO_4$, 2 g $CaCl_2$ in a total volume of one liter. The type of nitrogen sources however varied namely, KNO_3, glutamic acid, glycine, beef extract and corn steep liquor. The cultures were raised in 250 ml capacity Erlenmeyer flasks, containing low nitrogen media varying only in the type of the nitrogen source utilized, under stationary conditions. The cultures were oxygenated every third day with pure oxygen for 1 min using tygon tubing and Pasteur pipettes, under sterile conditions. Cupric suphate at a final concentration of 2 mM was added to the 4-day old cultures. The effect of various colored industrial effluents on the production of lignin-degrading enzymes as well as the ability of the fungus to decolorize these effluents *in vivo*, was studied by adding different effluents namely, textile mill effluent A and B (TEA and TEB), molasses spent wash (MSW) from alcohol distillery and black liquor (BL) from a paper and pulp mill, to the 6-day old fungus grown in each nitrogen source. The controls for the experiment were the cultures grown in varying nitrogen sources without any added effluent. The effluents were added to a final concentration of 10% were added to 6-day old culture. Textile effluent A (from Atul Ltd., Gujarat) contained mainly azodye-20 and had a *p*H of 8.9 with 0.34% carbonate. The textile effluent B from the same source had a *p*H of 2.5 with 21,700 color units. The black liquor obtained from Seshasayee Paper Mills, Erode, Tamil Nadu, was from a bagasse and wood chip-based newsprint manufacturing unit. As per the data provided by the mill, the effluent had COD of 416 mg l^{-1} and BOD of 190 mg l^{-1}. Raw untreated molasses spent wash obtained from Rhea Distilleries Ltd., Goa was reported to have a *p*H of 4.3, BOD of 42,000 mg l^{-1} and COD of 80,000 mg l^{-1}.

The cultures were allowed to grow for another 6 days after the addition of the effluents. The biomass

of each of the 12-day old cultures was obtained by filtering the contents through oven-dried, pre-weighed Whatman No. 1 filter paper discs. The dry weight of the fungus was determined as the difference in weight after drying the filter papers at 60°C until a constant weight.

The lignin-degrading enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase as well as the amount of decolorization that had occurred in the filtrate obtained from these cultures were estimated. The activity of LiP was determined[15] by measuring the rate of oxidation of veratryl alcohol to veratryldehyde in the presence of H_2O_2. Manganese peroxidase was determined[16] by measuring the rate of oxidation of Mn^{+2} to Mn^{+3} in the presence of H_2O_2. Laccase activity was assayed[17] using ABTS (2,2′-azino-bis-3-ethyl benzothiazoline-6-sulphonic acid) (Sigma, USA) substrate at *p*H 3.0. The enzyme units were expressed as units per liter of culture filtrate ($U\ l^{-1}$).

2.2 In vivo decolorization of effluents

Decolorization of the effluents on day 12 was determined by monitoring the absorbance at their absorbance maxima. The absorbance obtained immediately upon addition of the effluent was considered to be 100%. The extent of decolorization was recorded as percentile residual color or percentile decolorization. Decolorization of TEA and TEB were monitored at their absorbance maxima of 505 and 667 nm respectively and MSW and BL were monitored at 475 and 317 nm respectively. All experiments were carried out in triplicates and the average values are presented.

2.3 In vitro decolorization of effluents

The enzyme source for the decolorization of effluents, *in vitro* was the five-fold concentrated culture filtrate obtained from the culture grown in various nitrogen sources without the added effluent. Thus, five different enzyme sources corresponding to the different nitrogen sources were used for the *in vitro* decolorization of each of the effluents. The concentration of the culture filtrates was by ultra filtration using Centricon tubes with a 10 kDa cut-off membrane (Millipore, USA) at 5,000 rpm in the cold (4°C). Each of the concentrated culture filtrates with 10 U of laccase activity was incubated at 30°C at a final concentration of 1000 color units of the effluents. Color units of the various effluents were determined by measuring its absorbance maximum in a UV/Visible spectrophotometer (Shimadzu, Japan). One color unit is defined as the amount of colored material in 1 ml giving an optical density of 1.0 in a path length of 1.0 cm at its absorbance maximum[18]. Decolorization of various effluents *in vitro* was monitored at 6, 12, 24, 36 and 48 hours after addition of the enzyme to the effluent. Decolorization achieved was calculated with reference to the zero hour reading.

2.4 Effect of nitrogen source on the laccase isozyme pattern

The culture filtrates from the 12 day-old cultures grown in different nitrogen sources as well as those supplemented with effluent were concentrated by ultra filtration with nanosep centriguge tubes (Pall Gellman, USA) having 3 kDa cut-off membrane at 5,000 rpm (4°C). After estimating the laccase activity in the concentrates, non-denaturing SDS-PAGE using 12% resolving and 7% stacking gel was carried out with a constant voltage of 60 volts at 6°C. One unit of laccase from each sample was loaded in each well. The gels were observed for laccase activity by staining them with 2.5% guaiacol (SD-Fine Chemicals Ltd., Mumbai). The molecular weights of the laccase isozymes were determined by silver-staining of the corresponding gels[19].

3. Results and Discussion

3.1 Growth of NIOCC # 2a in media with different N sources and effluents

Fungal growth was best in the presence of glutamic acid as the nitrogen (N) source. When supplemented with black liquor (BL), the fungus showed enhanced growth in all the nitrogen sources except glutamic acid. Fungal biomass was more in KNO_3 and beef extract (BE) supplemented with molasses spent wash (MSW) than in other N sources. No significant difference was observed between glutamic acid and the same supplemented with MSW. On the other hand, growth was inhibited in corn steep liquor (CSL) and glycine supplemented with MSW. The fungus showed enhanced growth in all of the TEA-supplemented media (Table 1) whereas, growth was inhibited in media containing TEB except in the presence of glycine.

3.2 Effect of N source and effluent on the production of lignin-degrading enzymes

Among several of the carbon sources tested, maximum laccase activity was detected when fructose

was used as the carbon source. Among all the nitrogen sources, glutamic acid supported maximum laccase production (Table 1). This trend was maintained even in the presence of TEA and MSW. On the other hand, TEB and BL supported maximum laccase production when glycine was the N source (Table 1). Glutamic acid and glycine being amino acids not only serve as a nitrogen source but also as a readily available source of carbon. This carbon in addition to the carbon supplied by fructose probably aided in biomass build-up which in turn positively affected laccase production. Among, the two amino acids, glutamic acid supplies more carbon than glycine for a constant amount of nitrogen. This is reflected in the difference in the amount of biomass obtained (Table 1). Irrespective of the nitrogen source used, laccase production was inhibited when supplemented with BL and TEA.

Production of manganese peroxidase (MnP) was enhanced by several fold in the presence of BL in all of the N sources (Table 1). To a certain extent, TEA and MSW also enhanced the production of MnP in some of the nitrogen sources. Similarly, production of LiP was enhanced by several fold in the presence of BL and to a certain extent in the presence of MSW, followed by TEA and TEB (Table 1). Fungal biomass and lignin-degrading enzyme production did not show any correlation.

Table 1—Effect of nitrogen source and effluents on the production of biomass and lignin-degrading enzymes by NIOCC # 2a.

Nitrogen Source	Effluents				
	Control (without effluent)	TEA#	TEB#	MSW#	BL#
	Biomass (mg/ 20 ml)				
KNO_3	106.5	141.6	80.4	149.7	118.2
Glycine	81.2	104.8	111.5	53.9	154.2
Glutamic acid	144.7	150.5	96.5	142	106.2
Beef extract	77.4	89.3	74.9	190.4	138.5
Corn steep liquor	95.4	99.I	94.6	61.7	146.1
	Laccase Activity (U L^{-1})				
KNO_3	16,687	2,224	10,385	6,652	2,622
Glycine	34,659	3,702	49,628	5,644	12,210
Glutamic acid	47,567	9,756	20,47I	26,552	2,287
Beef extract	10,346	1,979	8,254	8,320	1,445
Corn steep liquor	12,973	620	12,172	2,553	2,058
	MnP Activity (U L^{-1})				
KNO_3	0	353	0	0	2421
Glycine	12	508	30	0	1621
Glutamic acid	0	0	38	394	1679
Beef extract	25	493	0	450	2372
Corn steep liquor	46	1760	222	604	2178
	LiP Activity (U L^{-1})				
KNO_3	26	266	150	85	913
Glycine	2	343	65	98	1114
Glutamic acid	17	75	103	180	2591
Beef extract	25	473	141	0	671
Corn steep liquor	26	29	0	75	0
	Percentile Decolorization				
KNO_3	—	64	78	30	0
Glycine	—	56	88	61	5
Glutamic acid	—	64	70	36	1
Beef extract	—	28	77	49	0
Corn steep liquor	—	53	92	71	0

TEA =Textile effluent A; TEB =Textile effluent B; MSW =Molasses spent wash; BL = Black Liquor

Mansur *et al.*,[20] showed that fructose induced 100-fold increase in laccase production in the basidiomycetous fungus CECT 20197. Stajic *et al.*,[21] demonstrated the effect of inorganic and organic nitrogen sources on laccase production in different species of *Pleurotus*. Our results showed that well defined organic N sources such as glutamic acid and glycine were better than beef extract and corn steep liquor for laccase production. Elisashvili *et al.*,[22] observed highest laccase activity in the medium with ammonium sulfate as the N source in *Cerrena unicolor* IBB 62.

3.3 Effect of N source on (in vivo) decolorization of colored effluents

Among the effluents, BL was least decolorized, irrespective of the N source used, whereas TEB was decolorized equally well in all the media (Table 1). Decolorization of TEA and MSW was achieved to a moderate extent in media with different N sources. As the volume and not the color units of the effluent added (10% v/v) was kept constant, decolorization of the individual pollutants varied vastly. The black liquor at the same volume gave intense color with much higher color units than the other effluents. Textile effluent B was less turbid than the other effluents and had a *p*H of 2.5, the *p*H at which most of the lignin-degrading enzymes show their optimum activity[23]. Textile mill effluent A with a *p*H of 8.9 showed less decolorization since the lignin-degrading enzymes show[23] negligible activity at alkaline *p*H.

Although MnP and LiP production was enhanced in the presence of black liquor, this did not result in its decolorization. On the other hand, mutants of *Phanerochaete chrysosporium*, a well-known lignin-degrading white-rot fungus that lacked the ability to produce LiP but produced MnP, showed about 80% decolorization of bleach plant effluent, suggesting that MnPs play an important role in decolorization of bleach plant effluent[24]. Black liquor enhanced the growth of the fungus, whereas, TEB inhibited its growth. In spite of this, TEB was decolorized up to 70-90%, whereas, black liquor was decolorized only up to 5%. Maximum laccase was produced in the presence of TEB and similarly TEB was decolorized to the maximum extent (Table 1) suggesting that laccases play a key role in decolorization of textile effluents. The important role of laccases in textile dye decoloriztion has been reported[25].

3.4 Decolorization of the effluents in vitro

All the effluents were decolorized to the maximum by 48 hours *in vitro*. The process might have continued, but the experiment was terminated at 48 h in the present study (Fig. 1). Enzymes from media containing only KNO_3 as N source performed continuous decolorization of TEA (Fig. 1A). On the other hand, enzymes from all the N sources, decolorized TEB continuously (Fig. 1B) without any repolymerization. A similar phenomenon was observed in the case of MSW where continuous decolorization occurred only when enzyme from beef

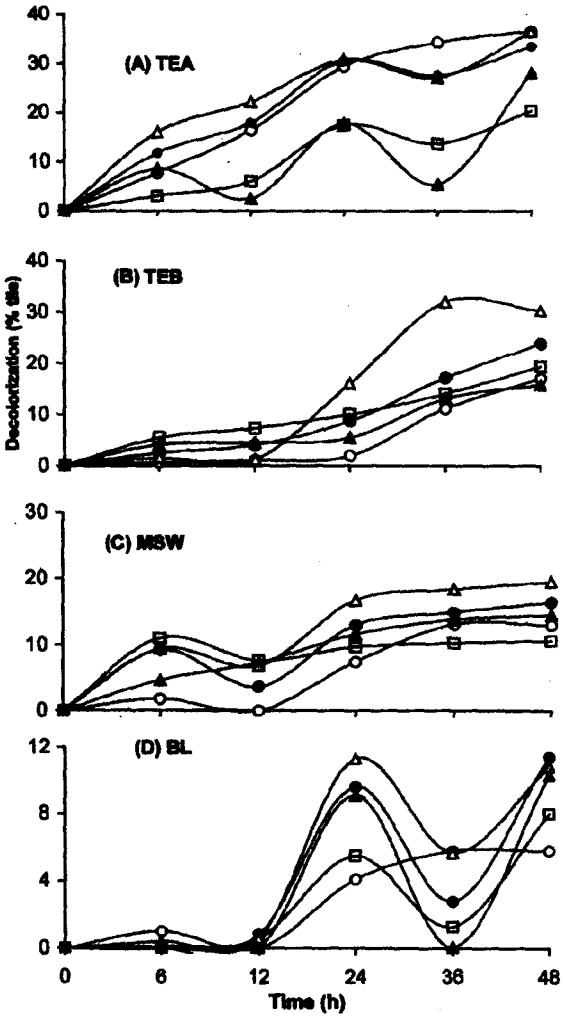

Fig. 1—*In vitro* decolorization of effluents with 1000 color units each, using 10 U of laccase obtained from different nitrogen sources namely, KNO_3, glycine, glutamic acid, beef extract and corn steep liquor. Decolorization was monitored from 0 to 48 h. KNO_3= O ; Glycine ●; Glutamic acid Δ; Beef extract ▲; Corn steep liquor □.

extract as N source was used (Fig. 1C). Continuous depolymerization of black liquor occurred, when the enzyme source from KNO_3 was used (Fig. 1D). In the presence of enzymes obtained from media containing other N sources, an initial decolorization of BL was followed by increase in color, which is probably due to its repolymerization (Fig. 1D). The decolorization was not the effect of *p*H, as there was no change in *p*H at the end of 48 h in any of the reaction mixtures. These results led us to hypothesize that the N source in the medium regulated the production of lignin-degrading enzymes and this in turn affected its decolorization ability. However, no direct correlation between enzyme units and percentage decolorization was observed in any of the N sources. Therefore, it appears that besides the lignin-degrading enzymes and the source of N, the composition of the effluents plays an equally important role in decolorization.

3.5 Influence of N source on the isozyme pattern of laccase

To study the production of different isoenzymes of laccase as a function of the type of nutrient nitrogen in the presence as well as absence of effluents, samples containing one unit of laccase were analyzed by non-denaturing SDS-PAGE. The results showed that the nitrogen source not only affects laccase production quantitatively but also the type of laccase isozymes produced (Figs. 2-4). One of the reasons for differential decolorization of various effluents in the presence of constant laccase units may be attributed to differential isozyme patterns and amount of laccase produced under different N sources. The isozymes may have altered substrate affinity which may affect its decolorization potential. In case of the enzyme source from media without effluent supplemention, The 47 kDa laccase was present irrespective of the N source used in the medium and those supplemented with the effluents except black liquor (Fig. 2). Incidently, black liquor (BL) showed the lowest decolorization. A faint band of 27 kDa was observed in KNO_3 alone. A 55 kDa laccase was induced in the presence of glycine, beef extract and corn steep liquor, whereas a 25 kDa laccase was induced only in the medium containing glutamic acid as the N source. The 47 kDa laccase was present in all the N sources supplemented with various effluents except BL (Fig. 2). The 55 kDa laccase was present in media containing glycine, beef extract and corn steep liquor as N source (Fig. 2). It was induced only in medium containing KNO_3 supplemented with MSW, whereas it was inhibited in glycine and corn steep liquor (Figs. 2, 3). Only one laccase of 47 kDa was detected in medium with glycine as N source supplemented with TEB (Figs. 2, 4) where maximum laccase

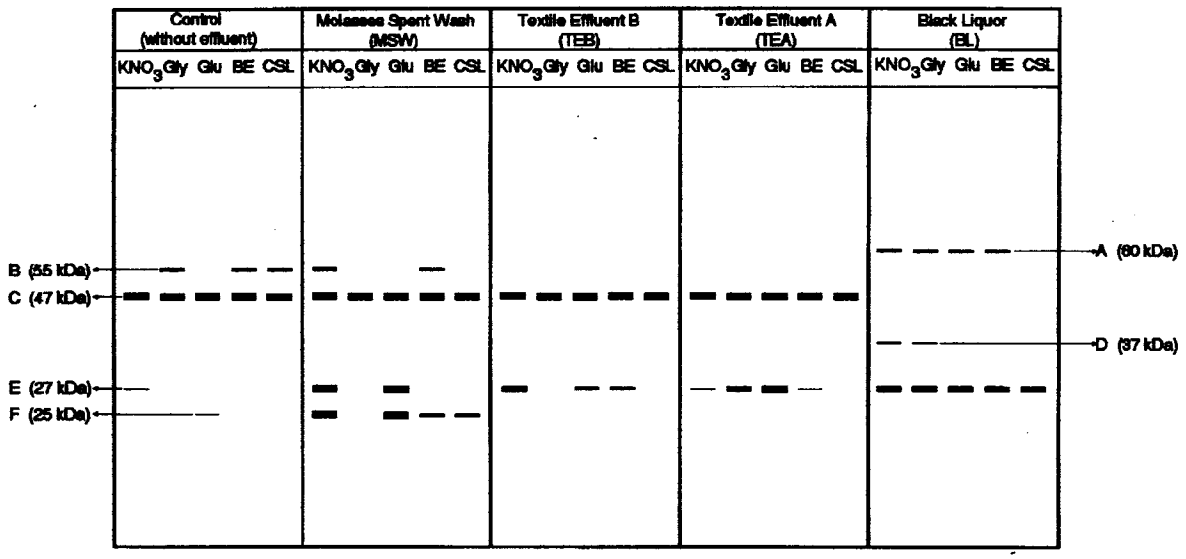

Fig. 2—Schematic representation of the laccase isozyme pattern obtained with non-denaturing PAGE using 12% resolving gel stained with 2.5% guaiacol. The figure shows isozyme pattern of laccase induced in different nitrogen sources, with and without effluent supplements. The nitrogen sources were KNO_3, Gly (Glycine), Glu (Glutamic acid), BE (Beef Extract) and CSL (Corn Steep Liquor). Bands A – F represent the different laccase isozymes induced and their corresponding molecular weights are given within parenthesis. The figure is drawn to the original scale.

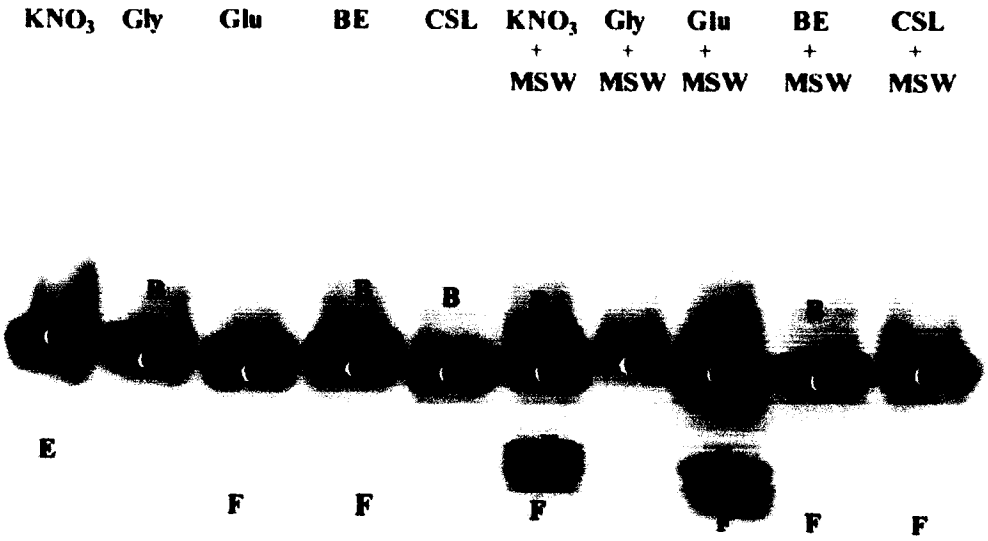

Fig. 3—Non-denaturing PAGE using 12% resolving gel stained with 2.5% guaiacol for detecting laccase isozymes induced in different nitrogen sources and these supplemented with molasses spent wash (MSW). [Band B = 55 kDa; C = 47 kDa; E = 27 kDa and F = 25 kDa].

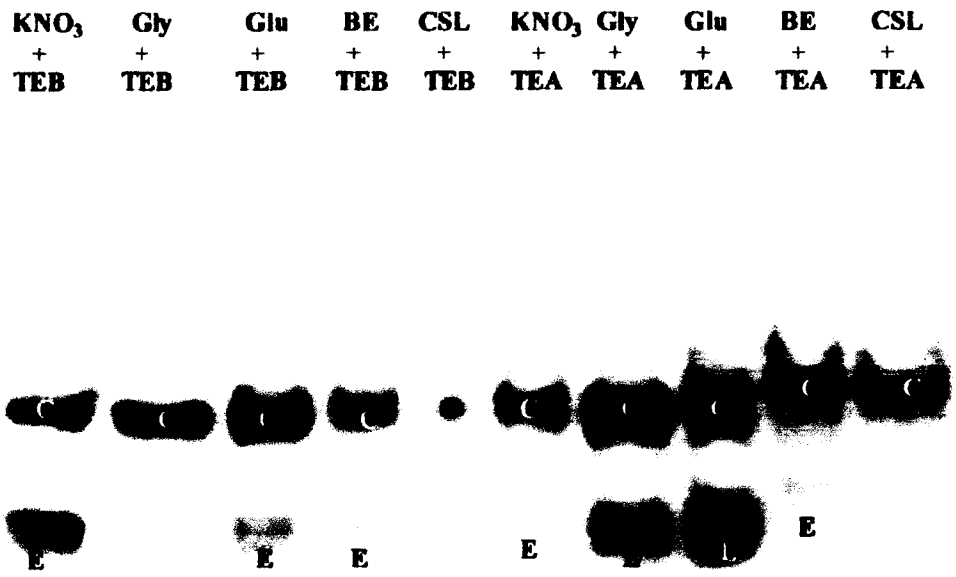

Fig. 4—Non-denaturing PAGE using 12% resolving gel stained with 2.5% guaiacol for detecting laccase isozymes induced in different nitrogen sources supplemented with TEB and TEA. [Band C = 47 kDa and E = 27 kDa].

activity was detected (Table 1). Therefore, the amount of decolorization achieved is dependant on the type of laccase isozymes induced as well as their concentration. The 27 kDa laccase was induced in several other combinations of N source and effluents (Figs. 2-4). The 25 kDa isozyme was present in all the nitrogen sources only when supplemented with MSW, except in glycine (Figs. 2, 3). A 37 kDa isozyme was specific to black liquor supplemented-KNO_3 and glycine media (Fig. 2). Similarly, a 60 kDa isozyme unique to BL-supplemented media was observed except when CSL was the N source (Fig. 2).

Differential regulation of laccase-encoding genes in response to culture conditions has been documented in the terrestrial fungus *Phanerochaete chrysosporium*[11]. Effect of copper on induction of laccase isoenzymes was demonstrated in the white-rot fungus *Pleurotus ostreatus*[13]. We have demonstrated here the effect of the type of N source as well as type of effluent used on the laccase isoenzyme production. Western blot analyses using specific anti-laccase isozyme-antibodies will help in confirming this activity to protein production. Genes that encode laccase isoenzymes in *P. ostreatus* have been cloned and sequenced[26, 27], but very little is known about the regulation of the laccase gene expression.

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Treatment of Colored Effluents with Lignin-Degrading Enzymes: An Emerging Role of Marine-Derived Fungi

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Some of the industries that discharge highly colored effluents are paper and pulp mills, textiles and dye-making industries, alcohol distilleries, and leather industries. Terrestrial white-rot basidiomycetous fungi and their lignin-degrading enzymes laccase, manganese-peroxidase and lignin peroxidases are useful in the treatment of colored industrial effluents and other xenobiotics. Free mycelia, mycelial pellets, immobilized fungi or their lignin-degrading enzymes from terrestrial fungi have been reported in treatment of several effluents. Marine obligate or facultative (marine-derived) fungi may have unique properties but have not been explored sufficiently for this purpose. This article presents a critical review of bioremediation potential of such fungi and their lignin-degrading enzymes in comparison with the state-of-the-art in terrestrial white-rot fungi.

Keywords Industrial effluents, decolorization, detoxification, laccase, peroxidases, marine fungi

1. INTRODUCTION

Paper and pulp mills, molasses based-alcohol distilleries, tanneries, dye-making units, and textiles are some of the major industries that produce and discharge highly colored effluents. Each of these industrial effluents creates some specific problem besides producing aesthetically unacceptable intense coloring of soil and water bodies. They block the passage of light to the lower depths of the aquatic system resulting in cessation of photosynthesis, leading to anaerobic conditions, which in turn result in the death of aquatic life causing foul smelling toxic waters.

Colored industrial wastewater is usually treated by physicochemical processes. These processes include flocculation, flotation, electro flotation, membrane-filtration, ion exchange, irradiation, precipitation, ozonation, and adsorption using activated carbon or biological adsorption using bacteria, fungi, algae, or plant biomass (Robinson et al. 2001; Husain 2006; Whiteley and Lee 2006 and references therein). Both living and dead cells have been used for bio-adsorption (Fu and Viraraghavan 2001).

Bioremediation is also possible with naturally occurring (Reddy 1995) or genetically modified organisms (Chen et al. 1999) for treatment of soil, ground or surface water for general protection of human health and the environment. This has to be primarily safe and comparatively less expensive than conventional treatments. Bacteria and fungi along with their products such as enzymes (Whiteley and Lee 2006) and exopolymeric substances (Liao et al. 2001) aid in bioremediation. The application of bioremediation has remained limited due to incomplete understanding of the degradation processes performed by organisms in natural systems and engineering of suitable systems for the optimum utilization of the organism is required (Pritchard et al. 1996). Maintaining the optimum catalytic activity of an organism for a long period of time under controlled conditions for treatment of massive volumes of effluents is a rather difficult task. Therefore bioremediation has not been as successful as physical and chemical methods (Pritchard et al. 1996).

A number of biotechnological approaches have been tried for the treatment of colored effluents and one of the most successful groups of organisms in this context has been the white-rot basidiomycetous fungi that are capable of extensive degradation of lignin under aerobic conditions. White-rot fungi are known to play a major role in mineralization of the lignin polymer to CO_2 and H_2O in the terrestrial environment. These fungi produce a wide range of lignin-degrading enzymes (LDEs), which in turn act on lignin and lignin-analogous compounds. The LDEs share common features such as broad substrate specificity, high redox potential, and are mostly extracellular in nature. The high redox potential and broad substrate specificity, increases the range of pollutants the enzyme is capable of degrading. These features combined with the fact that LDEs are mostly expressed under nutrient deficient conditions (which is usually the case in the nature) and their ability to oxidize substrates with low solubility have made them the preferred

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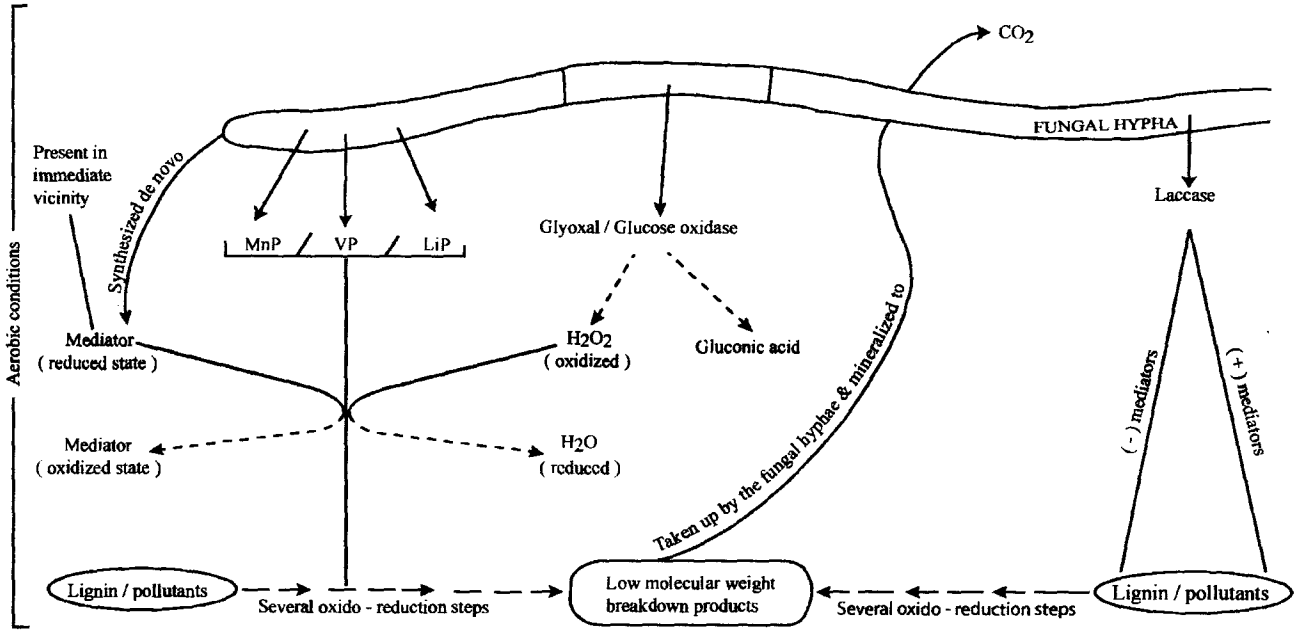

FIG. 1. Schematic diagram illustrating the role and activity of various extracellular lignin-degrading enzymes produced by fungi (Modified from Field et al. 1993). MnP = manganese-dependent peroxidase; LiP = lignin peroxidase; VP = versatile peroxidase.

candidates for bioremediation along with the fungi, responsible for their production (Reddy 1995).

The process of bioremediation can be monitored by measuring any of the following factors: (1) by measuring the redox potential, together with pH, temperature, oxygen content and concentrations of electron acceptor (s)/donor(s) and the breakdown products such as carbon dioxide or (2) by measuring chemical oxygen demand (COD) and biological oxygen demand. Biological oxygen demand (BOD) represents only the organic matter which is capable of being degraded/oxidized by microbes whereas COD represents all the oxidizable matter, including organic matter in any particular effluent (Marmagne and Coste 1996). For colored effluents, bioremediation is measured by estimating the reduction in color units of effluents and percentage of detoxification achieved besides measuring a few of the above-mentioned parameters.

2. LIGNIN-DEGRADING ENZYMES

Lignin-degrading enzymes (LDEs) belong to two classes *viz* the heme-containing peroxidases and the copper-containing laccases. A series of redox reactions are initiated by the LDEs, which degrade the lignin (or lignin-derived pollutants). The LDEs oxidize the aromatic compounds until the aromatic ring structure is cleaved, which is followed by further degradation with other enzymes.

Peroxidases are heme-containing enzymes that comprise manganese-dependant peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP). Lignin peroxidase (EC 1.11.1.14) requires H_2O_2 as the co-substrate as well as the presence of a mediator like veratryl alcohol to degrade lignin and other phenolic compounds (Fig. 1). Here H_2O_2 gets reduced to H_2O by gaining an electron from LiP (which itself gets oxidized). The oxidized LiP then returns to its native reduced state by gaining an electron from veratryl alcohol and oxidizing it to veratryl aldehyde. Veratryl aldehyde then gets reduced back to veratryl alcohol by gaining an electron from lignin or analogous structures such as xenobiotic pollutants. This results in the oxidation of lignin or the aromatic pollutant (ten Have and Teunissen 2001). MnP (EC 1.11.1.13). This too, requires H_2O_2 as its co-substrate and the presence of Mn^{2+} (naturally present in wood). It catalyses oxidation of Mn^{2+} to Mn^{3+}, resulting in an Mn^{3+} chelate-oxalate, which in turn oxidizes the phenolic substrates. The resulting Mn^{3+} chelate-oxalate is small enough to diffuse into areas inaccessible even to the enzyme, as in the case of lignin or analogous structures such as xenobiotic pollutants buried deep within the soil, which are not necessarily available to the enzymes (Tortella et al. 2005).

A novel enzyme which can utilize both veratryl alcohol and Mn^{2+}, versatile peroxidase (EC 1.11.1.16) has been recently described as a new family of ligninolytic peroxidases (Martínez 2002; Martínez et al. 2004). The most noteworthy aspect of versatile peroxidase (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). It

has an 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).

Laccase (EC 1.10.3.2) is a benzenediol:oxygen oxidoreductase (a multi-copper enzyme), present across the kingdoms from bacteria, e.g., *Azospirillum lipoferum* and actinomycetes like *Streptomyces*, to fungi to plants and even in insects (Baldrian 2006). This multi-copper oxidase has the ability to oxidize phenolic compounds. Unlike peroxidases, it does not contain heme as the cofactor but copper. Neither does it require H_2O_2 as the co-substrate but rather molecular oxygen. Laccase often sports a high degree of glycosylation, which confers a degree of self-resistance to attack by proteases (Yoshitake et al. 1993). The downside however, is that the redox potential although varying between different laccase isozymes, cannot be compared with that of the peroxidases, especially LiP. Although laccase does not have the exclusive requirement for the presence of mediators like veratryl alcohol and Mn^{+2}, their presence increases the effective range of substrates, which can be degraded by laccase. Laccase, can not only catalyze depolymerizing reactions but polymerizing reactions as well. Whilst depolymerization is obviously useful for the breakdown of pollutants, polymerization can also be useful, even though larger compounds are created. This is because sequestration is acceptable as a method for bioremediation. While forming a larger compound does not remove it from the environment, it can be rendered non-toxic thus negating the need for its removal (Ali and Sreekrishnan 2001).

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 CO_2 and 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 but mostly no laccase, has been the laboratory model for physiological and molecular biological studies of LDEs (Fu and Viraraghavan 2001). *Trametes versicolor* producing laccase as the major LDE has been studied widely for industrial application in biobleaching of paper pulp, treatment of effluents and various other industrial applications (Wesenberg et al. 2003). However, production of MnP in strains of *T. versicolor* has also been demonstrated recently (Snajdr and Baldrian 2007; Diorio et al. 2008).

3. WHAT AND WHY MARINE FUNGI FOR LIGNIN DEGRADATION?

Mangrove plants and seagrasses contain 50% lignocellulosic material as structural polymers and are the major contributors of lignocellulose substrate in coastal marine environment (Benner and Hodson 1985). Mangrove leaves, twigs, wood pieces, and seagrasses fallen into the intertidal zone are colonized by epibiotic bacteria and epi- and endobiotic fungi (Fig. 2). The term 'marine-derived' fungi, is used here since the marine ecosystem comprises of obligately marine as well as facultative marine fungi. The facultative forms although having counterparts in the terrestrial ecosystem, have adapted to the marine environment. Obligate and facultative marine fungi colonizing these substrates produce cell wall-degrading enzymes and are responsible for the production of dissolved organic carbon (DOC) and particulate organic carbon (POC) in water (Newell 1996). The resulting DOC is utilized by bacteria for biomass build up and the microbially colonized POC is utilized as feed by detritus-feeding larvae and other macroorganisms such as crabs and shrimps (Odum et al. 1979).

Fungi have been isolated and enumerated from mangrove leaves, seagrasses, and salt marsh grass (Sathe and Raghukumar 1991; Newell 1993; Raghkumar et al. 1995). Several reports have demonstrated active loss in weight of various timber blocks colonized by marine wood-degrading fungi (Nilsson et al. 1989; Pointing et al. 1998; Pointing and Hyde 2000; Bucher et al. 2004). Interestingly, most of these reported fungi belong to ascomycetes and a very few to basidiomycetes or white-rot fungi. Enumeration of fungi, their succession and decomposition of mangrove wood is reported from various tropical and subtropical parts of the world by numerous workers (Vrijmoed and Tan, 1990; Chinnaraj and Untawale 1992; Vishwakiran et al. 2001). Therefore, fungi growing under such marine conditions are expected to have adapted to grow under saline (ranging from 10–34 ppt) and alkaline conditions since the pH of seawater ranges from 7.5– 8.2. Such LDE-producing fungi should find application in bioremediation of lignin-based derivatives in colored industrial pollutants such as paper and pulp mills, tanneries, molasses-based distilleries, and textile mills. These effluents are mostly alkaline and have high salt content (Bartlett 1971) and therefore, marine fungi, facultative, or obligate that grows in the presence of saline and alkaline conditions perhaps are well suited for treatment of such effluents.

3.1. Lignin-degrading ability of marine fungi

Mineralization of ^{14}C (ring)-labeled synthetic lignin to $^{14}CO_2$ is considered the acid test for the lignin-degrading ability of any fungus (Kirk and Farrell 1987). Sutherland et al. (1982) demonstrated limited mineralization of ^{14}C-labeled maple and spruce lignin to $^{14}CO_2$ by a number of marine fungi. Only 5–6% of the labeled lignin was mineralized at the end of 30 days by these fungi. *Phaeospheria spartinicola*, an ascomycetous fungus growing on the decaying leaves of the salt marsh cord grass *Spartina alternifolia* was shown to degrade lignocellulose and contribute to dissolved DOC formation (Bergbauer and Newell 1992). After 45 days of incubation, only 3.3% of the lignin moiety was mineralized to $^{14}CO_2$ and 2.7% solubilized to $DO^{14}C$ by this fungus. An obligate marine fungus *Halosarpheia ratnagiriensis*

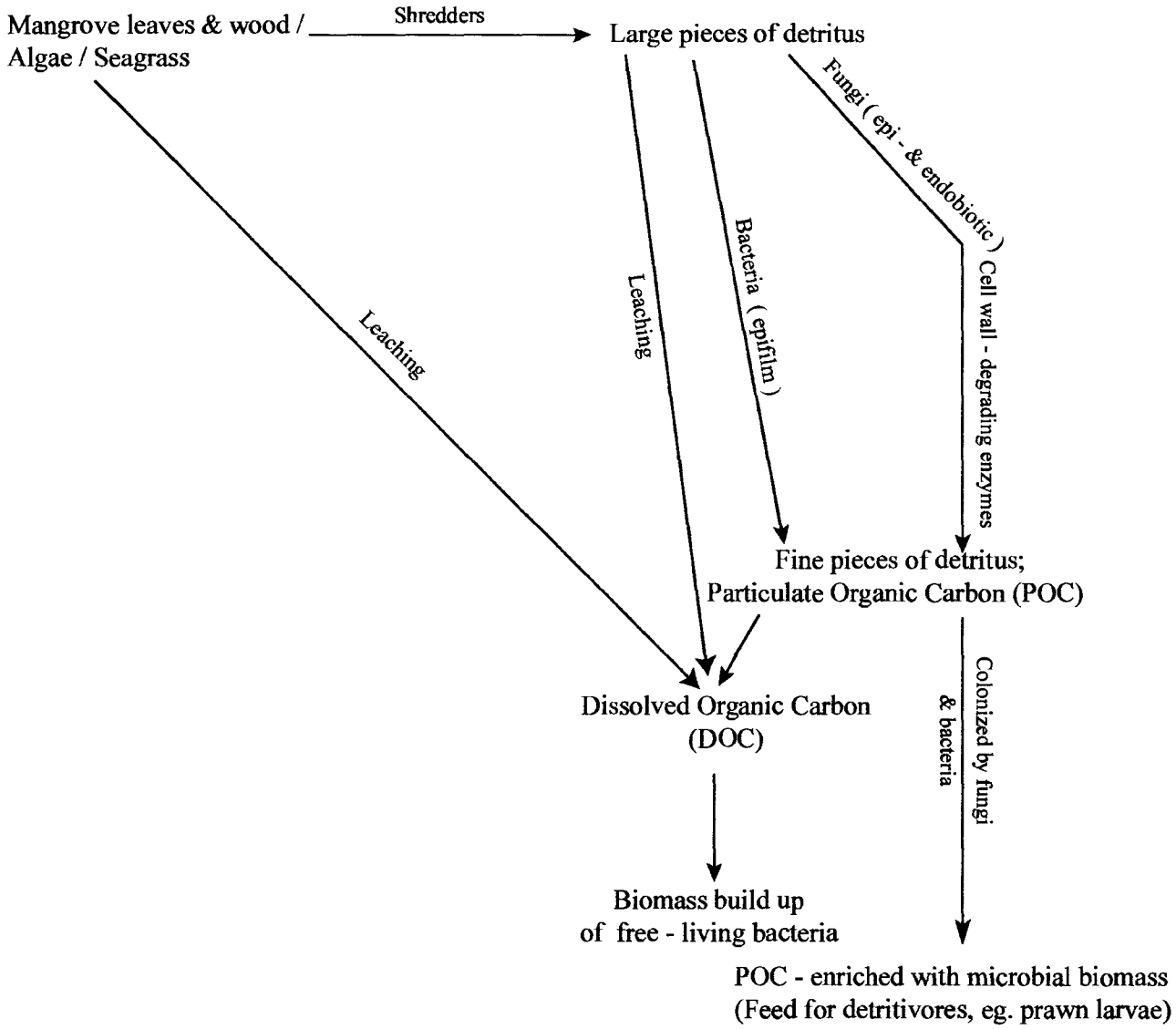

FIG. 2. Schematic diagram illustrating the process of degradation of plant material in the marine habitat.

(strain NIOCC #321) and one facultative marine fungus *Sordaria finicola* (NIOCC #298) mineralized about 9–10% of the U-ring ^{14}C-labeled lignin to $^{14}CO_2$ within 21 days (Raghukumar et al. 1996). A basidiomycete, NIOCC #312 isolated from decaying leaves of the sea grass *Thalassia hemprichii* on the other hand, mineralized 21% of the U-ring ^{14}C-labeled lignin to $^{14}CO_2$ within 21 days (Raghukumar et al. 1999). In the same experiment, the lignin-degrading terrestrial fungus *Phanerochaete chrysosporium* generally used as a benchmark for lignin-degradation was shown to mineralize about 21% of the ^{14}C-labeled lignin (DHP) to $^{14}CO_2$ within 21 days (Raghukumar et al. 1999). Thus, this marine-derived fungus, NIOCC #312 has been the only one reported among the marine fungi to match the efficiency of terrestrial white-rot fungi in lignin mineralization.

3.2. Lignocellulose-degrading enzymes in marine fungi

Lignocellulose-degrading enzymes; cellulase and xylanase have been detected in marine fungi isolated from salt marsh grass (Gessner 1980). Rohrmann and Molitoris (1992) have also reported the presence of laccase in addition to the above enzymes in marine fungi isolated from algae. Marine basidiomycetes and ascomycetes grown in seawater media showed higher laccase activity than those grown in fresh water media (Rohrmann and Molitoris 1992). Schaumann et al. (1986) demonstrated laccase activity in 65% of marine ascomycete *Lulworthia* sp. in substrates like guaiacol, naphthol, and benzidine. Subsequently, presence of laccase, cellulase, and xylanase activities in several facultative and obligate marine fungi isolated from mangrove and seagrass leaves and sediments from mangrove stands

TABLE 1
Comparison of the lignin-degrading enzyme production in low nitrogen medium (LNM) by the two marine-derived fungi, NIOCC #312 and NIOCC #2a

	Days						
	3	5	7	9	11	13	15
NIOCC #312							
MnP ($U L^{-1}$)	0	47	112	298	338	276	118
LiP ($U L^{-1}$)	8	12	29	52	15	0.1	0
Laccase ($U L^{-1}$)	0.8	0.24	30.5	1.4	2.3	0.6	0.6
NIOCC #2a							
MnP ($U L^{-1}$)	1.5	80	82	79	84	105	93
LiP ($U L^{-1}$)	0.3	0.5	0	0	0	0.4	0.6
Laccase ($U L^{-1}$)	571	8425	5097	6125	8946	12613	7415

were reported (Raghukumar et al. 1994). About 70% (12 out of 17 fungi screened) of these fungi showed laccase activity and ~80% of the fungi showed cellulase activity when grown in media prepared with half strength sea water. Among these, two of the marine ascomycetous fungi *Halosarpheia ratnagiriensis* (NIOCC #321) and *Sordaria fimicola* (NIOCC #298) secreted MnP and laccase in seawater media. Thus, these two fungi belong to the second category of lignin-degrading fungi, which are classified to produce MnP and laccase (Hatakka 1994). Pointing et al. (1998; 1999) reported presence of laccase, cellulose, and xylanase in several marine fungi from tropics. Although a thorough list of marine fungi in tropical America and Africa is available (Kohlmeyer and Kohlmeyer 1979) and other tropical countries (Kohlmeyer 1984), lignin-degrading activity of these fungi have not been investigated. On the other hand, large amount of information is available on biologically active natural product chemistry from marine and marine-derived fungi (Liberra and Lindequist 1995; Bugni and Ireland 2004) but not on lignin-degrading enzymes. Recently a number of filamentous fungi have been isolated from hypersaline environment of the Dead Sea (Molitoris et al. 2000). They were demonstrated to decolorize several synthetic dyes at various salinities but no information is available regarding presence of LDE system in these.

Lignin-degrading marine-derived fungi that do not fall into any of the categories described by Hatakka (1994) have also been reported. The basidiomycetous fungus NIOCC #312, isolated from decaying seagrass (*Thalassia hemprichii*) of the Lakshadweep island, India, produced all three LDEs, also does not conform to any of the above categories (Raghukumar et al. 1999). However, the major LDEs in this fungus were MnP and LiP whereas laccase was minimal (Table 1).

Another basidiomycetous fungus NIOCC #2a, isolated from decaying mangrove wood in Chorao island, Goa, showed laccase positive reaction when grown in LNM prepared with seawater containing 4 mM guaiacol or 2 mM ABTS (2,2'-azinobis-(3-ethyl benzothiazoline-6-sulphonic acid). Production of an intense brown color under and around the fungal colony in guaiacol-supplemented agar and a deep green color in ABTS-supplemented agar was considered a positive reaction for the presence of laccase activity (D'Souza et al. 2006) (Table 1). It produced MnP and LiP in LNM at negligible levels and thus too, does not fall into any of the reported categories of the lignin-degrading fungi. Several obligate marine fungi have been reported to produce only laccase (Pointing et al. 1998; Luo et al. 2005). Thus, it appears that lignin-degrading marine fungi may not be strictly classified in to groups as described by Hatakka (1994). However, it would be interesting to screen for the presence of all the LDE genes in marine fungi in general.

The test of adaptation of marine-derived fungi to their environment is growth and production of degradative enzymes in seawater media. Although, marine fungi showed growth and LDE activities in agar media (Raghukumar et al. 1994; Pointing et al. 1998) or in liquid media containing sea water (Raghukumar et al. 1999; Li et al. 2002a, 2002b; D'Souza et al. 2006) laccase and MnP activity of NIOCC #312 and NIOCC #2a, decreased drastically when seawater was added during enzyme assay (in vitro) studies (unpublished results). Purified laccase from NIOCC #2a was not inhibited in the presence of NaCl roughly up to 0.3 M, above which it was reversibly inhibited (unpublished results). Luo et al. (2005) reported that inhibitory effect of seawater on the laccase activity of two marine fungi tested was reversible. A basidiomycetous fungus *Phlebia* sp (strain #MG-60) isolated from mangrove stands was identified as a hypersaline-tolerant lignin-degrading fungus (Li et al. 2002a; 2003a) which participated in biodegradation of sugarcane bagasse, biobleaching of paper pulp and decolorization of dyes (Li et al. 2002b) in the presence of different concentrations of sea salts. Subsequently these authors showed production of hypersaline–tolerant MnP in #MG-60, in the presence of sea salt and NaCl (Li et al. 2003b). Raghukumar et al. (1999) and D'Souza et al. (2006) demonstrated growth and LDEs production in NIOCC #312 and NIOCC #2a respectively, in media prepared with 50% diluted seawater. These two

cultures also decolorized several synthetic dyes and industrial effluents when grown in seawater medium.

Since ascomycetes are more dominant than basidiomycetes in the marine environment (Kohlmeyer and Kohlmeyer 1979), it is to be expected that ascomycetes would play a major role as biomass degraders in marine habitats. *In vitro* production of cellulase and xylanase was reported among 47 ascomycetes obtained from mangrove stands of tropics (Bucher et al. 2004). Lignin-degrading enzyme production among these was comparatively less common. Most isolates were able to cause loss in birch wood mass, when used as substrate during a 24-week period. Five of these ascomycetous fungi solubilized lignin, with indices of lignin-solubilization comparable to terrestrial white-rot basidiomycetes. The authors conclude that to a certain extent, marine ascomycetes in the marine realm play a similar ecological role as that of terrestrial white-rot fungi (Bucher et al. 2004). Ascomyceteous species were shown to participate in the decay of dead plant biomass in salt marshes (Lyons et al. 2003).

Recent approach has been to screen for the laccase gene in marine environmental samples to assess their role in lignin degradation. Analysis of the fungal community in the salt marsh ecosystem using the diversity of the functional laccase gene indicated high diversity of laccase sequences in clones from environmental DNA and ascomyceteous fungi isolated from the decaying blades of *Spartina alterniflora* (Lyons et al. 2003).

3.3 Effect of nutritional parameters on production of lignin-degrading enzymes

Production of LDEs is affected by several culture conditions such as medium composition, carbon and nitrogen ratio, pH, temperature, and aeration. Production of LiP and MnP in several terrestrial white-rot fungi was reported in the presence of high carbon and low nitrogen medium, a condition found in plants. This resulted in the development of a special culture medium (Tien and Kirk 1988) termed low nitrogen medium (LNM). In contrast, several white-rot fungi were reported to produce LDEs in the presence of high nitrogen (Kuhad et al. 1997). Production of LDEs was reported to take place only in shallow undisturbed stationary cultures (Boominathan and Reddy 1992). Addition of surfactants such as Tween 20 or Tween 80 to the culture media helped in overcoming this problem and production of these enzymes in bioreactors and agitated cultures was reported (Gomez-Alarcon et al. 1989). Addition of manganese to culture medium induced MnP but suppressed LiP production in *Phanerochaete chrysosporium* (Boominathan and Reddy 1992). Addition of veratryl alcohol induced both, LiP and MnP production in several white-rot fungi (Gill and Arora 2003; Boominathan and Reddy 1992). Several natural substrates like wood chips and shavings from soft and hard wood have been used to induce production of both peroxidases (Niku-Paavola et al. 1990).

With increasing interest in laccase from fungi for bioremediation applications, efforts have been made to enhance the laccase titer. Addition of various aromatic compounds analogous to lignin or lignin derivatives, have induced laccase production (Gianfreda et al. 1999). Nitrogen source and concentration in the culture medium are known to influence laccase production (Gianfreda et al. 1999). Addition of copper has been reported to increase laccase production by several folds (Galhaup and Haltrich 2001; Galhaup et al. 2002). Recently, novel approaches to increase laccase production in white-rot fungi by addition of ethidium bromide and a range of vitamins, amino acids, and antibiotics to the culture medium have been reported (Dhawan et al. 2003; Dhawan and Kuhad 2002; Dhawan et al. 2005). To make the fermentation process cost-effective, optimizing the culture conditions is a prerequisite for large-scale production of these enzymes. In recent years, several statistical designs collectively under response surface methodology have been introduced into the fermentation field to replace the "one-factor-at-a-time" method (Levin et al. 2005). Using these methodologies, production of laccase has been optimized in several species of white-rot fungi (Levin et al. 2005).

In light of this scenario in terrestrial fungi, marine fungi have received a scant attention. The marine-derived fungus NIOCC #312 produced both, MnP and laccase in low as well as high nitrogen medium containing 2.4 mM and 24 mM ammonium tartrate respectively (Raghukumar et al. 1999). It produced all the three LDEs to varying extents when grown in sea water medium containing sugarcane bagasse fibers, pine and poplar wood shavings as carbon and nitrogen source (Table 2). Another marine-derived fungus, NIOCC #2a, produced laccase in low as well as high nitrogen medium prepared with seawater of 25 ppt (Table 2). Laccase production was enhanced by several folds on addition of copper or a combination of copper and guaiacol to 6-day-old culture growing in high nitrogen medium (peptone as the nitrogen source). Several phenolics and lignin-derivatives also enhanced laccase production in this culture (D'Souza et al. 2006). Glutamic acid and glycine were good sources of nitrogen in the presence of fructose as carbon source (Table 2) for enhancing laccase production in this fungus (D'Souza-Ticlo et al. 2006).

4. APPLICATION OF LIGNIN-DEGRADING ENZYMES

Growing public awareness of the environment is forcing several industrial units to practice stringent pollution treatment on a top priority. Several strategies including biological approaches besides physical and chemical methods are devised to restore polluted environments. Oxidoreductive enzymes play an important role in degradation and transformation of polymeric substances. The partially degraded or oxidized products can easily be taken up by microbial cells where they are completely mineralized. Lignin-degrading enzymes are one such group of oxidoreductive enzymes, which have practical application in bioremediation of polluted environment (Husain 2006).

TABLE 2
Production of lignin-degrading enzymes (U L^{-1}) by the two marine-derived fungi grown in seawater media. Maximum activity recorded in each media is shown

Culture	Media	MnP	LiP	Laccase
NIOCC #312	Low nitrogen medium (LNM)*	654	52	30
	High nitrogen medium (HNM)*	50	0	20
	Sugarcane bagasse*	450	20	15
	Pine shavings*	110	4	9
	Poplar shavings*	110	0	8
NIOCC #2a	LNM with Ammonium tartrate as N source	40	0	3428
	HNM (peptone as N source)**	13	1	921
	HNM + Cu**	37	0	42,933
	LNM with KNO_3 as N source***	0	26	16,687
	LNM with glycine as N source***	12	2	34,659
	LNM with glutamic acid as N source***	0	17	47,567
	LNM with beef extract as N source***	25	25	10,346
	LNM with corn steep liquor as N source***	46	26	12,973

*Source data: Raghukumar et al. 1999; ** source data: D'Souza et al. 2006a; *** source data: D'Souza et al. 2006b.

4.1. Decolorization of black liquor

The paper and pulp industry release large volumes of intensely colored black-liquors that contain toxic chlorinated lignin-degradation products. These products include chlorolignins, chlorophenols, and chloroaliphatics (Ali and Sreekrishnan 2001). Besides, these paper mill effluents are highly alkaline and alter the pH of the soil and water bodies into which they are discharged. A vast literature is available on the involvement of lignin-degrading fungi from terrestrial sources in treatment of such effluents (Garg and Modi 1999). Several white-rot fungi producing lignin peroxidases are shown to be involved in decolorization of black liquor (Thompson et al. 2001; Sahoo and Gupta 2005; Wu et al. 2005) but in a few instances non-ligninolytic fungi have also been used for this purpose (Nagarathnamma and Bajpai 1999; Sumathi and Phatak 1999). The relative important role of the peroxidases in decolorization of black liquor has been debated. Frederick et al. (1991) demonstrated negligible decolorization of bleach plant effluent from paper and pulp mills by a mutant of *Phanerochaete chrysosporium* that lacked the ability to produce the peroxidases. A mutant of the same fungus which produced only MnPs but not LiPs showed about 80% of the decolorizing activity exhibited by the wild type, indicating the relatively major role of MnPs in decolorization of this bleach plant effluent.

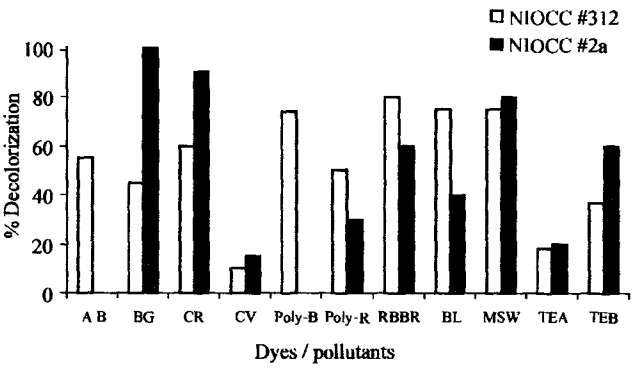

FIG. 3. Comparison of the efficiency of the two marine-derived fungi, NIOCC #312 and NIOCC #2a. The dyes and pollutants were added to the 6-day old cultures and % decolorization was estimated after 3 days. AB = Azure B; BG = Brilliant Green; CR = Congo Red; Poly-B = Poly-B 411; Poly-R = Poly-R 478; CV = Crystal Violet; RBBR = Remazol Brilliant Blue R; BL = Black Liquor; MSW = Molasses spent Wash; TEA = Textile effluent A; TEB = Textile effluent B. Standard deviation was < 5%.

Very few attempts have been made to tap the potential of marine-derived fungi in treatment of colored effluents from paper and pulp mills. The marine-derived fungus NIOCC #312 which produced the peroxidases was better than NIOCC #2a, which produced laccase as the major LDE, in decolorizing black liquor from paper and pulp mills (Fig. 3). Although the production of MnP was induced in the presence of black liquor in NIOCC #2a, it did not decolorize the effluent (D'Souza et al. 2006). Earlier studies demonstrated that marine fungi *Sordaria fimicola* (NIOCC #298) and *Halosarpheia ratnagiriensis* (NIOCC #321), which produced MnP and laccase, brought about 65–75% decolorization of bleach plant effluent within 8 days (Raghukumar et al. 1996). These results may indicate that MnP and not laccase plays an important role in decolorization of black liquor.

The pH of black liquor is always alkaline ranging from 8–11 and most of the lignin-degrading enzymes show optimum activity between pH 3–6. Therefore, black liquor is usually acidified prior treatment with these fungi (see references in Wu et al. 2005). These authors have reported optimum decolorization of black liquor at pH 8–11 using three terrestrial fungi. Raghukumar et al. (1996) reported decolorization of bleach plant effluent at pH 11 by the isolate NIOCC #312. These are the only reports demonstrating effective decolorization of black liquor at alkaline pH.

4.2. Decolorization of molasses spent wash

Molasses spent wash (MSW) is a by-product of sugar mills and alcohol distilleries, where the starting material is molasses. It contains mostly dark brown colored recalcitrant compounds, collectively termed as melanoidin, which are formed by the Maillard amino-carbonyl reaction (Wedzicha and Kaputo 1992).

These compounds are toxic to many microorganisms including those generally involved in wastewater treatment processes (Kitts et al. 1993). Anaerobic digestion of MSW-containing effluents is one of the treatments followed by distilleries and the resulting dark brown sludge is used as fertilizer. Dark brown color of these effluents remains a major problem for the distilleries.

Color removal from MSW using terrestrial white-rot fungi was shown to be MnP-dependent in *Phanerochaete chrysosporium* (Dehorter and Blondeau 1993) and laccase-dependent in *Trametes versicolor* (González et al. 2007). The process was sorbose oxidase- and glucose oxidase-dependent in mitosporic fungi *Aspergillus fumigatus* (Ohmomo et al. 1987) and *A. oryzae* (Ohmomo et al. 1988) and in the basidiomycete *Coriolus* sp. No. 20 (Watanabe et al. 1982). Raghukumar and Rivonkar (2001) demonstrated MnP-independent decolorization of MSW by the marine-derived fungus NIOCC #312. About 60% decolorization of MSW was brought about when added at 50% concentration in seawater medium. Subsequently it was demonstrated that decolorization was dependent on glucose oxidase levels in the culture medium (Raghukumar et al. 2004). The activity of MnP and percentage decolorization of MSW by the isolate NIOCC #312 did not correlate (Fig. 4A) but there was a direct co-relation between concentration of glucose oxidase and decolorization of MSW (Fig. 4B). It was suggested that H_2O_2 produced by glucose oxidase may act as a bleaching agent. In another marine-derived fungus NIOCC #2a, 80% of color removal was obtained by day 3 (Fig. 3). Glucose oxidase production in this fungus ranged from 8769 to 23,253 U L^{-1} but did not correlate with decolorization of MSW. It was further demonstrated that marine fungi are capable of decolorizing MSW effectively in the presence of seawater of 15–34 ppt salinity (Raghukumar et al. 2000; D'Souza et al. 2006). Studies to understand the mechanism of decolorization of MSW in marine fungi merit further attention.

4.3. Decolorization of synthetic dyes and textile effluents

Textile industries release highly colored effluents containing large amounts of a mixture of dyes, many of which are resistant to degradation leading to deleterious effects on the aquatic life (Rodriguez et al. 1999). Exhaustive reviews on decolorization of synthetic dyes (Wong and Yu 1999; Peralta-Zamora et al. 2003) and dye wastewaters using white-rot fungi and their lignin-degrading enzymes have appeared (Fu and Viraraghavan 2001; Wesenberg et al. 2003). Textile effluents besides containing dyes, also have extreme pH values and contains salts, often at very high ionic strength. Thus, in spite of the highly efficient terrestrial strains reported, marine fungi may find use in decolorization of industrial effluents with these added factors of salt content and extreme pH values. The marine-derived culture NIOCC #2a with laccase proved to be more efficient in the decolorization of textile effluents and synthetic dyes than the culture NIOCC #312 having MnP and LiP activity (Fig. 3). Two textile effluents, textile effluent A (TEA) with pH of 8.9 and the

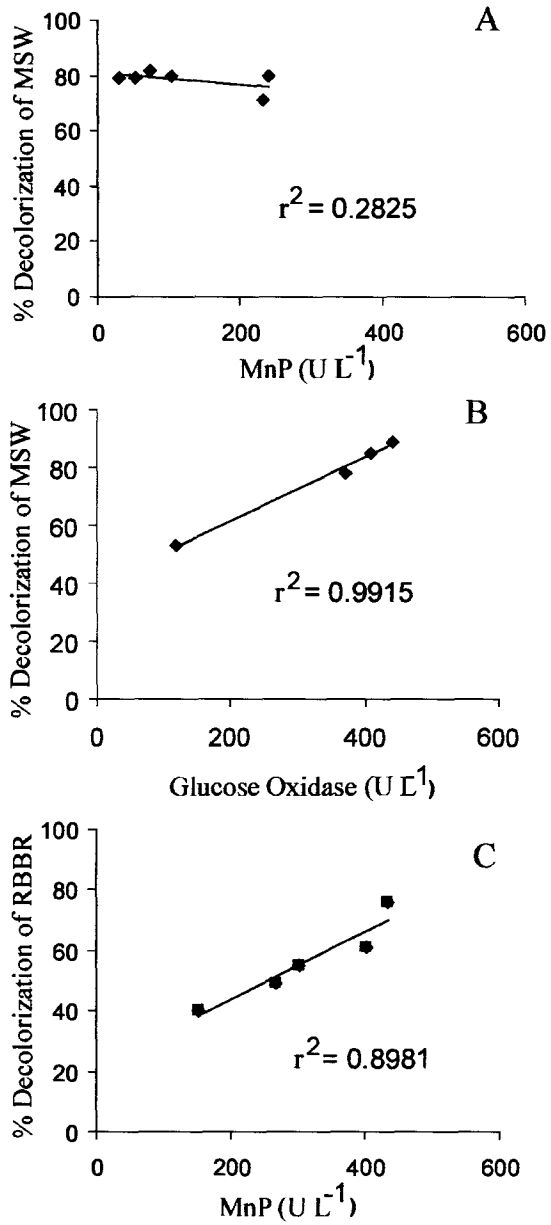

FIG. 4. (A) No correlation observed between MnP production and decolorization of MSW in NIOCC #312; (B) Correlation observed between glucose oxidase production and decolorization of MSW in NIOCC #312 (C) Correlation observed between MnP production and decolorization of Remazol Brilliant Blue R dye by NIOCC # 312.

textile effluent B (TEB) with pH 2.5 were added separately at 10% final concentration in the fungal cultures grown in seawater medium. Among the synthetic dyes, Brilliant Green and Congo Red were almost totally decolorized by NIOCC #2a, whereas Remazol Brilliant Blue R and Poly R-478 were better decolorized by NIOCC #312 than NIOCC #2a (Fig. 3, Raghukumar et al. 1999; D'Souza et al. 2006).

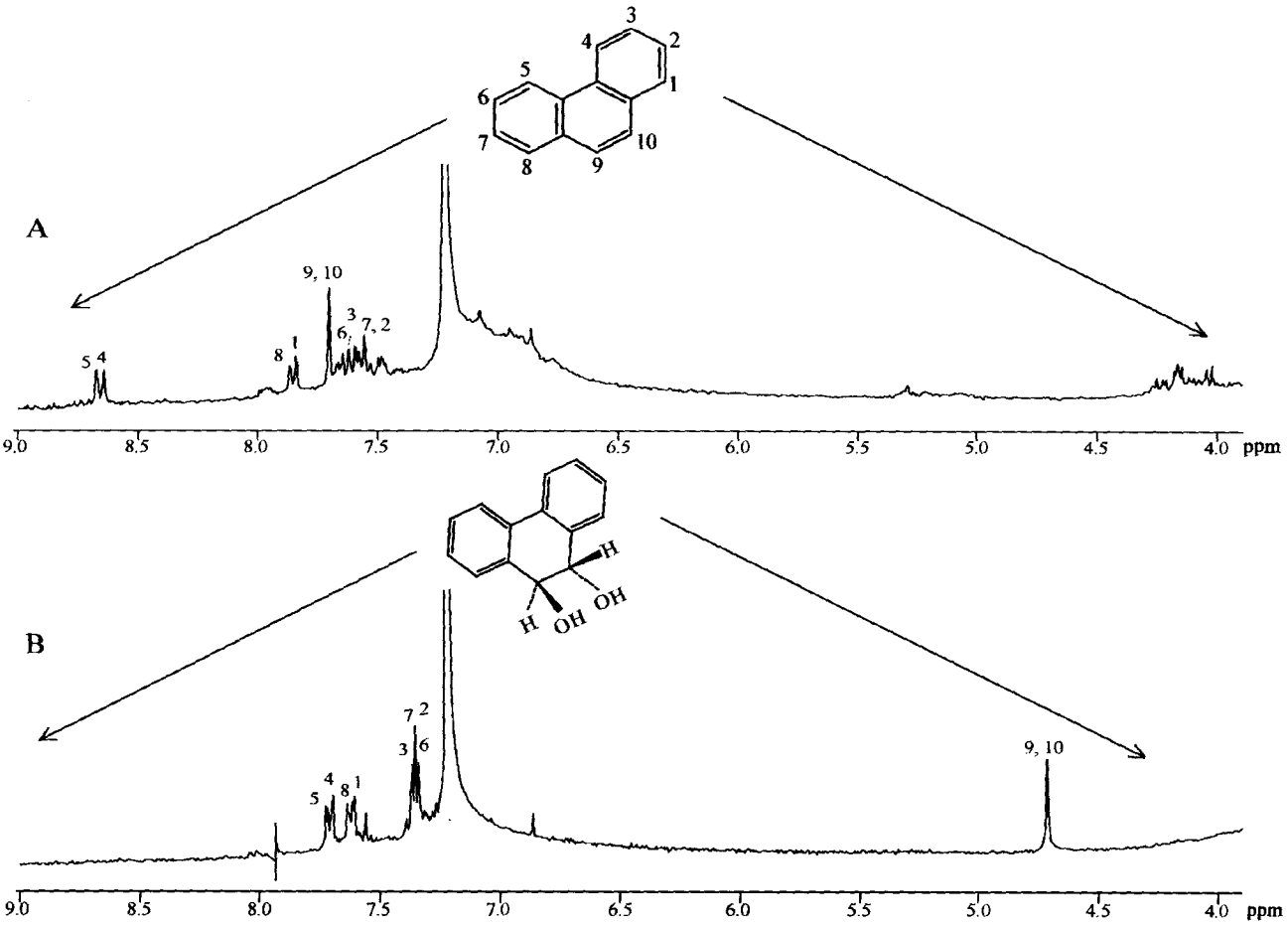

FIG. 5. (A) A 300-MHz Proton NMR spectra of the standard phenanthrene, (B) intermediate degradation product, phenanthrene (*trans*)-9,10-dihydrodiol extracted from the fungal biomass (NIOCC #2a) on day 6. The numbers on peaks correspond to the numbers shown in the chemical structures in the insets. The insets in (A) and (B) show the structure of phenanthrene and phenanthrene (*trans*)-9,10-dihydrodiol respectively.

The role of laccase in terrestrial fungi in decolorization of dyes and dye wastewaters is undisputed (Wong and Yu 1999; Fu and Viraraghavan 2001; Wesenberg et al. 2003). In the marine-derived fungus NIOCC #312, decolorization of the dye RBBR directly correlated with MnP concentration (Fig. 4C). On the other hand, decolorization of the effluents or dyes did not correlate with laccase concentration in the marine-derived fungus NIOCC #2a (D'Souza et al. 2006). A number of filamentous fungi including a halophilic new species of *Gymnoscella marismortui* isolated from the Dead Sea decolorized synthetic dyes belonging to four different groups (Molitoris et al. 2000). However, it is not known whether these fungi produced any of the lignin-degrading enzymes.

4.4. Degradation of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are formed during incomplete combustion of fossil fuels. They consist of analogs of benzene having two or more aromatic rings in various alignments. Most of the low molecular weight PAHs are very toxic and adversely affect aquatic life. Degradation of PAHs by MnP-producing terrestrial white-rot fungi has been demonstrated in *Irpex lacteus* (Baborova et al. 2006), *Nematoloma frowardii* (Sack et al. 1997), *Phanerochaete chrysosporium* (Moen and Hammel 1994) and several other fungi. Laccase-producing white-rot fungi such as *Trametes versicolor* (Collins et al. 1996), *Pleurotus ostreatus* D1 (Pozdnyakova et al. 2006) and *Coriolopsis gallica* (Picard et al. 1999) have been implicated in PAHs degradation.

The marine-derived fungi, NIOCC #312 and NIOCC #2a facilitated removal of phenanthrene, a PAH from the medium by adsorption on the fungal mycelium. Phenantherene was completely metabolized or transformed into more polar derivatives by NIOCC #312 by day 6 (Raghukumar et al. 2006). Phenanthrene added to the culture NIOCC #2a at 20 ppm concentration was metabolized to an intermediate compound (Fig. 5A, 5B). The NMR spectrum obtained was consistent with earlier data obtained with *Phanerochaete chrysosporium*, a terrestrial basidiomycete (Sutherland et al. 1991). The mass spectrum of this metabolite showed a molecular mass of 212 (data not shown).

TABLE 3

Effect of effluents on lignin-degrading enzyme production in NIOCC #312 and NIOCC #2a. All the effluents were added to 10% final concentration in the culture medium.

		Enzyme Activity (U L^{-1}) in NIOCC #312					
LDE	Treatment	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
MnP	Without effluent	47	112	298	338	276	118
	With MSW	51	78	263	226	225	142
	With BL	16	255	331	483	66	13
	With TEA	104	0	0	54	0	0
	With TEB	52	0	0	0	0	0
Laccase	Without effluent	0	0	0	0	0	0
	With MSW	0	0	16	56	56	40
	With BL	1	1	0	1	0	0
	With TEA	104	0	0	54	0	0
	With TEB	52	0	0	0	0	0
		Enzyme Activity (U L^{-1}) in NIOCC #2a*					
MnP	Without effluent	80	82	79	84	105	93
	With MSW	0	22	69	78	193	79
	With BL	197	513	516	683	530	367
	With TEA	86	53	94	0	0	0
	With TEB	8	14	8	48	13	0
Laccase	Without effluent	8425	5097	6125	8946	12613	7415
	With MSW	566	629	644	1266	720	704
	With BL	197	513	516	683	530	367
	With TEA	5603	3348	3278	3195	4279	2900
	With TEB	1728	799	412	676	1549	848

* Source data: D'Souza et al. 2006a. MSW = molasses spent wash; BL = black liquor from paper and pulp mill; TEA = textile effluent A; TEB = textile effluent B.

The comparison of the fragmentation pattern of this peak with the library established its identity to be phenanthrene (*trans*)-9,10-dihydrodiol (unpublished results). The fragment ions at m/z 194, 181, 166, 165, and 152 were consistent with mass spectrum of phenanthrene 9,10-dihydrodiol reported previously (Sutherland et al. 1991). In both the fungi, majority of phenanthrene removal and further metabolism was by the fungal mycelia. It was suggested that EPS that form a sheath around the fungal mycelium plays an active role in the adsorption and degradation of xenobiotic pollutants (Bes et al. 1987). We have shown the presence of EPS in the isolate NIOCC #312 (Raghukumar et al. 2006) and in NIOCC #2a (D'Souza et al. 2006). A part of the extracellular LDEs are likely to get trapped in the EPS surrounding the mycelium, which may further help in degradation of PAHs (Barassa et al. 1998).

5. EFFECT OF POLLUTANTS ON LIGNIN-DEGRADING ENZYMES

Several workers have demonstrated the production of LDEs especially laccase, in terrestrial fungi induced in the presence of aromatic compounds (Mai et al. 2000; Carbajo et al. 2002; Marques et al. 2004). This induction was associated with induced expression of laccase gene *cglcc*1 in the presence of tannic acid in the white-rot fungus *Coriolopsis gallica* and *lcc*1 and *lcc*2 in *Trametes* sp. I-62 (Carbajo et al. 2002; González et al. 2007).

We found that marine fungi were no exception to this. Raghukumar (2000) demonstrated increased MnP production in three marine fungi, *Sordaria fimicola* (NIOCC # 298), *Halosarpheia ratnagiriensis* (NIOCC #321) and marine-derived basidiomycete (NIOCC #312) in the presence of bleach plant effluent from a paper and pulp mill. Laccase production in these fungi was reduced in the presence of bleach plant effluent. In the culture medium containing either MSW or black liquor, production of MnP was generally enhanced, whereas laccase production was inhibited in NIOCC #312 (Table 3). In the isolate NIOCC #2a, black liquor and both the textile effluents in the culture medium induced laccase production whereas, MSW resulted in decreased laccase production (Table 3). D' Souza et al. (2006) demonstrated that textile dye effluent, black liquor and MSW enhanced laccase production in the same fungus. However, the media used in these two different studies differed in their nitrogen source. Subsequently, it was proven that laccase production in the presence of effluents was strongly influenced by the nitrogen source (D' Souza et al. 2006a).

Addition of phenanthrene (12 ppm) increased fungal biomass production in both NIOCC #312 (Raghukumar et al. 2006) and

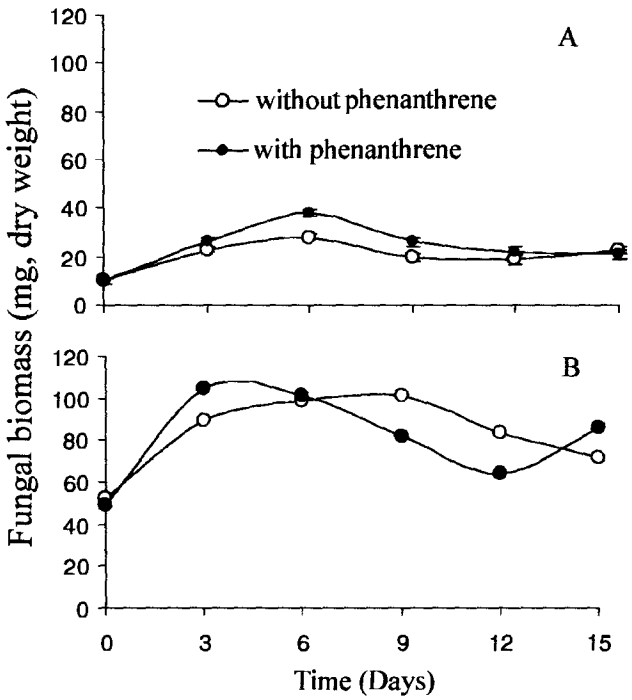

FIG. 6. Effect of phenanthrene on biomass production in (A) NIOCC #312 and (B) NIOCC #2a.

in NIOCC #2a. However in the presence of phenanthrene, maximum biomass was attained earlier in NIOCC #2a than in NIOCC # 312 (Fig. 6A, 6B).

6. METHODS OF TREATMENT OF EFFLUENTS

Several methods of treatment of industrial effluents with fungi for decolorization have been reported (Thompson et al. 2001). Whole cultures, mycelial pellets, immobilized fungi or their enzymes, free enzymes, and biofilm (Wu et al. 2005) are some examples of such treatments.

6.1. Treatment of effluents with whole versus immobilized cultures

Most of the experiments for decolorization of various effluents and xenobiotics with terrestrial basidiomycetes are carried out by addition of effluents to pre-grown, shallow, static cultures having substantial fungal biomass (Reddy 1995). Care should be taken not to disturb the fungal mat, as this affects LDE production. The efficiency of the fungal mat decreases on repeated usage. In nature, most of these fungi grow in solid-state conditions, in the near absence of free water. Such cultures when immobilized on a variety of solid supports have been shown to decolorize various effluents (Ohmomo et al. 1987; Shin et al. 2002; Šušla et al. 2007). Zhang et al. (1999) used immobilized a white-rot fungus strain F29 to decolorize Orange II, a toxic dye. Wu et al. (2005) used biofilm of white-rot fungi grown on a porous plastic rings for decolorization of paper mill effluent.

The marine-derived fungus NIOCC #312 when immobilized in polyurethane foam (PUF) cubes, decolorized MSW by 70% by day five and most of the high molecular weight compounds were removed by this treatment. The same batch of immobilized fungus could be reused up to four cycles for treatment of MSW. The immobilized fungus remained viable for a minimum of 75 days (Raghukumar et al. 2004). When NIOCC #2a, was immobilized in PUF, it was able to decolorize MSW to varying degrees and also reduce COD levels by 50% within 72 h (unpublished results). The porous nature of PUF increases the diffusion of oxygen, resulting in increased production of LDEs as well as enhanced activity. Since many of the white-rot fungi produce LDEs under oxygen-saturation conditions (Boominathan and Reddy 1992). Thus immobilized marine fungi offer an alternative technology for an effective environmental bioremediation.

6.2. Treatment of effluents with crude/purified enzyme and immobilized enzymes

Several studies with terrestrial white-rot fungi showed involvement of either mycelial-bound lignin-degrading enzymes or H_2O_2 generating mechanism for degradation of lignin and decolorization of several effluents (Wesenberg et al. 2003; Svabodová et al. 2008). It has been shown by immunochemical methods that sites of H_2O_2 production are located in the periplasmic space of the fungal mycelium in *P. chrysosporium* (Forney et al. 1982). By using an immuno-cytochemical technique Garcia et al. (1987) demonstrated that LiPs are located very close to the cell membrane in *Phanerochaete chrysosporium* and other white-rot fungi. Further, washed pellets were shown to retain a part of the lignin-degrading enzyme activity (Kurek and Odier 1990). Earlier studies on lignin-degradation therefore were concentrated using whole cultures of white-rot fungi showing lignin-peroxidases activity. As reports about laccase-producing white-rot fungi increased, lignin-degradation work and decolorization applications with partially purified or crude laccase gained importance (Wong and Yu 1999; Rodriguez et al. 1999; Kokol et al. 2007; Šušla et al. 2007). Laccase-mediated degradation of PAHs has also been widely reported (Pozdnyakova et al. 2006 and references therein).

Decolorization of various effluents by culture supernatant collected from liquid culture was possible with the marine-derived fungus NIOCC #2a (Table 4) whereas, the same by NIOCC #312 was not effective. It is possible that the mycelial-bound lignin-degrading enzymes or the H_2O_2-generating mechanism in NIOCC #312 is responsible for decolorization of various effluents. This isolate produces more peroxidases than laccase and the former require constant supply of H_2O_2, which is not possible practically while using mycelia-free culture supernatants.

Immobilized laccases have been extensively used for decolorization of textile effluents and synthetic dyes (Abadulla et al. 2000; Peralta-Zamora et al. 2003). Various supports such as alumina particles, chemically modified silica, amberlite, and

TABLE 4
Decolorization of effluents by fungus-free extracellular culture supernatant and extracellular polymeric substance (EPS) produced by the fungus NIOCC #2a (D'Souza et al. 2006a)

	(%) Decolorization by culture supernatant		(%) Decolorization by EPS	
Effluents (10%)	6 h	12 h	12 h	24 h
Textile Effluent A	9	11	11	100
Textile Effluent B	14	22	35	100
Molasses Spent Wash	34	33	12	100
Black Liquor	71	59	41	100

Culture supernatant (500 μl) with 9 U ml^{-1} laccase activity was incubated with an equal volume of diluted effluent at pH 6.0 and 60°C. Freeze-dried EPS (10 mg) of the fungus was incubated with effluents at 60°C. Decolorization of both was measured at the absorbance maxima specific to the effluents. The percentage decolorization was calculated based on the initial readings. All the values are mean of 2 replicates.

glass-ceramic have been used for this purpose (Abadulla et al. 2000; Peralta-Zamora et al. 2003). It has been demonstrated that treatment of azo dyes with free laccase enzyme results in darkening of the solution due to coupling of the degraded products with the unreacted dyes (Zille et al. 2005). Using immobilized enzyme would avoid these coupling reactions. This knowledge gained with terrestrial fungus, *Trametes villosa* should be considered while using laccases from marine-derived fungi for any bioremediation processes.

6.3. Treatment of effluents with exopolymeric substances (EPS) produced by white-rot fungi

Color removal by biosorption is an alternative to the economically disadvantageous physical and chemical methods of treatment (Namasivayam et al. 1996). Color removal by adsorption is an alternative option. Biological adsorbants include plant, fungal, and bacterial biomass, either live or dead (Robinson et al. 2001). Bioadsorption potential of microbial exopolymeric substance is well known (Wingender et al. 1999). Basidiomycetous fungi are reported to produce large amount of EPS (Smith et al. 2002; Maziero et al. 1999). These polymeric substances form a sheath around the fungal hyphae and may be water soluble or insoluble forms. The ligninolytic fungus *Phanerochaete chrysosporium* also produces polysaccharide sheath and dissolution of this sheath by addition of glucanase inhibited lignin degradation (Bes et al. 1987). This suggests an active involvement of EPS in lignin degradation process.

The participation of fungal EPS in degradation of xenobiotics has been assumed but not yet investigated. D'Souza et al. (2006) reported for the first time its use in decolorization of colored industrial effluents. Extracellular polymeric substance was produced by the marine-derived fungi NIOCC #2a and NIOCC #312. Freeze-dried EPS of NIOCC #2a was effective in decolorizing various industrial effluents (Table 4) but that of NIOCC #312 was not effective for this purpose. The EPS produced by a marine cyanobacterium *Cyanothece* sp. was reported to remove dyes from textile effluents by gelation under alkaline conditions (Shah et al. 1999). EPS are highly charged and thus absorb water and become gel-like (Whiteley and Lee 2006). EPS produced by basidiomycetes function as a supporting network in which some of the excreted enzymes get trapped (Ruel and Joseleau 1991). Biofilm prepared with EPS for treatment of effluent needs to be considered for the future bioremediation processes. Lignin-degrading enzymes immobilized in fungal EPS is another possible strategy for bioremediation purpose. As EPS production is reported to be NaCl-dependent in cyanobacteria (Philippis & Vincenzini 1998), marine fungi should be screened for EPS production.

7. DETOXIFICATION OF EFFLUENTS

It has been reported that effluents from paper and pulp mills and textile dye waste waters are toxic and mutagenic (Reddy 1995). Laccases are shown to render phenolic compounds in effluents, less toxic via degradation or polymerization reactions or by cross-coupling of pollutant phenols with naturally occurring phenols (Abadulla et al. 2000). Toxicity of several textile dyes, including azo compounds, was reduced by treatment with laccase from *Trametes hirsuta* (Abadulla et al. 2000). Eight white-rot fungi grown in green olives reduced phenolic content by nearly 70–75% but phytotoxicity was not reduced (Aggelis et al. 2002). All of these fungi produced laccase and some of them produced MnP. *Rhizomucor pusillus* strain RM7, a mucoralean fungus and a white-rot fungus *Coriolus versicolor* were shown to detoxify bleach plant effluent (Driessel and Christov 2001).

Molasses spent wash treated with the marine-derived fungus NIOCC #312 was detoxified as measured by serum sorbitol dehydrogenase (SSDH) assay (Raghukumar et al. 2004). Increased levels of SSDH are indicative of chemically induced liver damage in fish (Dixon et al. 1987). Accordingly, there was a drastic reduction in SSDH activity in fungus-treated MSW (Table 5). Toxicity test was also carried out by Comet assay, a technique to detect DNA damage in individual cells (Singh et al. 1988). In the presence of untreated MSW, 85% of the hepatic cells of an estuarine fish *Oreochromis mosambicus* showed damaged

TABLE 5
Detoxification of molasses spent wash by NIOCC #312 tested by various toxicity assays (Raghukumar et al. 2004)

Toxicity Test	Untreated MSW	Treated MSW
Serum sorbitol dehydrogenase	122 U ml^{-1}	2.6 U ml^{-1}
Comet assay (damaged nuclei)	85 %	9 %
Benzo (α)pyrene	3.8 μg ml^{-1}	1.2 μg ml^{-1}

nuclei as comets, whereas, in the presence of fungus-treated MSW only 9% of the cells showed presence of comets indicating a near total removal of toxic components after the fungal treatment (Table 5). There was a 30% reduction in benzo(α)pyrene, one of the toxic polycyclic aromatic hydrocarbons in MSW on treatment with the fungus (Table 5), which might be one of the toxic components present in MSW. A decrease in phenolics and PAHs might be responsible for cumulative reduction in toxicity of the fungus-treated MSW (Raghukumar et al. 2004). Reduction in COD as an indicator of detoxification of the effluents has been shown in several white-rot fungi (Table 6). Molasses-based pharmaceutical effluent in the presence of 1% glucose when added to NIOCC #2a and NIOCC #312 cultures, showed a 57 and 67% reduction in COD after 72 h respectively. The total phenolics were reduced by 20 and 35% respectively in the same treatment (unpublished results). Thus, besides decolorization, detoxification of wastewaters is an important parameter to be monitored.

8. ROLE OF MEDIATORS IN TREATMENT OF EFFLUENTS

Lignin-degradation by white-rot fungi that produce only laccase led to the discovery of low molecular weight enzyme mediators. These laccase-mediator systems (LMS) involves use of low molecular weight compounds that are oxidized by the enzyme to stable radicals which in turn act as redox mediators and oxidize other compounds that are not substrates of laccase. Lack of correlation between laccase activity and degradation of xenobiotic compounds further supports the role of LMS (Johannes and Majcherczyk 2000). These mediators can either be natural compounds produced by fungi or plants or synthetic compounds such as, ABTS, 1-hydroxybenzothiazole (HBT), violuric acid (VIO), and N-hydroxyacetanilide (NHA) (Camarero et al. 2005). Degradation products of lignocellulose such as acetosyringone, *p*-coumaric acid, syringaldehyde, and vanillin, can also act as mediators. A metabolite, 3-hydroxyanthranilic acid produced by the white-rot fungus *Pycnoporus cinnabarinus* was shown to act as a mediator in degradation of lignin (Eggert et al. 1996). Natural mediators have been extracted from black liquor of eucalyptus-based kraft pulping (Camarero et al. 2007). The redox potential of laccase alone is not high enough to break C-H aliphatic bonds. In the presence of a redox mediator, oxidation of such bonds becomes feasible. The enzyme oxidizes the mediator, which can diffuse away from the enzyme and oxidize a substrate. The reduced mediator is ready for the next cycle (Wells et al. 2006). Laccase-mediator system has found applications in paper pulp delignification (Camarero et al. 2007), degradation of PAHs (Johannes and Majcherczyk 2000) and industrial dyes (Camarero et al. 2005).

Preliminary results with the marine-derived fungus NIOCC #2a showed that treatment of sugarcane baggase with laccase brought about reduction in lignin content, as determined by kappa number. Lignin content was further reduced in the presence of ABTS, a mediator (unpublished results). Role of mediators in laccases from marine fungi has remained a totally ignored subject. As marine-derived fungi are a vast source of biological compounds, search for the presence of natural mediator compounds in them is an exciting new field.

9. CONCLUSION AND FUTURE PERSPECTIVES

Microbial decolorization and degradation of colored effluents is a cost-effective and promising green technology for treatment of such effluents. Reports of white-rot fungi that show lignin-degrading ability in saline conditions are very few. Industrial effluents are mostly alkaline and rich in carbonates, chlorides, and sulfates. In light of this, the marine fungi discussed here hold good promise for the application of bioremediation of colored effluents under saline conditions. The marine-derived fungi NIOCC #312 with MnP and LiP and NIOCC #2a with laccase as the major lignin-degrading enzyme were equally effective in decolorization of various effluents and degradation of PAHs under marine conditions. Marine-derived fungi are often more effective than terrestrial fungi in treatment of various colored effluents since they are better adapted to perform under such extreme conditions (Table 6).

Isolation of fungi from marine environment using media prepared with sea water of different salinities incorporated with lignin model compounds (Poly R 478) or laccase substrates, guaiacol, or ABTS is recommended for obtaining selective lignin-degrading fungi. Growth and enzyme production of such cultures under saline conditions is to be maintained by growing them in seawater. Media with low nitrogen and high carbon content is preferable for culturing these fungi.

Development of EPS-based bioremediation with marine fungi needs concerted efforts of basic and applied research. Dependency of EPS production on the concentration of NaCl in cyanobacteria is well known (Philippis and Vincenzini 1998). Similarly, production of EPS in obligate and facultative marine fungi in the presence of NaCl needs to be studied in detail to increase the production of EPS in such fungi to bring out a biotechnologically developed product in the field of bioremediation. Search for hyper-production of EPS in marine fungi is a challenging field.

Screening of marine-derived fungi as a source of new naturally occurring mediators for enhancing laccase production require to be carried out systematically on a large scale. Understanding the mechanism of degradation and detoxification of industrial effluents by marine lignin-degrading fungi are some of the key areas for future research.

Most of the LDEs show optimum activity around 30–35°C and at pH of 3–4. Industrial effluents generally have alkaline pH and temperatures above ambient. Therefore, fungi from the marine environment with LDE activity at alkaline pH preferably around 40°C should be selectively isolated for bioremediation purposes. Recombinant enzymes with these properties or protein

TABLE 6

Comparison of bioremediation potential of terrestrial white-rot fungi with that of marine-derived fungi,NIOCC #312 and NIOCC #2a

Effluent	Fungus	% Decolorization	% Phenol Reduction	% COD Reduction	Enzyme (s) involved	Technique	Reference
Alcohol Distillary Waste (3%)	*Pycnoporus coccineus*	50–55 (9 days)	70–80 (9 days)	193 (9 days)	MnP	Immobilised on Polyurethane foam	Charattanamankorn et al. 2005
Pulp and Paper Mill Effluent	Consortium containing: 1) *Merulius* (Syn. *Phlebia*) *aureus*. 2) An unidentified basidiomycete 3) *Fusarium Sumbucinum*	79 (4 days)	—	89 (4 days)	—	Static Liquid Culture	Malaviya & Rathore 2006
Textile Effluent	*Phanerochaete sordida*	90 (48 h)	—	—	MnP	Static Liquid Culture	Harazono & Nakamura 2005
Olive Mill Wastewater (20% & 50%)	*Phanerochaete chrysosporium*	93 (15 days)	90 (15 days)	74 (15 days)	MnP, LiP & Laccase	Static Liquid Culture	Kissi et al. 2001
Pulp Mill Effluent	*Trametes versicolor*	90 (7 days)	—	69 (7 days)	—	Mycellial Pellets	Meghna et al. 1995
Green Olive Wastewater	*Pleurotus ostreatus*	49 (30 days)	76 (30 days)	12 (30 days)	MnP, MnIP, Laccase	Static Liquid Culture	Aggelis et al. 2002
Olive Mill Effluent	*Penicillium spp.*	85–90 (20 days)	55–60 (20 days)	60–70 (20 days)	—	Static Liquid Culture	Robles et al. 2000
Paper Mill Effluent	*Trametes versicolor*	—	70 (3 h)	—	Laccase with mediator	Static Liquid Culture	Minussi et al. 2007
MSW	*Coriolus versicolor*	53 (10 days)	—	—	—	Static Liquid Culture	Fitz Gibbon et al 1998
Sugar Refinery Waste Water	*Phanerochaete chrysosporium*	55 (3 days)	63 (3 days)	48 (3 days)	—	Immobilised on PUF and Scouring Web	Guimaraes et al. 2005
MSW (6.25% v/v)	*Coriolus versicolor*	71.5 (10 days)	90 (10 days)	—	—	Shake Flask Technique	Kumar et al. 1998
Bleach Plant Effluent	*Rhizopus oryzae*	92–95 (24 h)	—	50 (24 h)	—	Immobilised on PUF	Nagarathnamma et al. 1999
Nitrocellulose Industry Effluent	*Aspergillus sp. Lentinus edodes*	83–95 (24-72 h)	56–79 (24-72 h)	—	—	Shake Flask Technique	Souza et al. 2005
MSW (10%)	(NIOCC #312)	75 (3 days)	50 (8 days)	50 (8 days)	—	Static Liquid Culture	Raghukumar & Rivonkar 2001
MSW (10%)	(NIOCC #312)	78–80 (7 days)	—	—	Glucose Oxidase	Immobilised on PUF	Raghukumar et al. 2004
TEA (10 %)	(NIOCC #312)	18 (3 days)	50 (3 days)	72 (3 days)	—	Static Liquid Culture	In this review
TEB (10 %)	(NIOCC #312)	37 (3 days)	22 (3 days)	79 (3 days)	—	Static Liquid Culture	In this review
Bleach Plant Effluent (2000 Color Units)	(NIOCC #312)	80 (8 days)	—	—	MnP	Static Liquid Culture	Raghukumar et al. 1996
BL (10%)	. (NIOCC #2a)	40 (72 h)	14 (72 h)	50 (72 h)	—	Static Liquid Culture	D'Souza et al. 2006
MSW (10%)	(NIOCC #2a)	71 (48 h)	20 (48 h)	50 (48 h)	laccase	Static Liquid Culture	D'Souza et al. 2006
TEA (10%)	(NIOCC #2a)	64 (6 days)	87 (6 days)	82 (6 days)	laccase	Static Liquid Culture	D'Souza et al. 2006
TEB (10%)	(NIOCC #2a)	92 (3 days)	69 (3 days)	85 (3 days)	laccase	Static Liquid Culture	D'Souza et al. 2006

engineering of the enzymes should be considered to achieve this goal. Increasing efficiency of these enzymes for electron transfer should be aimed at, by producing hybrid enzymes. Effect of carbon and nitrogen sources and pH of the effluent on the fungus and/or enzyme used for bioremediation should be studied in detail. The efficacy of the isolates in treating effluents from common effluent treatment (CET) plant which contain mixtures of effluents from various small-sized mills should also be assessed. Laccase can act alone or in the presence of low molecular weight mediators as well as in the absence of the fungal biomass. On the other hand, LiP and MnP require an H_2O_2-generating system from the fungal mycelium and thus they require presence of the live fungal biomass for lignin-degradation. Immobilized peroxidase-producing fungi may be tested for this purpose. A laccase-hyperproducing strain from marine environment using inexpensive growth medium would be a suitable alternative. Co-immobilization of all the three LDEs or fungal isolates that produce them could be a promising technology for treatment of colored effluents.

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ORIGINAL ARTICLE

A Thermostable Metal-Tolerant Laccase with Bioremediation Potential from a Marine-Derived Fungus

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Abstract Laccase, an oxidoreductive enzyme, is important in bioremediation. Although marine fungi are potential sources of enzymes for industrial applications, they have been inadequately explored. The fungus MTCC 5159, isolated from decaying mangrove wood and identified as *Cerrena unicolor* based on the D1/D2 region of 28S and the 18S ribosomal DNA sequence, decolorized several synthetic dyes. Partially purified laccase reduced lignin content from sugarcane bagasse pulp by 36% within 24 h at 30°C. Laccase was the major lignin-degrading enzyme (~24,000 U L^{-1}) produced when grown in low-nitrogen medium with half-strength seawater. Three laccases, Lac I, Lac II, and Lac III, of differing molecular masses were produced. Each of these, further resolved into four isozymes by anion exchange chromatography. The N-terminal amino acid sequence of the major isozyme, Lac IId showed 70–85% homology to laccases from basidiomycetes. It contained an *N*-linked glycan content of 17%. The optimum pH and temperature for Lac IId were 3 and 70°C, respectively, the half-life at 70°C being 90 min. The enzyme was most stable at pH 9 and retained >60% of its activity up to 180 min at 50°C and 60°C. The enzyme was not inhibited by Pb, Fe, Ni, Li, Co, and Cd at 1 mmol. This is the first report on the characterization of thermostable metal-tolerant laccase from a marine-derived fungus with a potential for industrial application.

Keywords Marine-derived fungus · Basidiomycete · Laccase · Thermostable · Metal-tolerance · Bioremediation

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Introduction

Laccases (EC 1.10.3.2: benzenediol/oxygen oxidoreductase) are copper-containing lignin-degrading enzymes. They use molecular oxygen to oxidize a wide range of aromatic compounds besides lignin. Unlike the other lignin-degrading enzymes, lignin peroxidase and manganese-dependent peroxidase, laccase can be used in vitro for depolymerization of lignin or similarly structured aromatic compounds. Laccases do not have oxidation potentials as high as those of the peroxidases, but in the presence of a suitable redox mediator 2,2′-azino-bis (3-ethylthiazoline-6-sulfonate) (ABTS), their effective redox potential is increased and they can oxidize non-phenolic lignin model compounds. Basidiomycetous fungi are the major laccase producers (Baldrian 2006). Potential applications of laccases are in denim bleaching, decolorization and detoxification of dye-containing textile effluents, effluents containing lignin-related compounds, and biobleaching of pulp for paper industries (Baldrian 2006). Many such effluents also contain various inorganic chemicals such as sulfides, sulfates, chlorides, carbonates, peroxides, chlorine bleach compounds, and heavy metals. Heavy metals are known to be toxic to white-rot fungi (Baldrian and Gabriel 1997) and have a negative effect on the activity of ligninolytic enzymes in vitro (Baldrian et al. 1996). Therefore, new sources of

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laccase through bio-prospecting with better properties such as high salt and heavy metal tolerance and thermostability for industrial applications are desired.

Obligately and facultatively, marine (marine-derived) fungi from lignocellulose substrates in the marine environment, particularly mangroves and sea grasses, are an important source of ligninocellulose-degrading enzymes (Raghukumar et al. 1994, 1999; Pointing and Hyde 2000; Bucher et al. 2004). They have been demonstrated to grow in the presence of high salt content (Raghukumar 2002, 2008; Raghukumar et al. 2008). A majority of fungi from mangroves were shown to produce enzymes such as cellulase, xylanase, and laccase in media prepared with seawater (Raghukumar et al. 1994; Pointing et al. 1998, 1999; Pointing and Hyde 2000). A marine-derived basidiomycete NIOCC #2a (MTCC 5159) isolated from mangrove wood produced laccase as the dominant lignin-degrading enzyme with negligible amounts of peroxidases in seawater medium (D'Souza et al. 2006). These authors reported enhanced production of laccase by this isolate in the presence of textile effluent (~85,000 U L^{-1}) and synthetic dyes. Various colored industrial effluents such as textile mill wastewater, molasses spent wash from alcohol distillery, and black liquor from paper and pulp industry were decolorized in the presence of live fungal culture and culture supernatant of MTCC 5159 (D'Souza et al. 2006; D'Souza-Ticlo et al. 2006). As these effluents are often contaminated with heavy metals and have temperatures above ambience, the heavy metal tolerance and thermostability of purified laccase from this marine-derived fungus MTCC 5159 was investigated. This enzyme was also further characterized and the results are presented here.

Materials and Methods

Organism, Culture Conditions, and Identification The basidiomycetous fungus NIOCC #2a was isolated from decaying mangrove wood, Chorao Island in Goa, India. It was maintained on Boyd and Kohlmeyer agar medium (Kohlmeyer and Kohlmeyer 1979) prepared with half-strength seawater (15–17 ppt) and was routinely checked for purity by light microscopy. The fungus was deposited in the Microbial Type Culture Collection (MTCC, Chandigarh, India) under the accession number MTCC 5159 under the Budapest treaty for patent culture deposition. As telomorphic stage of this fungus was not obtained in culture, it was identified by molecular methods. The fungus was identified using the D1/D2 region of 25-28S ribosomal DNA (rDNA; MIDI Labs, DE, USA). For ITS1-5.8S-ITS2 region, primers pITS1 (Fwd) (TCCGTAGGTGAACCTGCGG) and pITS4 (Rev) (TCCTCCGCTTATTGATATGC) were used (White et al. 1990). For 18S rDNA, two sets of universal primers were used to cover the entire length of the gene (White et al. 1990), the first set of primers being NS_1F (GTAGTCA TATGCTTGTCTC) and NS_4R (CTTCCGTCAATTCCTTT AAG) and the second set being NS_3F (GCAAGTCTGGTG CCAGCAGCC) and NS_8R (TCCGCAGGTTCACCTA CGGA). The amplification was performed in DNA Engine Thermal Cycler (BioRad, NSW, Australia). The polymerase chain reaction products were purified and sequencing was carried out by Microsynth Laboratories (Microsynth AG, Switzerland).

Dye Decolorization and Biobleaching Studies The laccase-hyperproducing NIOCC #2a was tested for its ability to decolorize synthetic dyes. These dyes, Congo Red, Methylene Blue (0.02 %), Trypan Blue, and Aniline Blue (0.04 %) were incorporated in low nitrogen (LN) agar medium. The plates were inoculated with plugs of this fungus and observed for decolorization up to 10 days.

Biobleaching of sugarcane bagasse pulp at 5% consistency was carried out with 100 U of laccase from this isolate at 30°C for 24 h. Reduction in lignin content was determined by estimating the kappa number in untreated and laccase-treated bagasse. The kappa number is an indication of the lignin content or bleachability of pulp. It is determined by the amount of 0.1 N $KMnO_4$ (in mL) which is absorbed by 1 g of oven-dried pulp under specific conditions and is then corrected to 50% consumption of $KMnO_4$. Its value indirectly indicates the lignin content or bleachability of pulp lignin, usually with yields of 70% or more. Kappa number was determined using $KMnO_4$-based method T236 (Anonymous 1988). Lignin content was obtained by multiplying the kappa number by a factor of 0.15. However the kappa number measurement is inflated by the presence of hexenuronic acids in the pulp. These compounds are formed from hemicelluloses during the chemical pulping process. Thus, the Kappa number measurement does not only represent the residual lignin in the pulp.

Culture Conditions LN medium (Tien and Kirk 1988) was modified to include glycine and fructose at 0.5% and 3.75% respectively, as the nitrogen and carbon sources instead of glucose and ammonium chloride. Among the trace metals, $CuSO_4$ was replaced with $CdCl_2$ at 0.08 g L^{-1}. The modified LN medium was prepared with half-strength seawater. The fungus was raised in the same medium for 6 days, homogenized in Omni Macro-homogenizer (model no. 17505, Marietta, GA, USA) for 5 s and the mycelial suspension was used as the inoculum. Erlenmeyer flasks (250 mL) containing 50 mL LN medium were inoculated with 5 mL mycelial inoculum (10%, *v/v*). The fungus was incubated at 30°C under static conditions. On day 6, $CuSO_4$ at 2 mM final concentration was added to the fungus under

aseptic conditions to stimulate laccase production. For large-scale cultivation, 3-L Haffkine flask with 1 L of LN medium was inoculated with 100 mL of mycelial suspension and incubated as described above.

Responses Measured The cultures were vacuum-filtered to remove the mycelium and the filtrate was stored at 4°C for enzyme estimation and purification. Responses were measured as fungal biomass, protein concentration, and laccase activity.

Fungal biomass was measured as dry weight. The fungus was filtered through pre-weighed Whatman no. 1 filter paper and dried at 60°C until a constant weight was obtained. The difference in weight was considered as the fungal dry weight and estimated in terms of grams per liter.

Protein concentration was determined using the Bradford reagent (Sigma, St. Louis, USA) and expressed as milligrams protein.

Laccase activity was estimated by measuring oxidation of 1 mM ABTS (2,2′-azino-bis-(3-ethylbenzothazoline-6-sulfonate) buffered with 0.2 M glycine–HCl buffer, pH 3, at 405 nm (Niku-Paavola et al. 1988). Activity was expressed in units defined as 1 μmol of product formed per minute per liter of culture supernatant (U L^{-1}).

All spectrophotometric measurements were carried out using UV–Vis 2450 spectrophotometer (Shimadzu, Japan). Values represent the mean of three measurements from two independent experiments.

Purification of Extracellular Laccase On day 12 of cultivation when laccase activity reached its maximum, the culture filtrate from Haffkine flask was collected after filtering the mycelium through GF/F and subsequently through 0.22-μm filter (Whatman Asia Pacific, Singapore). It was frozen at −20°C and all subsequent purification steps were carried out at 4°C. The precipitated exopolymeric substance produced by the fungus was removed from the thawed culture filtrate by centrifugation at 14,000 rpm for 15 min. The culture supernatant was then concentrated by ultrafiltration using YM3 membrane (Millipore, USA). The concentrate after filtering through 0.22-μm sterile filter was applied to High Load 16/60 Superdex 75 preparative grade column and eluted with 0.2 M Na acetate buffer (pH 4.5) containing 1 M KCl at a flow rate of 1 mL min^{-1} using a fast protein liquid chromatography system (Amersham Biosciences, Sweden). The molecular markers (Amersham Pharmacia, Sweden) used were bovine serum albumin (66 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa), and α-lactoalbumin (14.2 kDa). Out of the three major laccase peaks of differing molecular masses obtained during this size exclusion chromatographic step, the peak showing maximum absorbance at 280 nm (molecular mass ~56 kDa) and laccase activity was collected. It was concentrated using Amicon Ultracentrifugal filter tubes with a 5-kDa cutoff (Millipore, MA, USA). The concentrate was then applied to Mono Q™ 10/100 GL (Amersham Biosciences) column of 10×100 mm size and eluted with Tris–HCl buffer (pH 8) containing 0.5 M KCl at a flow rate of 0.3 mL min^{-1}. Out of several laccase peaks obtained with differing surface charges, the peak showing maximum laccase activity and absorbance at 280 nm was concentrated as above. This laccase fraction was termed as Lac IId and was used for further characterization. Purification and yield of laccase was carried out in three independent experiments.

Homogeneity of Lac IId was confirmed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) and 2D PAGE.

Determination of N-terminal and Internal Peptide Sequence of Lac IId For N-terminal amino acid (AA) sequence determination, Lac IId was electroblotted from an SDS-PAGE gel onto a polyvinylidene fluoride membrane (BioTrace, Pall Pharmalab Filtration, India) using mini trans-blot electrophoretic transfer cell (Bio-Rad, CA, USA). The AA sequence was then determined using an automated protein sequencing system (Procise HT-491, Applied Biosystems, CA, USA) at National Institute of Immunology, New Delhi, India.

For determination of the internal peptide sequence, the protein stained Lac IId from 2D-PAGE was digested in-gel by trypsin; the peptides were extracted by sonication and spotted on the α-cyano-4-hydroxycinnamic acid matrix for a matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis. The sequencing was carried out at the Proteomics Facility, The Centre for Genomic Applications (TCGA), New Delhi using MALDI-TOF (Ultra Flex, Germany).

Determination of the Glycosylation Content of Lac IId For estimating the *N*-linked glycan content, 0.6 μg Lac IId was treated with 60 mU of endoglycosidase H (endo H, from *Streptococcus plicatus*, Sigma, MA, USA) overnight at 37°C. A non-denaturing 12% SDS-PAGE of the Endo H-treated and untreated Lac IId was then carried out. The gel was silver-stained for protein and with Schiff's reagent (Sigma) for glycoprotein (Coll et al. 1993; Xiao et al. 2003).

Isoelectric focusing, 2D PAGE, and Activity Staining Isozyme pattern of laccase in concentrated culture filtrate was determined by non-denaturing isoelectric focusing (IEF) with IEF strips of pH gradient 3 to 10 and 4 to 7 in IEF Cell (BioRad, CA, USA). The gel was stained for activity with guaiacol (5%) dissolved in citrate phosphate buffer (pH 6). For Lac IId, denaturing IEF was performed with pH gradient of 4 to 7 and p*I* was determined by silver staining (Heukeshoven and Dernick 1985).

For activity staining of laccase, non-denaturing SDS-PAGE (12%) was carried out and the laccase bands were visualized after staining with guaiacol. For determining the molecular mass of Lac IId, standard protein molecular markers of the medium range 14.3–97.4 kDa (PMWM, Bangalore Genei, India) were used and visualized by silver staining. A 2D PAGE (Bio-Rad) was run for confirming the p*I* and molecular mass of Lac IId.

Determination of pH and Temperature Optima of Lac IId To determine the pH and temperature optima of Lac IId, activity was measured with 1 mM ABTS in 0.2 M glycine–HCl (pH 2.5, 3), 0.2 M sodium citrate phosphate buffer (pH 3–6), 0.2 M Tris–HCl (pH 7–9). Each of the above assays (at varying pH) was performed in triplicates at 10° intervals from 20°C to 90°C.

Determination of pH and Temperature Stability of Lac IId To determine the pH stability, Lac IId was pre-incubated in triplicates at pH 2.5–9 for 1 h at 30°C and the residual activity estimated with ABTS, pH 3, at 70°C (at its optimum pH and temperature). For testing temperature stability, Lac IId was incubated in Tris–HCl buffer, pH 9 (at its maximum stability), in triplicates for various incubation periods at 50°C, 60°C, and 70°C, and the residual activity was estimated similarly.

Determination of Enzyme Kinetics Thermal characteristics for enzymatic reactions are described in accordance with kinetic parameters such as activation energy (E_a) which expresses the temperature dependence of the k value as indicated in the Arrhenius relationship: $\ln k = -E_a/RT + \ln A$. Hence, $k = A\left(e^{-E_a/RT}\right)$. The temperature coefficient Q_{10} value is the change in the rate of a reaction that occurs with a 10°C change in temperature. Thus, Q_{10}= [rate at temperature T+10°C]/[rate at T]. The Q_{10} value can be related to the Arrhenius equation as, $Q_{10}=e^{10E_a/RT}$, where E_a is independent of temperature provided that the conditions are appropriate and Q_{10} is dependent on temperature.

The effect of temperature on Lac IId activity was determined in triplicates by the Q_{10} value (Nelson and Cox 2004). If the rate of reaction is completely temperature-independent, the resulting Q_{10} will be 1. If the reaction rate increases with increasing temperature, Q_{10} will be greater than 1. Thus, the more temperature-dependent a process is, the higher will be its Q_{10} value.

Energy of activation was calculated from the Q_{10} values (Nelson and Cox 2004). The K_m constant and V_{max} of Lac IId were determined from Lineweaver–Burke plot using the substrate ABTS and syringaldazine (at their optimum pH and temperature). From these results, K_{cat} and specificity constant of Lac IId with ABTS were derived (Das et al. 2001).

Substrate Specificity and Inhibitors of Lac IId Oxygen uptake during reaction of Lac IId with substrates, non-substrates, mediators, and inhibitors was measured using an oxygen electrode (Oxygraph, Hansatech, Norfolk, England) at 30°C using 1-mL reaction chamber with 0.2 M citrate phosphate buffer at pH 4. The reaction mixture for inhibition measurements contained 1 U of Lac IId and 1 mM ABTS. Oxygen consumption was monitored for 15 min. The rate of oxygen uptake was measured in terms of nanomoles O_2 per minute per unit Lac IId. The results are expressed in terms of percent relative activity, considering laccase activity solely in the presence of ABTS as 100%.

Effect of Metal Ions on Lac IId Activity Effect of various metal ions at 1 mmol on Lac IId activity was measured spectrophotometrically with 1 mM ABTS at its optimum pH (3) and temperature (70°C). The activity in the absence of metal ions was considered as 100%, and the activity in the presence of metal ion was expressed as percent relative activity.

Results

Dye Decolorization and Biobleaching The fungus NIOCC #2a showed very good decolorization of the dyes Congo Red, Trypan Blue, Methylene Blue, and Aniline Blue in plate assay (Fig. 1). Laccase from this fungus also reduced lignin content of sugarcane bagasse by 36% within 24 h at 30°C. Based on these preliminary results, NIOCC #2a was used for further detailed studies.

Identification and Growth Characteristics of the Fungus NIOCC #2a had 99% and 98% identity to *Cerrena unicolor* (accession no. AY850007) with the 18S and 25–28S rDNA region, respectively. The ITS1-5.8S-ITS2 rDNA of NIOCC #2a showed homology to two strains of *C. unicolor* (EF577058 and DQ056858), which were the closest taxonomically identified hits with 91% homology. However, NIOCC #2a showed greater homology to basidiomycetes such as some unidentified marine-sponge-derived fungi (EF029829, EF029823, and EF029817) and an endophyte (AY456192) with 99% identity. These unidentified basidiomycetes could possibly be marine-adapted forms of *C. unicolor*. Hence, the fungus NIOCC #2a has been tentatively identified as a *C. unicolor* strain. The sequences of the ITS1-5.8S-ITS2, D1/D2, and the 18S regions of the rDNA are deposited in NCBI database under accession numbers AY939879, AY939878, and EF059806, respectively. The fungus was deposited in MTCC Chandigarh, India under accession no. MTCC 5159. Henceforth, this fungus is referred as *C. unicolor* MTCC 5159.

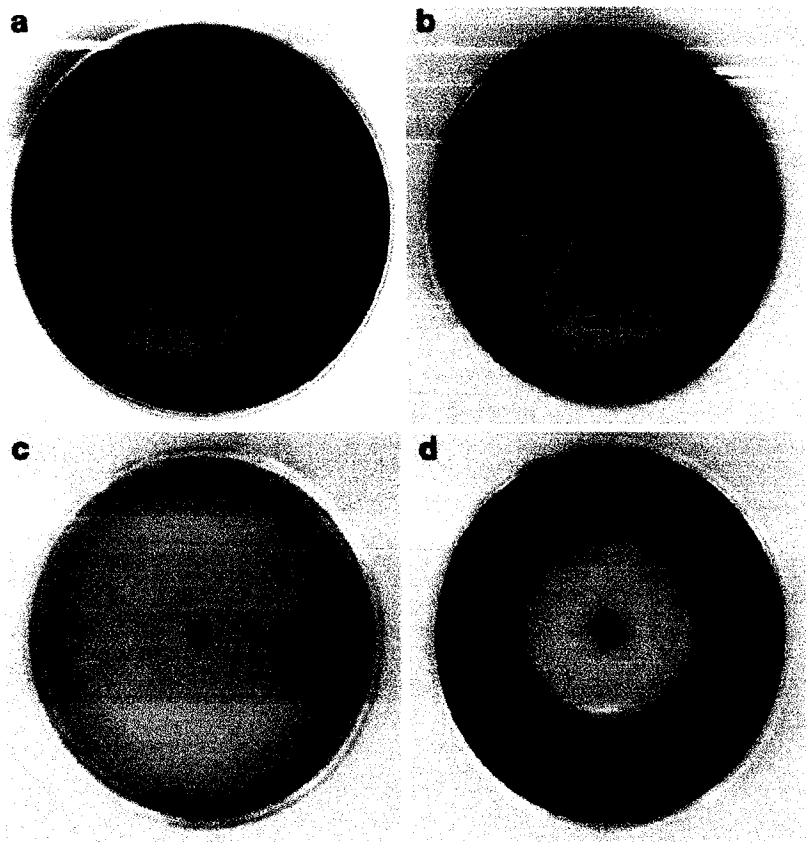

Fig. 1 Decolorization of Congo Red (**a**), Trypan Blue (**b**), Methylene Blue (**c**), and Aniline Blue (**d**) by MTCC 5159 in plate assay

As *Cerrena* is known to be a terrestrial fungus, its growth on mangrove wood could be a result of physiological adaptations, and therefore, its growth and laccase production in seawater medium were ascertained. *C. unicolor* MTCC 5159 showed growth and laccase production in LN medium prepared with seawater containing fructose and glycine as C and N source (Fig. 2). Laccase activity reached a maximum on day 12 (~12,600 U L^{-1}). Addition of catalase to the reaction did not affect rate of oxidation of ABTS, indicating that the entire oxidation of ABTS was contributed by laccase alone and not by any of the peroxidases. Lignin peroxidase and manganese-dependent peroxidase were negligible in this medium.

Purification of Laccase The fungus was harvested from 3-L Haffkine flasks on day 12 when laccase production reached its maximum, yielding 23,714 U L^{-1} with a specific activity of 330 U mg^{-1} protein. The concentrate when subjected to size exclusion chromatography (Superdex-75 column), yielded three major laccase peaks corresponding to molecular masses of 82, 56, and 45 kDa, and were termed Lac I, Lac II, and Lac III (Fig. 3a) respectively, among which Lac II showed maximum laccase activity and absorbance at 280 nm. By anion exchange chromatography (Mono Q column), Lac II further resolved into four major laccase peaks with differing surface charges (Fig. 3b). Lac I and III also resolved into four major laccase peaks, each with very low laccase activity (data not shown). This indicated that all the three molecular mass laccase isozymes also contained isozymes differing in their surface charges. Presence of

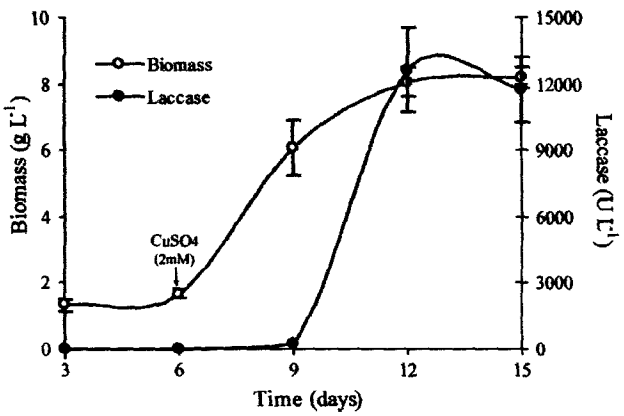

Fig. 2 Fungal biomass (*open circle*) and laccase production (*closed circle*) by *C. unicolor* MTCC 5159 in low nitrogen medium prepared in half-strength seawater containing fructose and glycine as C and N source, respectively. The fungus was maintained under shallow stationery conditions

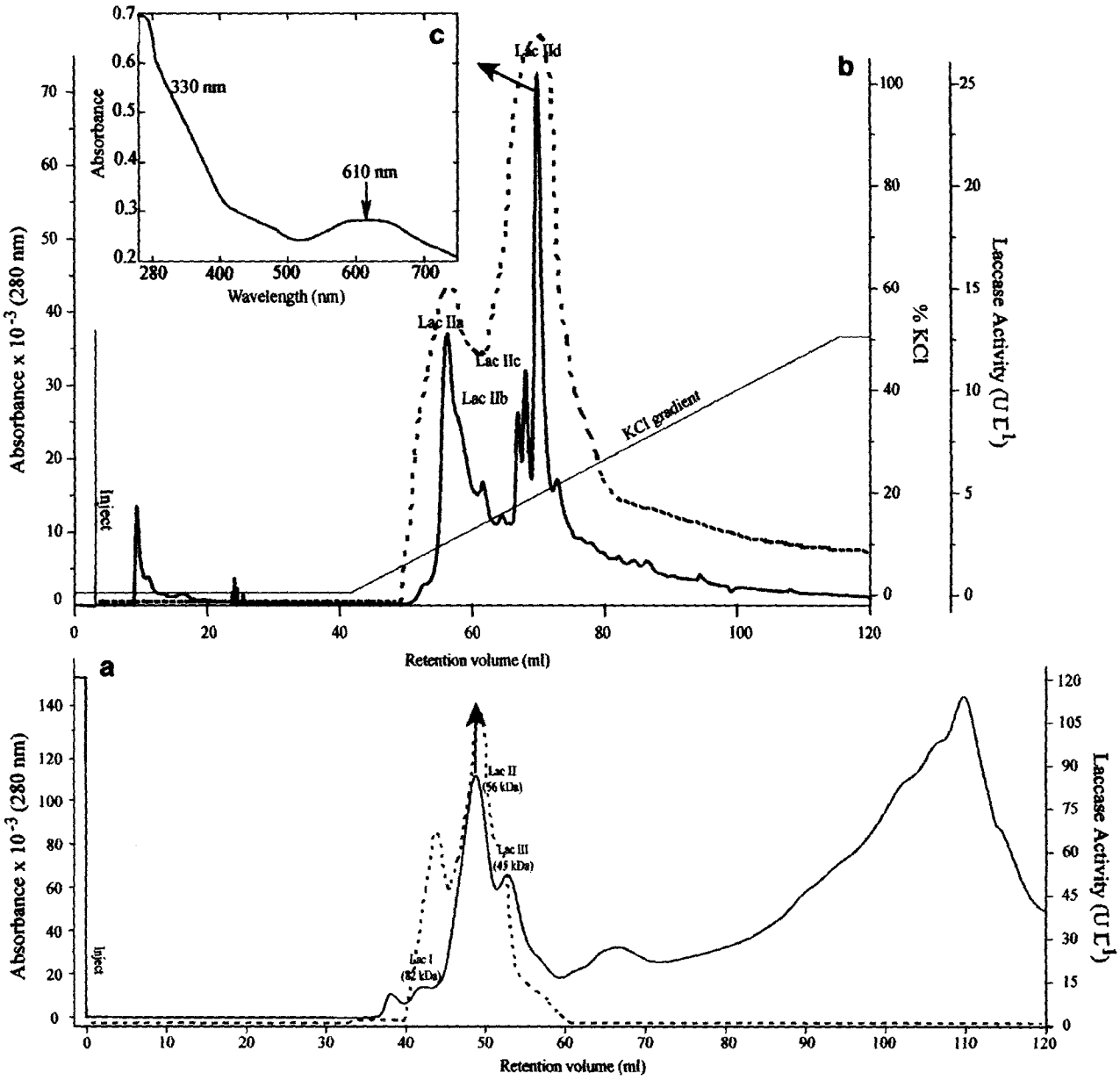

Fig. 3 **a** Size exclusion chromatography of the concentrated crude culture filtrate of a 12-day-old culture of *C. unicolor* MTCC 5159 (Superdex 75 column) showing three major laccase peaks (Lac I, Lac II, and Lac III, *broken line*). **b** Anion exchange chromatography of Lac II (Mono Q column) with KCl gradient of 0 to 0.5 M. Lac II resolved into four major laccase peaks termed Lac IIa, IIb, IIc, and IId (*broken line*). **c** A UV–visible spectrum of Lac IId showing a peak at 610 nm corresponding to type 1 Cu, typical of a blue laccase, and a shoulder at 330 nm corresponding to type 3 binuclear Cu pair

several isozymes (>20) with varying p*I*s in the concentrated crude culture filtrate was also confirmed by running a non-denaturing IEF (pH range 4–7) and subsequent staining for laccase activity (data not shown). From the anion exchange chromatography of Lac II, the peak which showed maximum laccase activity also corresponded to maximum absorbance at 280 nm (Fig. 3b). This was designated as Lac IId and selected for further characterization. Lac IId showed a molecular mass of 59 kDa and p*I* of 5.3 when analyzed by 2D PAGE. The UV–visible spectrum of Lac IId showed a peak at 610 nm, typical for the type 1 Cu(II) that is responsible for the blue color of the enzyme and a shoulder at 330 nm, which suggests the presence of the type 3 binuclear Cu(II) pair (Fig. 3c). Non-denaturing SDS-PAGE also confirmed the single band to be of 59 kDa (Fig. 4a, lane 4), which stained with guaiacol (Fig. 4b, lane 4) and had a p*I* of 5.3 (Fig. 4c) by IEF. A 33-fold purification was achieved with a final yield of 17% (Table 1).

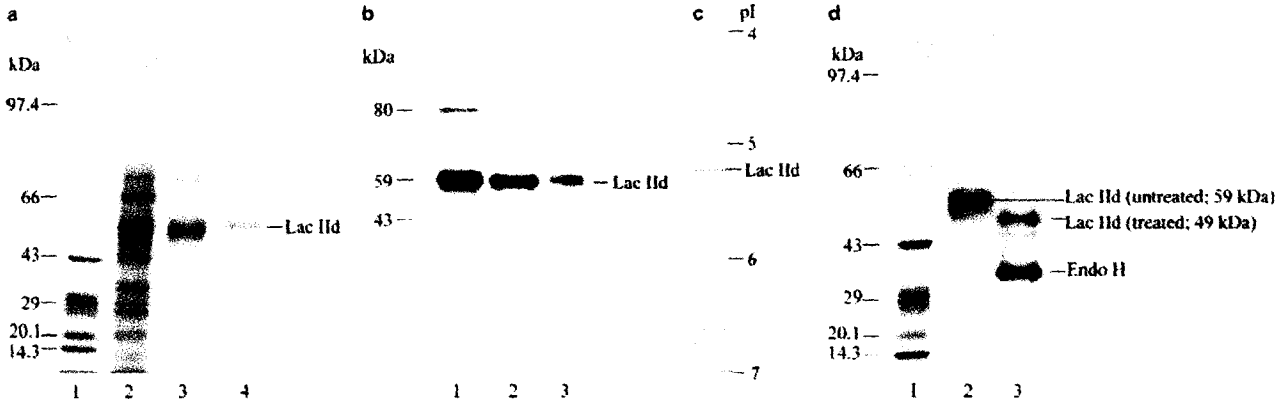

Fig. 4 **a** Non-denaturing SDS-PAGE (12%) of laccase from *C. unicolor* MTCC 5159. *Lane 1* PMWM marker; *lane 2* concentrated crude culture filtrate obtained by ultra filtration; *lane 3* Lac II (size exclusion chromatography); *lane 4* Lac IId (anion exchange chromatography). **b** Activity staining of laccase. *Lanes 1*, *2*, and *3* correspond to *lanes 2*, *3*, *4*, respectively, of **a**. **c** Silver staining of Lac IId on IEF strip showing a p*I* of 5.3. **d** Glycosylation status of Lac IId. *Lane 1* PMWM marker; *lane 2* Lac IId; *lane 3* Lac IId treated with endoglycosidase H

N-terminal and Internal Peptide Sequencing of Lac IId The N-terminal AA sequence of Lac IId showed 70–85% similarity with laccases of basidiomycetous fungi (Table 2). Maximum similarity was observed with laccases from the white-rot basidiomycetes, *Schizophyllum commune* and *Spongipellis* sp. The AA sequence was deposited under the accession number P85430 in the universal protein resource database, Uniprot (http://www.pir.uniprot.org).

From the MALDI-TOF analysis, the sequence of only one internal peptide of Lac IId could be positively identified (Table 2). This internal peptide sequence was also deposited in the universal protein resource database, Uniprot under the same accession number, P85430. A comparative study of various internal peptide sequences homologous to P85430 using the HSSP (Homology Derived Secondary Structure of Proteins, v. 1.1, 2001) was conducted after in silico digestion of the databases (protein, translated DNA, and expressed sequence tag) with trypsin. The internal peptide of P85430 matched with several known laccases. The maximum score of 51% at $p=0.05$, using the algorithm MASCOT (http://www.matrixscience.com/), was with a peptide "Q69FX1_9AGAR" present in Laccase 2 of *Volvariella volvacea* (AAR03581, from NCBI database). The predicted molecular mass and p*I* for AAR03581 was ~59 kDa and 5.4, respectively. This is very close to the experimentally determined molecular mass 59 kDa and p*I* 5.3 of Lac IId from this fungus.

Glycosylation Status of Lac IId On treatment with endoglycosidase H (Endo H), the molecular mass of Lac IId was reduced to 49 kDa (Fig. 4d, lane 3), revealing that it contained an *N*-linked glycan content of 17%. The untreated Lac IId stained for glycoprotein with Schiff's reagent, whereas the endo-H-treated sample did not.

Properties of Lac IId Lac IId showed optimum activity with ABTS at pH 3 and 70°C. With guaiacol and syringaldazine, it showed optimum activity at pH 6 (data not shown). It was most stable at pH 9 for 1 h at 30°C by retaining 100% of the activity (Fig. 5a). At pH 9, more than 60% of the activity was retained up to 180 min at both, 50°C and 60°C (Fig. 5b). About 50% residual activity was detected up to 90 min at 70°C (Fig. 5b). When the enzyme was incubated at its most stable pH of 9 in Tris–HCl buffer for 1 h at varying temperatures, it retained about 60% of its activity at 70°C (Fig. 5c). It retained 67% and 100% of its activity at 4°C and −20°C, respectively, for over 35 days. There was no loss in activity up to 1 year when stored at −20°C.

Table 1 Purification of extracellular laccase from *C. unicolor* MTCC 5159

Purification step	Volume (mL)	Total laccase (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification factor (fold)
Culture filtrate	1,000	23,714	71.9	330	100	1
Ultra filtration (5-kDa cutoff)	50	129,810	11.7	11,126	547	34
Size exclusion chromatography (Superdex 75)	2.5	3,039	0.92	3,315	2	10
Anion exchange chromatography (Mono-Q)	0.625	531	0.05	10,918	17	33

The results shown here are average of three independent experiments

Table 2 Alignment of the N-terminal and an internal peptide sequence of Lac IId (P85430) with other fungal laccases using T-Coffee version 5.0 (http://www.tcoffee.org)

N-terminal AA sequence	1 10 20 30	Similarity (%)	E value
Cerrena unicolor MTCC 5159 (P85430)	GIGPVADLHIIN LSPDGFQ PTVVAGGG	100	6e-19
Schizophyllum commune (BAA31217)	ALGPVGNLPIVN EIAPDGFS PTVLANG	85	8e-06
Spongipellis FERMP 18171 (BAE96003)	AIGPVADLHIVDVSIAPDGFS PAVLAGGT	85	5e-06
Panus rudis (AAW28932)	AIGPVTDLHIVNLNIAPDGFT AAVLAGGI	81	8e-06
Rigidoporus microporus (AAO38869)	AIGPVADLHISNANISPDGFT AAVLAGGS	81	6e-06
Volvariella volvacea (AAR03582)	AIGPVIELQIVNDEIAPDGFS GSVLANGA	77	0.018
Trametes I-62 (AAQ12270)	AVGPVTDLIISNANISPDGFQ AAVVANGG	75	3e-05
Ganoderma lucidum (AAR82930)	AIGPVANLIISLDIAPDGFT AAVVVNGV	70	0.25

Internal peptide	1 5 10 15	Signal peptide (AA)	Start Position (AA) including signal peptide	Similarity (%)	E value
Cerrena unicolor MTCC 5159 (P85430)	DVVSIG --AGDNVTIAF			100	--
Volvariella volvacea (AAR03581)	DVVSIG --AGDNVTIAF	1-22	471	100	3e-07
Rigidoporus microporus (AAO38869)	DVVSIGN--AGDNVTIAF	1-21	444	94	2e-05
Flammulina velutipes (BAE91880)	DVVSIGA--AGDNVTIAF	--	423	94	2e-05
Laccaria bicolor (XP 001886681)	DVVSIGG--AGDNVTIAF	1-17	439	94	4e-05
Pholiota nameko (ABR24264)	DVVSIGG--AGDNVTIAF	1-18	444	94	4e-05
Panus rudis (AAW28932)	DVVSTGT--AGDNVTIAF	1-21	445	88	3e-04
Trametes sp I-62 (AAQ12270)	DVVSTGTPAAGDNVTIAF	1-25	448	--	0.008
Ganoderma lucidum (AAG17009)	DVVSTGTPAAGDNVTIAF	1-21	444	--	0.008

The Q_{10} values of Lac IId which were >1 at its optimum pH 3 did not change drastically between 20°C and 70°C. In contrast, the Q_{10} values for temperatures above 70°C were <1. The energy of activation derived from Q_{10} values between 60°C and 70°C at this pH was 2.5 kJ mol^{-1}, whereas from the Arrhenius plot, it was 8.15 kJ mol^{-1}.

The K_m, V_{max}, and K_{cat} of Lac IId were compared using ABTS and syringaldazine; maximum specificity constant (K_{cat}/K_m) of 120 min^{-1} μM^{-1} was observed with ABTS at 70°C and pH 3 (Table 3). The kinetic parameters of Lac IId suggest that it has a higher affinity towards ABTS at 70°C than at 30°C. The K_m value suggests that at 30°C, syringaldazine is a better substrate than ABTS.

Substrate Specificity and Inhibitors of Lac IId The substrate specificity of Lac IId was measured by O_2 uptake (nanomoles per minute per unit Lac IId) in the presence of various phenolic compounds (Table 4). Among the substrates, ABTS was found to be the best followed by ferulic acid and guaiacol. Tyrosine, vanillic acid, 2,5-dimethyl aniline, *p*-anisidine, and violuric acid were not oxidized by Lac IId alone. However, in the presence of ABTS, most of the compounds, which previously did not get oxidized (with the exception of vanillic acid and tyrosine; Table 4), were oxidized. This indicates the mediating role of ABTS in the oxidation of these compounds when exposed to Lac IId. When ABTS was used as substrate, four of the known mediators were effective in increasing the laccase activity, confirming their role as mediators for Lac IId, whereas 4-hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl did not elucidate any response and 1-hydroxybenzotriazole was antagonistic.

Among the various inhibitors tested for Lac IId, most of its activity was inhibited in the presence of sodium azide, SDS, and mercaptoethanol (Table 5). It was not inhibited by 4-hexyl resorcinol, a differential inhibitor of tyrosinase, a polyphenol oxidase very similar to laccase. The enzyme was not inhibited by L-cysteine and dithiothreitol.

Effect of Metal Ions on Lac IId Approximately 56% and 48% of Lac IId activity was inhibited in the presence of Cr and W, whereas in the presence of Sn, Ag, and Hg, the inhibition was only ~32–37% (Table 5). The other metal ions did not show significant inhibition.

Discussion

Obligate marine fungi grow and sporulate exclusively in the sea; on the other hand, facultative marine fungi or marine-derived fungi are capable of growth in the sea, having

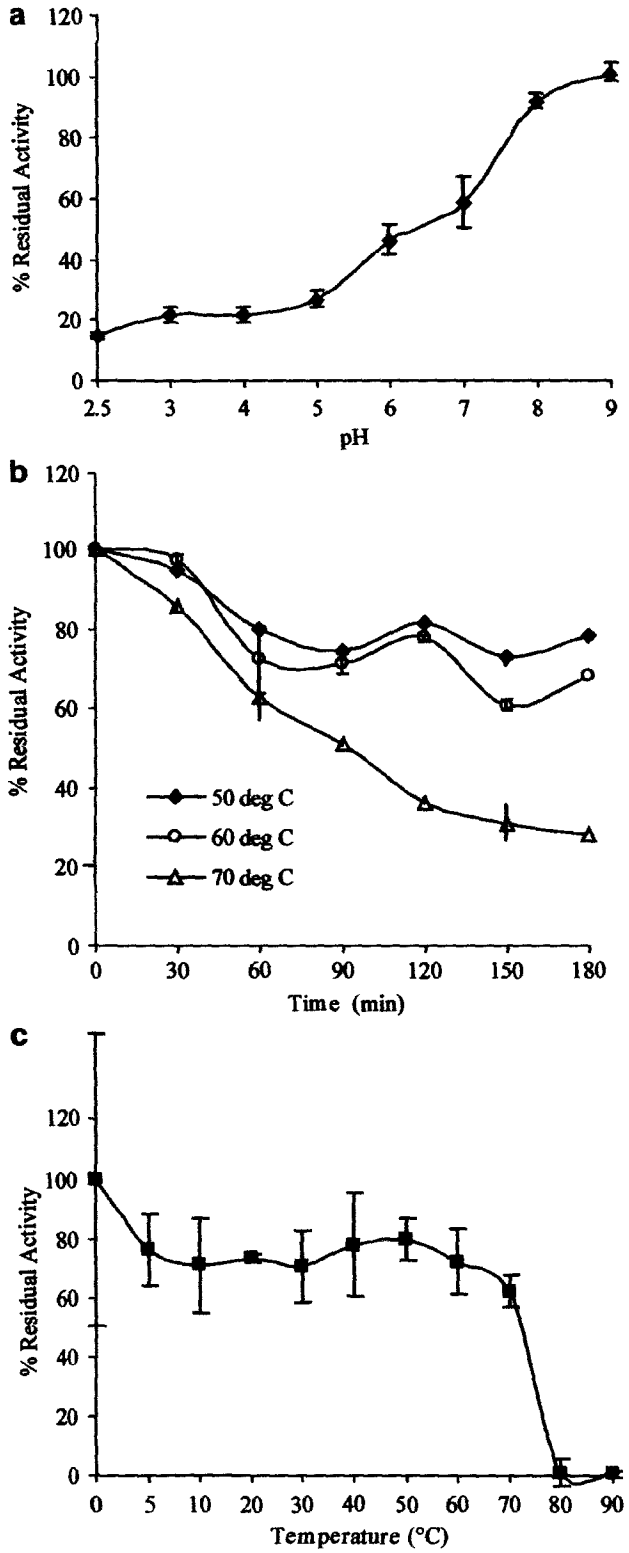

Fig. 5 Properties of Lac IId. **a** pH stability determined after incubation for 1 h at 30°C. **b** Thermostability at 50°C, 60°C, and 70°C at optimum pH stability (9). **c** Thermostability after incubation at various temperatures for 1 h at optimum pH (9) stability

become adapted to this environment. Several such fungi have been isolated from marine habitats and are reported to produce novel secondary metabolites and enzymes not seen in their terrestrial counterparts (Jensen and Fenical 2000; Raghukumar 2008). The present isolate MTCC 5159 appears to be one such highly potent marine-derived fungus. Although the isolate did not show any morphological features by which it could be identified, the 18S rDNA sequence identified it to be the basidiomycetous fungus *C. unicolor* (99% identity). It showed only 91% identity to *C. unicolor* in its ITS rDNA sequences but showed 99% identity to basidiomycetes associated with marine sponges, thus confirming its marine origin.

Although *C. unicolor* is a terrestrial fungus, the isolate MTCC 5159 obtained from mangroves appears to be a marine-adapted cryptic strain of the terrestrial species. This was evident by its growth and laccase production in media containing seawater (see Fig. 1). It could also decolorize dyes and colored effluents in seawater medium (D'Souza et al. 2006). The purified enzyme, Lac IId, was not inhibited in the presence of 1 mmol NaCl (see Table 5), and in the presence of half-strength seawater, it retained 75% of its activity (data not shown). Isoelectric focusing of the partially purified culture supernatant of this isolate grown in distilled water showed the major laccase isozymes around p*I* 4, whereas when grown in the medium containing full-strength seawater, these were around p*I* 7 (data not shown).

This isolate produced a titer of ~23,700 U L^{-1} laccase with a specific activity of 330 U mg^{-1} protein in low nitrogen medium prepared with seawater. In the presence of textile mill effluent (at 1%), MTCC 5159 produced a laccase titer of 85,829 U L^{-1} (D'Souza et al. 2006), whereas the terrestrial isolate of *C. unicolor* strain 137 was reported to produce only 18,700 U L^{-1} (Michniewiez et al. 2006). Several other terrestrial strains of *C. unicolor* were reported to produce much lower laccase titers (Gianfreda et al. 1998; Stepanova et al. 2003) than MTCC 5159. Although MTCC 5159 is known to produce LiP and MnP in low nitrogen medium, in the modified LN medium in the presence of $CuSO_4$ and $CdCl_2$, laccase was produced almost exclusively, making this medium ideal for the production, purification, and characterization of laccase. This strain produced a high titer of laccase in low as well as high nitrogen medium prepared with half-strength seawater.

The fungus produced three distinct laccases, Lac I, Lac II, and Lac III. Each one of these again yielded several isoforms. Presence of several guaiacol-stained laccase isozymes in the range of pH 4–7 during non-denaturing IEF of crude culture filtrate confirmed the existence of several isozymes differing in their p*I* in this isolate. From Lac II, the isoform which showed the maximum laccase activity, Lac IId has been purified and characterized. N-terminal amino acid sequencing, MALDI-

Table 3 Kinetic parameters of the Lac IId

Substrate[a]	K_m (μM)	V_{max} (μmol min^{-1})	K_{cat} (min^{-1})	Specificity constant (K_{cat}/K_m) (min^{-1} μM^{-1})
ABTS (70°C)	54.1	2.2	6,507	120
ABTS (30°C)	57.1	1.8	5,327	93
Syringaldazine (30°C)	19.2	0.7	2,112	110

Amount of Lac IId was held constant (0.02 μg protein) in all the assays carried out in triplicates

[a] Assay was carried out at optimum pH

TOF data, and 2D PAGE confirmed this to be a single isoform. The terrestrial isolate of *C. unicolor* strain 137 was reported to produce Lacc I and Lacc II, and on purification, together, they gave a yield of 22% (Michniewiez et al. 2006). The terrestrial *C. unicolor* strain T143 was reported to have one laccase which was purified to obtain 9.1% yield (Gianfreda et al. 1998). Kim et al. (2002) reported single laccase in the terrestrial *C. unicolor* strain CFC-120 which was purified to recover 23%. On the other hand, the marine-derived *C. unicolor* MTCC 5159 produced three major laccases differing in their molecular masses, Lac I, Lac II and Lac III, each of these resolving into four isoforms differing in their surface charges. A single isoform, Lac IId alone gave a yield of 17%. These results suggest physiological distinctiveness of this laccase-hyperproducing marine-derived isolate.

The Lac IId from MTCC 5159 has all the characteristics of a typical blue laccase: (1) blue color due to its absorbance at A_{610}, (2) a shoulder at A_{330} which is representative of type 3 binuclear copper pair, (3) N-terminal amino acid sequence

Table 4 Oxidation of various substrates, non-substrates, and mediators by Lac IId measured by oxygen uptake

	% Relative activity (SD)
Substrates (1 mM)	
ABTS	100 (27)
Ferulic acid	64 (9)
Guaiacol	53 (7)
Syringaldazine	42 (9)
L-Ascorbic acid	33 (13)
N,N-Dimethyl-*p*-phenylenediamine	21 (2)
Pyrocatechol	15 (3)
Indulin (0.25%)	15 (7)
p-Anisidine	0 (0)
2,5-Dimethyl aniline	0 (0)
Violuric acid	0 (0)
Vanillic acid	0 (0)
Tyrosine[a]	0 (0)
Non-substrates (1 mM)	
Control (only ABTS)	100 (11)
p-Anisidine	161 (12)
2,5-Dimethyl aniline	132 (16)
Violuric acid	122 (16)
L (−) Tyrosine	82 (7)
Vanillic acid	67 (8)
Mediators (1 mM)	
Control (only ABTS)	100 (11)
Hydroquinone	160 (21)
3,5-Dimethoxy-4-hydroxyacetophenone	128 (23)
Syringic acid	122 (10)
3,4-Dimethoxy benzyl alcohol	109 (13)
4-Hydroxy-2,2,6,6-tetra-methylpiperidin-1-oxyl	100 (15)
1-Hydroxybenzotriazole	86 (11)

Non-substrates and mediators were estimated in the presence of the substrate ABTS at 1 mM in triplicates [a] Tyrosine is not a substrate of laccase, it was included as a negative control

Table 5 Effect of inhibitors and metal ions on the activity of Lac IId in presence of 1 mM ABTS as substrate carried out in triplicates

	% Relative activity (SD)
Inhibitors (1 mM)	
Control (only ABTS)	100 (11)
DL-Dithiothreitol	160 (7)
4-Hexyl resorcinol	117 (10)
Cysteine	115 (9)
EDTA	104 (4)
Cysteine (0.1 mM)	104 (7)
o-Coumaric acid	101 (5)
DL-Dithiothreitol (10 mM)	80 (3)
8-Hydroxyquinoline	77 (10)
4-Nitrophenol	73 (7)
CTAB	69 (5)
Kojic acid	62 (3)
Tropolone	58 (9)
2-mercapto ethanol	23 (5)
SDS (1%)	23 (0)
Sodium azide (0.1 mM)	5 (3)
Metal ions (1 mmol)	
Control (only ABTS)	100 (9)
Mo^{+5}	103 (4)
Na^{+}	98 (5)
Ni^{+2}	97 (4)
Zn^{+2}	95 (1)
Cd^{+2}	95 (3)
Co^{+2}	94 (5)
Al^{+3}	94 (3)
Ca^{+2}	93 (2)
Cu^{+2}	93 (5)
Ba^{+2}	92 (2)
Mg^{+2}	90 (12)
As^{+3}	89 (7)
Pb^{+2}	84 (12)
Mn^{+2}	81 (6)
Li^{+2}	75 (3)
K^{+}	72 (2)
V^{+5}	72 (7)
Fe^{+3}	71 (2)

Inhibitors were measured by oxygen uptake. Metal ions were measured spectrophotometrically.

showing 70–85% homology to other basidiomycete laccases and the internal peptide showed 100% homology to a laccase from the basidiomycete, *V. volvacea*, (4) its inability to oxidize tyrosine is in concurrence with the fact that laccase is known for its inability to oxidize tyrosine, and (5) its non inhibition by 4-hexyl-resorcinol, a differential inhibitor of tyrosinase (Dawley and Flurkey 1993).

The Q_{10} value at its optimum pH (3.0) was >1 and did not change drastically between 20°C and 70°C. This indicates that up to 70°C, the activity of Lac IId was not negatively affected, which attests its thermostability. The optimum temperature for laccase activity in crude culture filtrate was 60°C (D'Souza et al. 2006), whereas for purified Lac IId, the optimum temperature was found to be 70°C. Laccase from terrestrial strains of *C. unicolor* showed optimum temperature of activity at 40°C and 60°C (Stepanova et al. 2003; Michniewiez et al. 2006). Lac IId of MTCC 5159 had a half-life of 90 min at 70°C. In contrast, Lacc I from terrestrial *C. unicolor* strain 137 lost its total activity in less than 10 min at 70°C, and Lacc II had a half-life of only 10 min at 70°C (Michniewiez et al. 2006). Energy of activation for Lac IId between 60°C and 70°C (2.5 kJ mol^{-1}) was much lower than at 20–30°C (13.4 kJ mol^{-1}), indicating that the enzyme is more efficient at higher temperatures. High turnover numbers for oxidation of ABTS by Lac IId at 70°C (K_{cat} and K_{cat}/K_m values) further indicates its thermostable character. High-temperature active laccase with thermostability at high temperature is desirable in biobleaching of pulp (Wong et al. 2000) and possibly also in treatment of colored industrial effluents (Asgher et al. 2008).

It is common for laccases from basidiomycetes to have optimum pH of activity in the acidic range and stability at neutral or alkaline pH (Xu et al. 1996). In concordance, Lac IId showed optimum activity at pH 3 and 70°C and was most stable at pH 9.

Lac IId of this isolate was not inhibited by several compounds that are generally inhibitory to laccase. *Pycnoporus cinnabarinus* laccase was totally inhibited by 1 mM L-cysteine and DTT (Eggert et al. 1996), whereas Lac IId from MTCC 5159 was neither inhibited in the presence of 0.1 nor 1 mM L-cysteine nor 1 mM DTT. Further, EDTA also did not inhibit its activity as was observed with the thermostable laccase from an unidentified basidiomycete (Jordaan et al. 2004). Sodium azide, an inhibitor of metalloenzymes (Heinzkill et al. 1998), maximally inhibited Lac IId. However, Lac IId was not affected by the addition of heavy metals such as Pb, Fe, Ni, Li, Co, and Cd at 1 mmol. Only Cr and W appeared to inhibit its activity by 48% and 56%, respectively. One of the problems of decolorization of colored industrial effluents is the presence of heavy metals. These are reported to have a negative effect on the action of lignin-degrading enzymes of white-rot fungi (Baldrian 2003).

Besides decolorization of dyes and effluents (D'Souza et al. 2006; D'Souza-Ticlo et al. 2006), the crude culture filtrate also reduced the lignin content in sugarcane bagasse. This process can be further improved by addition of natural mediators (Johannes and Majcherczyk 2000) to utilize this enzyme in biobleaching processes along with hemicellulases. Fungi producing lignin-degrading enzymes for

conversion of lignocellulose waste to create wealth in the form of biofuel are currently in great demand in the developing countries for meeting their growing energy demands (Howard et al. 2003). The basidiomycete MTCC 5159, with its high laccase titer and proven lignin-degrading ability, may find application in the pretreatment of lignocellulose waste. The tolerance of Lac IId to heavy metals, its high optimum temperature for activity and thermostability, as well as high laccase titer even in the presence of seawater makes the marine-derived *C. unicolor* MTCC 5159 a suitable candidate for application in treatment of effluents containing dyes, lignin-related compounds, chlorides, and sulfates.

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