Production and Characterization of Lignin peroxidases from Mangrove Ascomycetes

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Cochin University of Science and Technology
in partial fulfilment of the requirements of the degree of
Doctor of Philosophy in **Biotechnology**

by

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Dedicated to my parents

DECLARATION

I hereby declare that the work presented in this thesis is based on the original work done by me under the guidance of Dr. P. Prema, Scientist EII, Biochemical Processing Division, Regional Research Laboratory, Trivandrum and that no part of this has been included in any other thesis submitted previously for the award of any degree.

Shamla Ahammed

Trivandrum
05-03-2002

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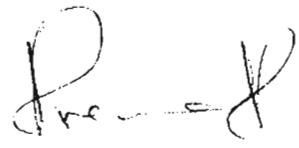
CERTIFICATE

This is to certify that the work presented in the thesis entitled, "Production and Characterization of Lignin Peroxidases from Mangrove Ascomycetes." is based on the original research done by Mrs. Shamla Ahammed under my guidance and supervision at the Biochemical Processing Division and no part of this work has been included in any other thesis for the award of any degree.



Dr. P. Prema

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LIST OF ABBREVIATIONS

psi	:	per square inch
V_{max}	:	maximum reaction velocity
mM	:	millimolar
°C	:	degree celsius
g %	:	gram percentage
pH	:	hydrogen ion concentration
g	:	gram
%	:	percentage
µ		micron
mg	:	milligram
EDTA	:	ethylene diamine tetra acetic acid
'	:	minutes
M	:	molar
DEAE	:	diethyl aminoethyl
pKa	:	pH at which maximum buffering occurs
Co.	:	company
kDa	:	kilo daltons
cm	:	centimetre
ml	:	millilitre
nm	:	nanometre
V	:	volts
SDS	:	sodium dodecyl sulphate
PAGE	:	polyacrylamide gel electrophoresis
µl	:	microliter
K_m	:	Michaelis constant
ε		extinction coefficient
µM		micromolar
SmF		submerged fermentation
SSF		solid state fermentation
g/kg		gram per kilogram
lb		pound
rev. min^{-1}		revolutions per minute

<	:	less than
>	:	greater than
wt.	:	weight
U	:	units
sp.	:	species
w/v	:	weight by volume
mg/l	:	milligram per litre
pI	:	isoelectric point
N	:	normal
μm	:	micrometre
λ_{max}	:	wavelength of maximum absorbance
U/L	:	units per litre
nkatals/L	:	nanokatals per litre
DHP	:	dehydrogenation polymer
wt.	:	weight
~	:	approximately
Å	:	angstrom
w/w	:	weight by weight
syn.	:	synonymn
&	:	and
e.g.	:	example
i.e.	:	that is
ppm	:	parts per million
g/l	:	grams per litre
M_r	:	molecular radius

Preface

The global interest in microbial lignin degradation can be gauged by its immense applications. The only microorganisms known to extensively degrade lignin are white rot fungi. Soft rot fungi belonging to ascomycetes preferentially degrade the carbohydrates of the wood while some of them have demonstrated significant ability to mineralize lignin. A majority of marine fungi, especially from the mangrove swamp, belong to ascomycetes and deuteromycetes. However, information till-date has been sparse on the type of lignin-modifying enzymes present in marine ascomycetes and deuteromycetes.

Three oxidative enzymes commonly found extracellularly in ligninolytic cultures of white rot fungi are (1) Lignin peroxidase (LiP), a heme peroxidase with an unusually high redox potential, low optimum pH, little substrate specificity which requires hydrogen peroxide for activity; (2) Manganese peroxidase (MnP), another heme peroxidase shows a strong preference for Mn(II) as its reducing substrate; (3) Laccase, a copper containing oxidase which does not require hydrogen peroxide. The bioligninolytic systems find application in biopulping and biobleaching of paper pulp; degradation of xenobiotic compounds in industrial effluents; digestibility enhancement of fodder; lignin bioconversion and desulphurization of coal and petroleum.

In this thesis, the production and characterization of ligninolytic enzymes using the fungi isolated from mangrove area are studied and presented in six chapters. Chapter 1 is an introduction with a review of literature on all aspects of the ligninolytic systems of various fungi. As studies on marine fungi capable of producing lignin degrading enzymes are rare and the potential applications of these fungi due to its salt tolerant nature are tremendous, the present study is considered to be of much value. Chapter 2 deals with the isolation and screening of lignin degrading enzyme producing microbes from mangrove area. The identification of the most potent fungal isolates and characterization of LiP from these are also done. Chapter 3 is concerned with the optimization of culture and nutritional parameters for maximum LiP production from *Aspergillus* sp. SIP 11, the most potent of the fungal isolates. Chapter 4 is on the solid substrate fermentation of *Aspergillus* sp. SIP 11 on wheat bran in order to check its effectiveness in LiP production. The growth conditions during SSF are also optimized. Chapter 5 deals with the purification and characterization of lignin peroxidase from *Aspergillus* sp. SIP 11. Dye decolorization studies using the purified LiP was undertaken. Chapter 6 gives a summary of the work done pinpointing the important conclusions drawn, which is followed by the Bibliography.

Chapter 1

Introduction and overall review of microbial lignin degrading system

1.1. INTRODUCTION

Microbial degradation of lignin has received considerable attention in recent years. The biological significance of lignin combined with the commercial utility of lignocelluloses have generated widespread interest in understanding the biochemistry of lignin degradation. The importance of lignin degrading fungi and their ligninolytic enzymes have been well appreciated globally because of their potential applications. For quite sometime lignin peroxidase has been considered as a key enzyme for primary attack of lignin and an impressive work has been done for their characterization (Kirk & Farrell, 1987).

Lignin is the most abundant renewable aromatic polymer on earth and is known to be one of the most recalcitrant materials. It plays an important role in the carbon cycle of the biosphere conferring the quality of rigidity and durability that makes woody plants "woody". It makes up about 30% of softwood and about 20% of hardwood. It is found in the cell walls in a complex with cellulosic and hemicellulosic polysaccharides (Crawford, 1980; Fengel & Wegener, 1983). In this natural composite material, the cellulose fibril provide tensile strength while the hemicellulose and lignin provide cross-linking, binding the structure together.

Lignin is biosynthesized by the polymerization of phenyl propanoid precursors. There are three of these precursors, differing in the number of methoxyl groups on the aromatic ring (Fig. 1). Softwood lignin contain mostly guaiacyl units, while hardwood lignin is formed of roughly equal amounts of guaiacyl and syringyl units. Grass lignins contain all the three types (Higuchi, 1985).

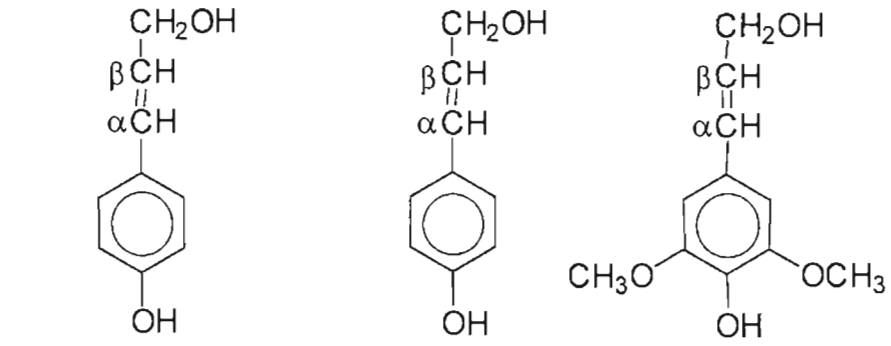
Lignin polymerization occurs in cell walls after the polysaccharides are deposited, and is initiated by the enzymatic oxidation

of the precursors to phenoxy radicals. These radicals can couple with each other and with the growing lignin polymer in numerous ways to form a complex cross-linked network (Fig. 2).

The most common interunit linkage is an ether bond formed between the middle carbon of a propanoid side chain and the phenolic group of another unit (β-O-4 bond). The benzyl (α) carbon of the side chain can also participate in ether bonds, and diaryl ethers form at lower frequency. Direct C-C bonds are formed by coupling between the β-carbon and the 5- or 6-portions of a ring, another β-carbon, or the 1-position of a ring (displacing the propanoid side chain). Direct C-C bonds are also formed between the 5-positions of two rings. Because the 5-position of syringyl unit is occupied by a methoxy group, these units cannot participate in any of the linkages involving that position. Some interunit linkages occur together, such as the combination of the β-5 and α-O-4 links to form a phenyl-coumaran structure or the combination of a β-β-bond with 2α-O-γ links to form a pinoresinol structure. Occasional ether bonds to sugars in hemicelluloses are also present (Higuchi, 1985).

1.1.1. Microbial lignin degradation: The biodegradation of lignin occupies a significant portion in the global carbon cycle since lignin constitutes the second largest sink for fixed carbon after cellulose (Eriksson et al., 1990). The microbes involved in this process and the conditions under which they bring about degradation of lignin are given below.

1.1.1.1. Anaerobic conditions: Lignin is apparently not biodegraded anaerobically. The very limited anaerobic metabolism of lignin-labeled plant tissues by various microflora during extensive incubations were attributed to non-lignin components or to low molecular weight materials freed abiotically (Benner & Hodson, 1985).



p-coumaryl alcohol Coniferyl alcohol [Guaiacyl unit] Sinapyl alcohol [Syringyl unit]

Fig. 1 Structures of the three lignin precursors

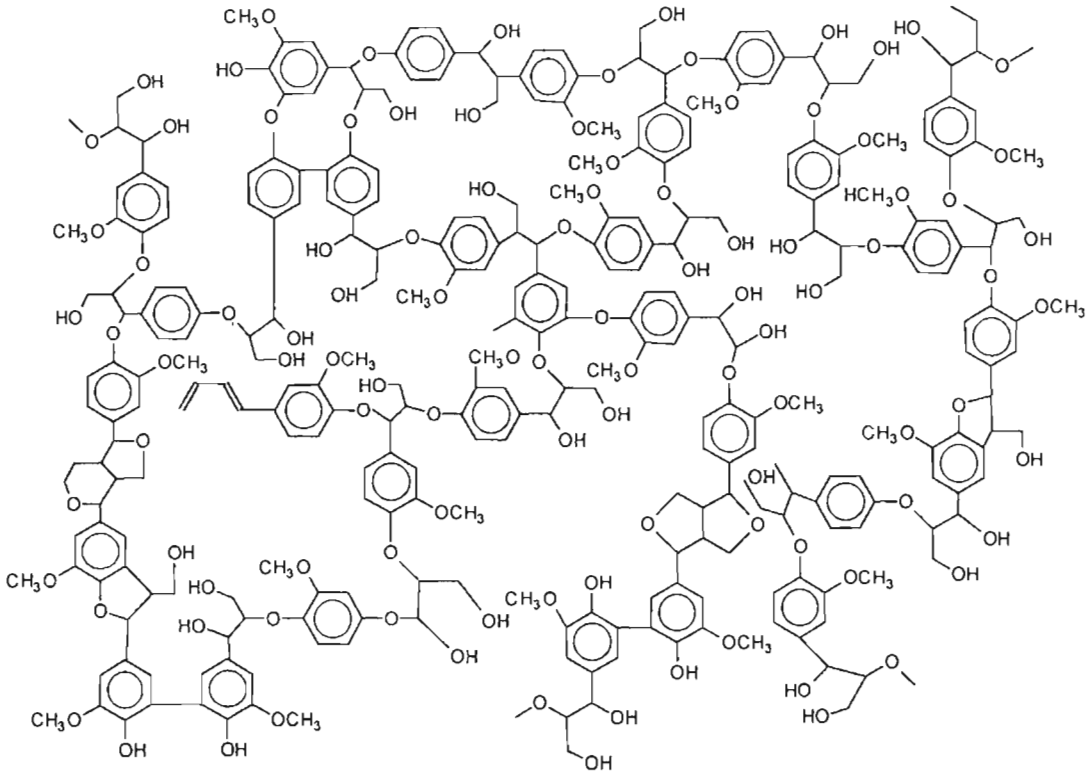


Fig. 2 Schematic structure of a portion of guaiacyl lignin polymer

1.1.1.2. Aerobic conditions: The only organisms known to extensively degrade lignin are fungi (Kirk & Farrell, 1987). White rot fungi, the most effective lignin degraders, are basidiomycetes. They occur predominantly on hardwoods and generally decay hardwood better than softwood because of the presence of syringyl lignin in the hardwood (Faix, et al., 1985). White rot fungi were able to completely mineralize both lignin and carbohydrate components of wood. Some species which are described as simultaneous rots removed lignin and carbohydrate at the same proportional rate while selective white rot species removed lignin faster than cellulose (Blanchette, 1991).

Brown rot fungi, in contrast to white rotters, depolymerized and removed the polysaccharides from wood while leaving the lignin as a crumbly brown residue. The lignin in brown rotted wood was demethylated, partially oxidized and depolymerized but not completely degraded (Kirk & Adler, 1970). Brown rot is most common in softwood. These fungi are basidiomycetes and apparently evolved from the white rot fungi (Gilbertson, 1980).

A third type of wood decay is soft rot caused by ascomycetes fungi (Blanchette, 1995). These fungi are thought to preferentially degrade the carbohydrates of the wood, although some of them demonstrated significant ability to mineralize lignin. Unfortunately, lignin degradation by soft rot fungi have been little studied.

Marine fungi capable of lignin degradation are found in decaying lignocellulosic substrates in the intertidal region of mangrove stands. They are concerned with the degradation and mineralization of mangrove leaves, wood, seagrasses and other lignocellulosic materials. Thus, the fallen leaves and wood of mangroves are a significant primary source in the food web of the tropical marine ecosystem (Pomeroy, 1980). Ascomycetes and Deuteromycetes, which are known to cause soft rot

decay of wood, are important wood degraders in aquatic environments as they require moist conditions and cause softening of the surfaces of the woody tissue. Several workers had shown that soft rot fungal activity resulted in loss of weight of wood blocks and that it was targeted towards the syringyl propane units of lignin (Nilsson et al., 1989). Sutherland et al. (1982) demonstrated decomposition of ^{14}C-labeled maple and spruce lignins by a number of species of marine fungi.

1.1.2. Lignin degrading enzymes: The initial steps in lignin biodegradation, an insoluble polymer, must be extracellular while the final steps culminating in the release of CO_2 takes place inside the fungal hyphae. Therefore, the extracellular reactions must break lignin into fragments that are able to diffuse into the hyphae and cross the cell membranes. These low molecular weight fragments are, however capable of repolymerization. Ligninolytic fungi are able to tip the balance between polymerization and depolymerization in favor of fragmentation, possibly by removing the low molecular weight pieces from the reaction mixture (Hammel et al., 1993) or by glycosylation (Kondo et al., 1990) or methylation (Chen & Chang, 1985).

Lignin has a complex and irregular structure due to the random polymerization process that formed it. The diversity of the interunit linkages, magnified by the presence of both enantiomeric forms at the asymmetric α- and β-carbons, and the irregularity of their arrangement make it difficult for a ligninolytic fungus to produce enzymes that could recognize and cleave all of them. The solution that evolved in the white rot fungi was to produce enzymes of low specificity that initiated, but did not direct, oxidative reactions in lignin. Kirk and Farrell (1987) termed this process "enzymatic combustion". This means that the enzyme

activated the lignin to overcome an energy barrier, thereby beginning a thermodynamically favored oxidative fragmentation without further control of the reaction pathway by the enzyme.

Three oxidative enzymes are commonly found extracellularly in ligninolytic cultures of white rot fungi. Different combinations of the known enzymes are produced by various lignin degrading fungi, suggesting that there was more than one successful strategy for lignin biodegradation. The oxidative enzymes involved in lignin degradation are the following.

1.1.2.1. Lignin peroxidase (LiP, EC 1.11.1.14): Once called ligninase, this enzyme is a heme peroxidase with an unusually high redox potential and low optimum pH (Tien, 1987; Umezawa & Higuchi, 1991). It has wide substrate specificity reacting with varied lignin model compounds and even unrelated molecules (Barr & Aust, 1994). Besides, it has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group. This generated cation radicals which further reacted by a variety of pathways, including $C_\alpha - C_\beta$ cleavage and ring opening. First discovered in 1983 from *Phanerochaete chrysosporium* (Tien & Kirk, 1983; Glenn et al., 1983), it was produced by many, but not all white-rot fungi (Hatakka, 1994; deJong et al., 1994) and required H_2O_2 for its activity. LiPs are oligomannose type glycoproteins with one or more N-glycosylation sites and a number of possible o-glycosylation sites. The LiP family contain multiple isozymes with a molecular weight range of 38 kDa to 43 kDa and isoelectric points ranging from 3.3 to 4.7 (Kirk et al., 1986; Glumoff et al., 1990). The mechanism of action of LiP is shown in Fig. 3.

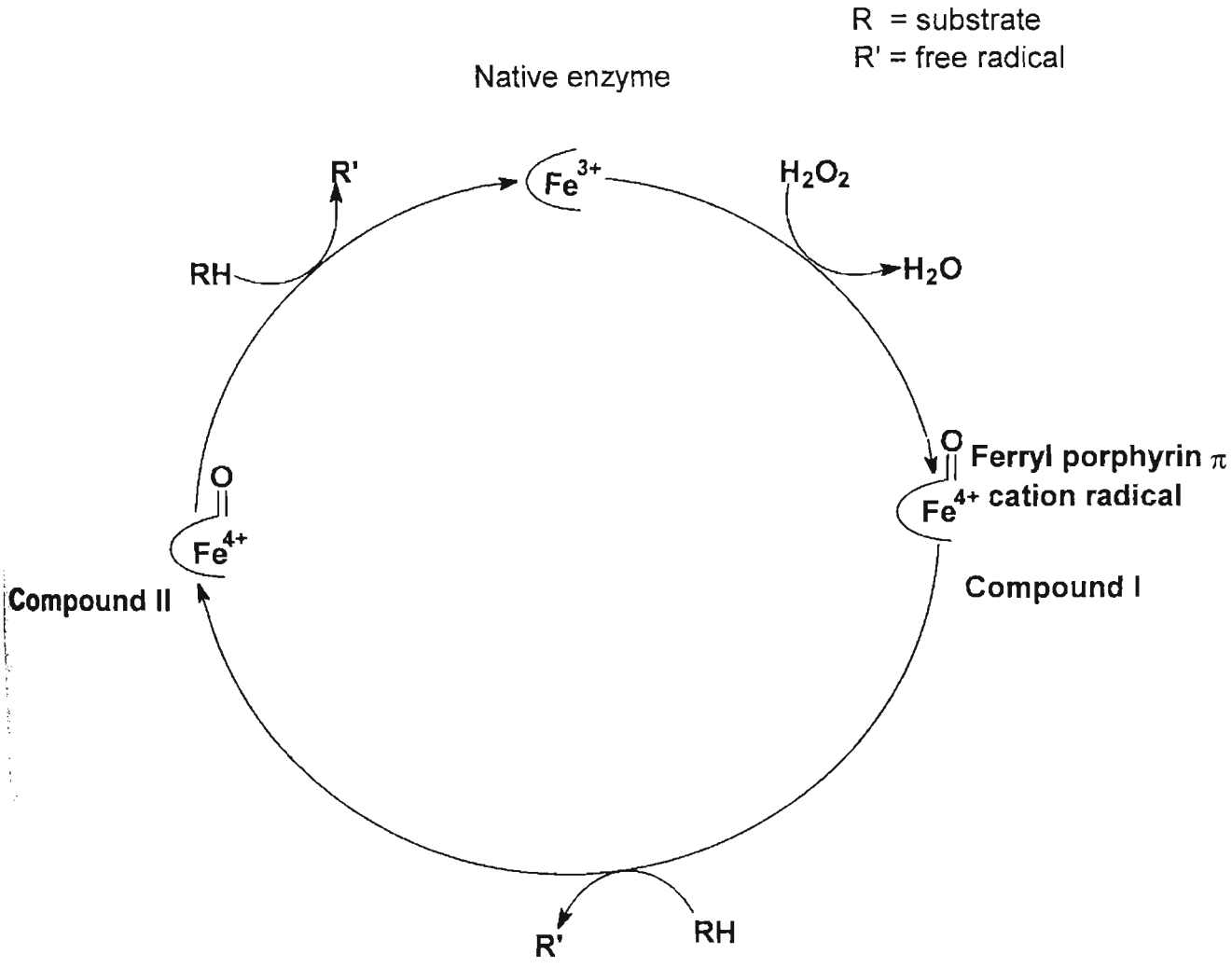


Fig. 3 The peroxidase catalytic cycle (Barr and Aust, 1994)

Veratryl alcohol (3,4-dimethoxy benzyl alcohol) is a fungal secondary metabolite produced subsequent to LiP formation. LiP oxidizes veratryl alcohol to yield veratraldehyde. Veratryl alcohol is the natural substrate for LiP and plays an important role in lignin biodegradation. The actual role of veratryl alcohol and probable binding site on the protein remains unclear. Harvey et al. (1986) was first to propose that veratryl alcohol acted as a redox mediator between LiP and lignin. They postulated that veratryl alcohol was oxidized enzymatically to veratryl alcohol cation radical that was able to mediate peroxidatic reactions and functioned as an electron transfer mediator in the catalytic reaction of LiP. Valli et al. (1990) reported that veratryl alcohol stimulated oxidation by preventing enzyme inactivation. Compound II of LiP could react with H_2O_2 to form a catalytically inactive form of the enzyme, Compound III. This frequently occurred when a substrate reacted more readily with Compound I of LiP than with Compound II. In such situations, the formation of Compound III occurred in a manner that was dependent on the concentration of H_2O_2. On the other hand, veratryl alcohol reacted readily with compound II of LiP and returned it to the resting ferric state (Tien & Kirk, 1984). Koduri & Tien (1994) suggested that veratryl alcohol acted as a preferred substrate for the one electron reduction in the enzyme's catalytic cycle.

1.1.2.1.1. Active site of LiP: The three dimensional structure of LiP is depicted in Plate I. The crystal structure of LiP is also known (Poulos et al., 1993; Piontek et al., 1993) and shown in Plate II. There is striking similarity between cytochrome c peroxidase and LiP, despite the fact that the sequence identity was < 20%. There are eleven helical segments with 343 aminoacid residues, four S-S bridges, two Ca^{2+} -- one proximally and other distally, 133 water molecules and seven sugar residues originating from a N-respectively o-glycosylation site. The enzyme is divided into

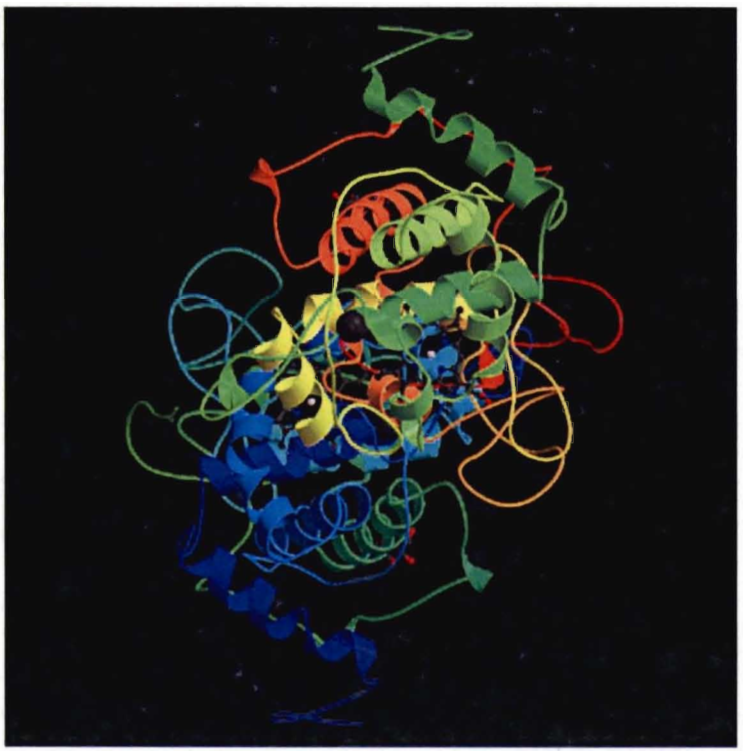


Plate I Three dimensional structure of LiP
(Poulos et al., 1993)

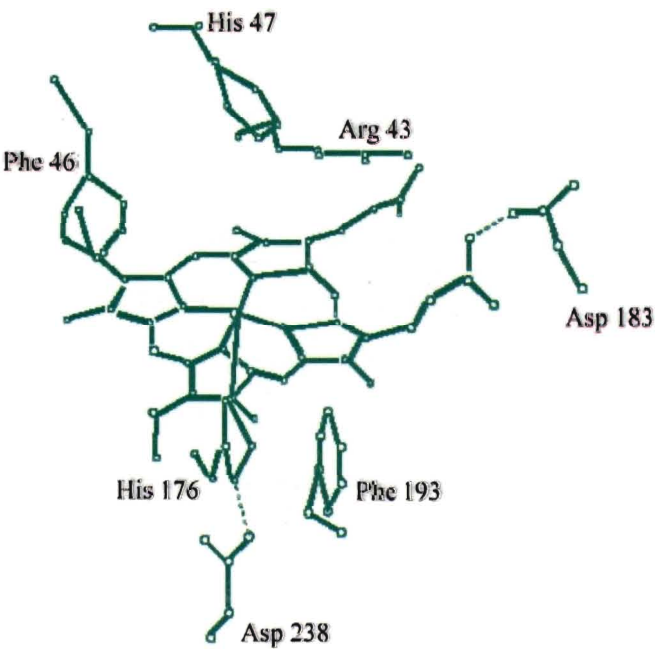


Plate II Crystal structure of LiP
(Piontek et al., 1993)

two domains. Each domain is located on either side of the heme plane. The heme is sandwiched between proximal and distal helices. One heme edge is situated at the bottom of a crevice formed by the surfaces of both N & C terminal domains. But this crevice is smaller in LiP due to side chain interactions, which make it less attackable by hydrazines. This small opening connecting the surface of the enzyme to the distal pocket is the way by which substrates like veratryl alcohol interacted with LiP. There was active site water molecules, one water molecule sits directly over the heme iron atom at a distance of 2.5 Å. All eight cysteine residues in LiP formed disulphide bridges and gave the protein a high degree of rigidity. The heme iron was in the high-spin pentacoordinate ferric state with histidine as the fifth ligand.

The distal pocket or H_2O_2 binding pocket has histidine and arginine residues. In the proximal pocket, the histidine is hydrogen bonded to a buried aspartic acid side chain. This hydrogen bond imparted a greater anionic character to the histidine ligand providing additional stability to the Fe^{4+} iron in compound I. Most striking difference in the active site was the substitution of tryptophan residues in both the proximal and distal pockets of cytochrome c peroxidase with phenyl alanine in LiP. That was why LiP compound I had a porphyrin π cation radical.

The rate of compound I formation was relatively insensitive to pH over a wide range. But the reduction of compound I was pH sensitive and exhibited an optimum near pH 3.0. There are three possibilities for such a low pH optimum which are as follows:

i) one heme propionate hydrogen bonded with an aspartic acid residue, thus giving a carboxylate-carboxylate hydrogen bond. At higher pH, this hydrogen bond would be disrupted leading to some subtle changes in the heme pocket critical to binding or electron transfer.

ii) His-82 and Glu-146 formed hydrogen bonding interaction in the substrate pocket. Veratryl alcohol binding led to hydrogen bonding interaction between the substrate and His-82. With higher pH, disruption of this interaction by protonation of Glu-146 would affect substrate binding directly.

iii) Redox potential of compound I increased as pH decreased, thus making compound I a better oxidizing agent.

Investigations on the veratryl alcohol binding sites of LiP from *P. chrysosporium* suggested the occurrence of more than two substrate binding sites. Veratryl alcohol was probably bound to Trp 17 or its vicinity as a reducing substrate and as an enzyme bound mediator. It was also strongly suggested that veratryl alcohol was bound to a different site when it reacted with LiP III for the reversion reaction (Johjima et al., 2002).

1.1.2.1.2. Location of LiP: During ligninolysis, the fungus produced an extracellular mucilaginous sheath, composed largely of β-1,3-1,6-linked glucan, which was closely associated both with the hyphae and with regions of the woody cell wall that was undergoing decay. LiPs were present in this mucilage (Garcia et al., 1987; Blanchette et al., 1989). At early stages of decay, the enzymes were found at the surface of the lignified cell wall, but were evidently unable to penetrate it. As decay progressed, the cell wall swelled and LiPs were found within its degraded regions.

1.1.2.2. Manganese peroxidase (MnP, EC 1.11.1.13): This is another heme peroxidase, but it showed a strong preference for Mn(II) as its reducing substrate (Glenn & Gold, 1985). The product Mn(III) formed a complex with organic acids and diffused away from the enzyme to oxidize other materials like lignin. The redox potential of the MnP-Mn system was lower than that of lignin peroxidase and it did not oxidize

nonphenolic lignin models. Phenolic substrates were oxidized to phenoxy radicals, which can react further by demethylation, alkyl-phenyl cleavage, C_α oxidation, or $C_\alpha - C_\beta$ cleavage (Tuor et al., 1992). MnP was very widespread among white-rot fungi (Hatakka, 1994; deJong, et al., 1994).

1.1.2.3. Laccase (EC 1.10.3.2): This enzyme is a copper containing oxidase which did not require hydrogen peroxide (Thurston, 1994). Like MnP, it normally oxidized only those lignin model compounds with a free phenolic group, forming phenoxy radicals. However, in the presence of artificial substrate ABTS, 2,2' - azino - di - (3 - ethyl - benzo thiazoline - 6 - sulphonic acid), laccase could also oxidize certain non-phenolic compounds (Bourbonnais & Paice, 1990) and was produced by most white rot fungi (Hatakka, 1994; deJong et al., 1994).

1.1.2.4. Hydrogen peroxide producing enzymes: The activity of extracellular peroxidase depended on a supply of H_2O_2. Glucose-1-oxidase (EC 1.1.3.4) (Kelley & Reddy, 1986), glucose-2-oxidase (EC 1.1.3.10) (Daniel et al., 1994), glyoxal oxidase (Kersten & Kirk, 1987), aryl alcohol oxidase (EC 1.1.3.7) (deJong et al., 1994) and methanol oxidase (EC 1.1.3.13) (Nishida & Eriksson, 1987) have all been proposed as H_2O_2 sources. Sugars needed as substrates for the glucose oxidases could be produced by the action of cellulases and hemicellulases on wood polysaccharides. Methanol could be released from methoxyl groups during lignin degradation (Paice et al., 1993). Glyoxal, aryl alcohols, and ethanol were excreted metabolites of white rot fungi (Kersten & Kirk, 1987; Reid & Deschamps, 1991).

1.1.2.5. Other enzymes: Besides the above, the following enzymes are considered to be involved in lignin degradation.

1.1.2.5.1. Aryl alcohol dehydrogenase (EC 1.1.1.91): It is an intracellular enzyme produced mainly by *P. chrysosporium* which bring

about aromatic ring cleavage and reduction of C_α aldehydes. This enzyme was postulated to act synergistically with LiP on the degradation of non-phenolic β-o-4 lignin models. The possible need for aryl alcohol dehydrogenase was due to the non-accessibility of C_α-oxo compounds for LiP due to their high electropotential. On the other hand, this enzyme was involved in the synthesis of veratryl alcohol performing the last reduction step (Buswell & Eriksson, 1979; Muheim et al., 1991).

1.1.2.5.2. NAD(P)H : quinone oxidoreductase (EC 1.6.99.2): This is also an intracellular enzyme produced by *P. chrysosporium* and other white rot fungi which reduced quinones, a product of lignin degradation (Buswell & Eriksson, 1988a). Constam et al., 1991 isolated and characterized this enzyme. It represented a good example for the fact that an essential product from the primary attack could be easily processed upto mineralization.

1.1.2.5.3. Cellobiose : quinone oxidoreductase (EC 1.1.5.1): This enzyme is concerned with the reduction of quinones and cellobiose degradation. It was discovered by Westmark and Eriksson (1974a) during cultivation of *Trametes versicolor*. Later the enzyme was purified from cellulose cultures of *P. chrysosporium* and shown to be an FAD enzyme. It had a molecular weight of 58 kDa and a pH optimum of 4.5 to 5.0. It reduced a great number of quinones and oxidized cellobiose to cellobiono-1,5-lactone.

1.1.2.5.4. Aromatic acid reductase: This enzyme bring about reduction of aromatic acids. Leisola and Fiechter (1985) reported this enzyme from *P. chrysosporium* while it was detected in *Phlebia radiata* by Lundell et al. (1990).

1.1.2.5.5. Vanillate hydroxylase: This enzyme catalyze the oxidative decarboxylation of vanillic acid. It was reported from *P. chrysosporium* by Buswell and Eriksson (1988b).

1.1.2.5.6. Dioxygenase : This enzyme produced by *P. chrysosporium* cultures is concerned with aromatic ring cleavage (Buswell & Eriksson, 1979).

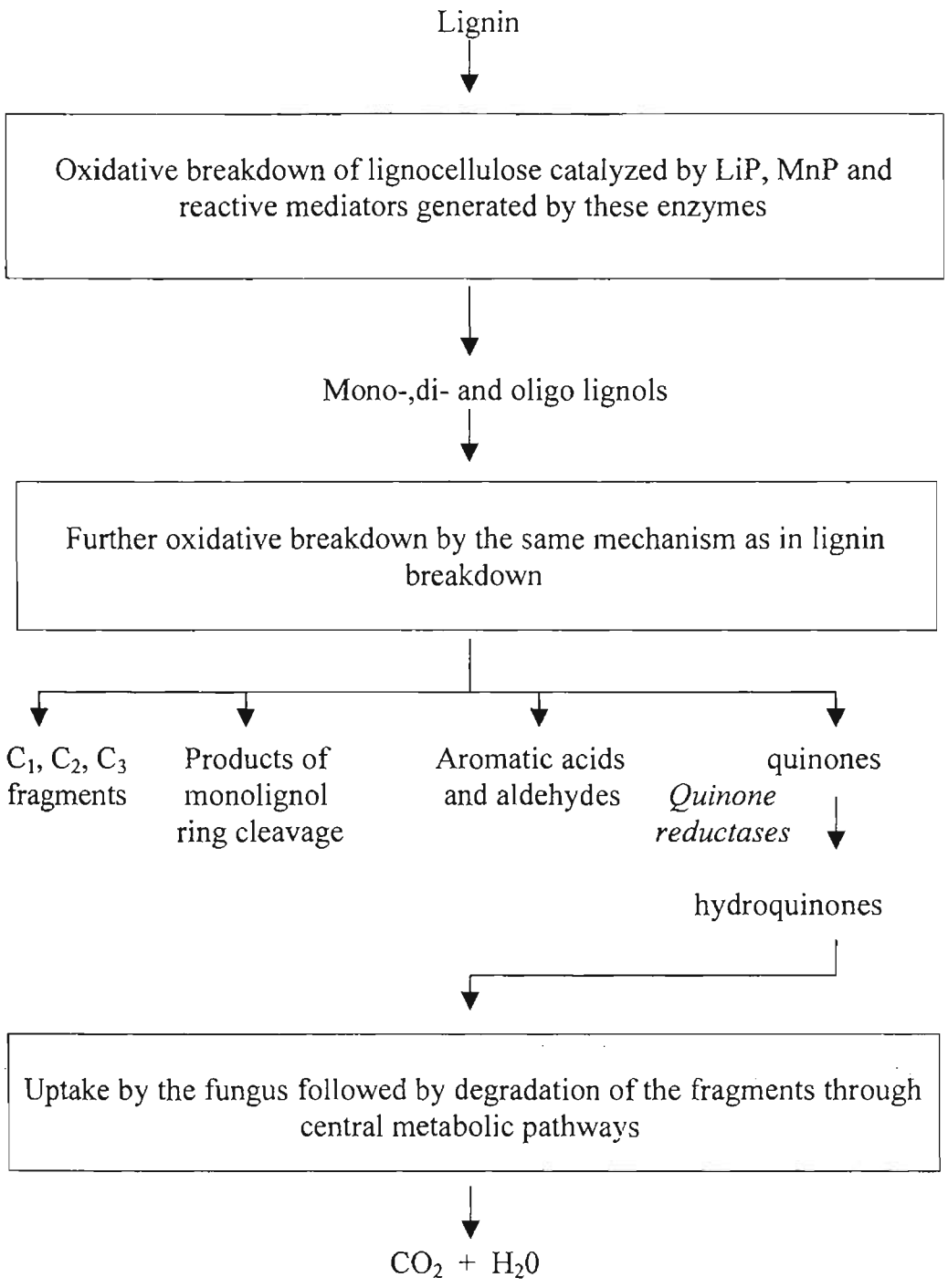
1.1.2.5.7. Catalase (EC 1.11.1.6): It is involved in the conversion of H_2O_2 to water and oxygen thereby preventing the inactivation of the enzyme by excess H_2O_2 (Kwon & Anderson, 2001). It was produced under nutrient limited growth conditions by *P. chrysosporium* and was not coordinately produced with ligninolytic metabolism.

A schematic representation of the major steps in lignin degradation by ligninolytic cultures of *P. chrysosporium* is given in Fig. 4 (Eriksson et al., 1990).

1.1.3. Potential applications of bioligninolytic systems: Microbial lignin degrading enzyme system find varied applications mainly because of its non- specificity of substrates. Some of the important applications to which this system could be put to use are given below.

1.1.3.1. Pulping: Making wood into pulp requires separating the fibres from each other and making them flexible, either by dissolving out the lignin in chemical pulping or by physically tearing apart the liquefied fibres in mechanical pulping (Reid, 1991). Treating the wood with selectively lignin degrading fungi decreased the energy input required for mechanical pulping and also improved the bonding ability of the fibres, leading to stronger paper (Akhtar et al., 1993). Biological delignification of wood was also reported to aid subsequent chemical pulping by the sulfite (Messner & Srebotnik, 1994) and Kraft processes (Oriaren et al., 1990).

Fig. 4 Schematic representation of the major steps in lignin degradation by ligninolytic cultures of *Phanerochaete chrysosporium*



1.1.3.2. Bleaching: Kraft pulps contain upto 5% of modified, dark colored residual lignin. Certain white-rot fungi could solubilize much of this lignin, decreasing the amount of chemicals required to bleach the pulp (Reid & Paice, 1994). Eventhough direct physical contact between fungus and pulp was not required for limited delignification, extensive bleaching by a cell free enzyme system was difficult to demonstrate (Kondo et al., 1994).

1.1.3.3. Pollution abatement: The low specificity and strong oxidative abilities of fungal lignin degradation systems allow them to be applied to the degradation of many organic pollutants. White rot fungi do not require preconditioning to a particular pollutant. Because the degrading system was induced by nutrient deprivation, limiting the nutrient source could initiate degradation. Furthermore, repression of enzyme synthesis did not occur when the concentration of a chemical was reduced to a level that was ineffective for enzyme induction (Bumpus & Aust, 1986). Ferrer et al. (1991) reported the use of LiP in the color removal of kraft mill effluents. The immobilized form of enzyme was in all cases more efficient than the free form. White-rot decolorized the chlorolignin in effluents from chlorine bleaching of chemical pulps (Roy-Arcand & Archibald, 1991; Yui et al., 1990). Color elimination from molasses wastewater by *Aspergillus niger* was reported by Miranda et al. (1996). There was 80% decolorization of synthetic and spentwash melanoidins using *P. chrysosporium* (Dahiya et al., 2001). Removal of phenol compounds from olive mill wastewater using *P. chrysosporium*, *Aspergillus niger*, *A. terreus* and *Geotrichum candidum* (Garcia et al., 2000) open up new methods in effective effluent treatment systems. Selected white rot fungi could also be used for the bioremediation of soils contaminated with a wide variety of aromatic pollutants like dioxins, polychlorinated biphenyls, synthetic dye pollutants and

polyaromatic hydrocarbons (Barr & Aust, 1994). Deguchi et al (1997) had reported the biodegradation of nylon by the white rot fungus IZU-54.

1.1.3.4. Digestibility enhancement of fodder: Lignification protected polysaccharides from hydrolytic enzymes and limited the digestibility of lignocellulosic materials by ruminant animals or by cellulases applied in vitro. Delignification of the raw materials by solid state fermentation with selective white rot fungi increased their value as fodder for ruminant animals and as substrates for enzymatic saccharification and fermentation (Reid, 1989). On using steam exploded wheat straw for SSF of *P. chrysosporium*, the partly degraded hemicellulose acted as the carbon resource for the microbe leading to 60% degradation of lignin on the fifth day of incubation (Chen et al., 2002).

1.1.3.5. Lignin bioconversion: Large quantities of lignin was available as byproducts from chemical pulping and will become available if lignocellulose were utilized as a source of sugars for fermentation. The available markets for these lignins are mostly low valued. Lignin degrading fungi or their enzymes could be used to tailor the properties of these lignin and thus increase their value, although the low specificity of the known enzymes limited the control that could be exercised. Laccase was used to polymerize lignin as a binder in particle boards (Haars et al., 1986). It was also used to incorporate organosolv and Kraft lignins into graft copolymers (Milstein et al., 1994). A new method for production of wood-based plastic involved solid state fermentation of cotton plant wastes with basidiomycetes (patent, Cherkason V.P., 1999). Hardwood and fibreboard were manufactured by biotransformation of wood waste by a culture liquid of higher basidiomycetes. Modification of natural polymers forming wood cells by enzymes of white rot fungi resulted in required molding characteristics and stickiness of material, which

enabled compressing the material without adding traditional carbamide or phenol formaldehyde toxic resins (Bolobova, 1999).

1.1.3.6. Desulphurization of coal and petroleum: White rot fungi and their enzymes were used for the development of practicable biological processes for the beneficiation of low-rank coal and its conversion to specific low molecular weight organic molecules and novel fluid fuels (Catchesida & Ralph, 1999; Temp et al., 1999).

The biodegradation of lignin is a special talent, especially of white rot fungi, which possess a powerful extracellular oxidative system with low substrate specificity. LiP, MnP and laccase were components of this system in various fungal species, other constituents remain to be discovered. Strategies are now being devised for improving ligninolytic enzyme activities in bioreactors (Rodriguez et al., 2001). The coming decade would see the application of these fungi and their enzymes in the pulp and paper, agricultural, and bioremediation industries.

1.2. REVIEW OF LITERATURE

In 1983, two research groups using the basidiomycete *P. chrysosporium*, independently isolated a lignin peroxidase (LiP) which was thought to play an important role in lignin degradation (Tien & Kirk, 1983; Glenn et al., 1983). Later a manganese dependent peroxidase (MnP) was also discovered (Kuwahara et al., 1984). In *P. chrysosporium*, these two iron porphyrin containing enzymes worked together to degrade lignin but their function in this connection was not well understood. Laccase, a copper containing enzyme, also was implicated in lignin biodegradation although the enzyme in vitro caused both depolymerization and polymerization of lignin (Kawai et al., 1988).

In the 1970s the main focus was to define laboratory conditions under which white rot fungi, especially *P. chrysosporium*, maximally

mineralized lignin. In the 1980s, the biochemistry of lignin-modifying enzymes were predominantly studied, due to the discovery of LiP. In the 1990s, in addition to the detailed studies on catalytic and enzymatic properties of lignin modifying peroxidases and their molecular biology, major lines of research have involved applications of enzymes in biopulping, pulp bleaching, and a research for the enzymes responsible for lignin degradation in more selective lignin degraders (Blanchette, 1991; Eriksson et al., 1990).

1.2.1. Classification of fungi based on their lignin degradation ability: The extent of lignin degradation brought about by fungi formed the basis for their classification into white rot, soft rot and brown rot which are dealt with separately.

1.2.1.1. White rot fungal lignin degraders: These are the most efficient lignin degraders in nature. According to their typical production patterns of extracellular enzymes, white-rot fungi might be divided into three main groups (i) LiP-MnP group (ii) LiP-laccase group and (iii) MnP-laccase group although overlaps and exceptions occur.

1.2.1.1.1. LiP-MnP group: *P. chrysosporium* belong to this group. It is a very efficient degrader of lignin, and some strains also possessed industrially appreciated properties like selective lignin degradation to promote biopulping (Blanchette et al., 1992). It lacked laccase, typically produced by white rot fungi. *P. chrysosporium* produced variable amounts of LiP and MnP isoenzymes depending on the culture conditions and separation methods (Paice et al., 1993; Eriksson et al., 1990; Blanchette et al., 1992). Farrell et al. (1989) described 6 LiPs and 4 MnPs in *P. chrysosporium* BKM F-1767. The main isoenzyme when the fungus was cultivated under oxygen atmosphere in N-limited or C-limited medium supplemented with veratryl alcohol was H8.

Chrysosporium purinosum, considered to be a separate strain of *P. chrysosporium*, similarly produced LiP and MnP activities (Waldner et al., 1988). *Phlebia radiata* was a white rot fungus which also readily produced LiPs, laccase and MnPs (Niku-Paavola et al., 1990; Hatakka et al., 1992). Extracellular enzymes of two strains of *P. radiata* 79 and L12-41 were separated (Hatakka et al., 1992) and the main isoenzymes of LiP and MnP characterized. Apparently, this enzyme system in which all three components – laccase, LiPs and MnPs – were readily produced allowed *P. radiata* to very efficiently degrade lignin according to studies using both ^{14}C (Ring) labeled synthetic lignins (Hatakka et al., 1983) and various ^{14}C (lignin) – labeled lignocelluloses (Hatakka & Uusi-Rauva, 1983).

Coriolus versicolor (Syn. *Trametes, Polyporus, Polystictus versicolor*) produced laccase (Eriksson et al., 1990), LiPs (Waldner et al., 1988) and MnPs (Rogalski et al., 1991), but probably utilized primarily laccase and MnPs in lignin degradation. An interesting laccase (Laccase III), which apparently degraded lignin model compounds and caused depolymerization of lignin, had been described in *Coriolus versicolor* (Morohoshi, 1991). Prior to *P. chrysosporium*, this fungus was considered as a standard white rot fungus and was frequently used in studies. *C. versicolor* started to degrade ^{14}C – (Ring) – DHP even more rapidly than *P. chrysosporium* (Leatham & Kirk, 1983). *C. versicolor* degraded lignin non-selectively i.e. it degraded wood carbohydrates and lignin at the same proportional rate (Eriksson et al., 1990). MnP was considered an important and essential enzyme in demethylation and delignification of pulp by *C. versicolor*. *Trametes trogii* also produced the three major classes of ligninolytic enzymes besides cellobiose quinone oxidoreductase, glucose oxidase and glyoxal oxidase (Levin & Forchiassin, 2001).

Phlebia tremellosa PRL 2845 was able to produce extracellular LiPs in shake flasks with large mycelial pellets, and in bioreactor cultivations. Under these conditions, 1-3 LiPs, MnP and laccase were produced (Hatakka et al., 1992). From *P. tremellosa* strain 77-51, one LiP and a laccase were separated and characterized (Hatakka et al., 1993). LiP production have been observed in all 5 strains of *Phanerochaete* studied, and in *P. flavidoalba*, *P. magnoliae*, *Lentinula edodes*, *Phellinus pini* and *P. radiata* (Bonnarme & Jeffries, 1990). *Trametes hirsuta* and *Trametes gibbosa* produced LiP, MnP and laccase (Nerud et al., 1991). However, controversial results have been obtained using *Bjerkandera adusta* CBS 595.78. Waldner et al. (1988) did not find MnP or LiP activity, whereas deJong et al. (1992) detected MnP and laccase in the same strain. However, cDNA and gene encoding LiP have been isolated in another *B. adusta* (Kumuora et al., 1991). *B. adusta* CBS 595.78 degraded ^{14}C – (Lignin) – labeled wheat straw to $^{14}CO_2$ upto 40% (Waldner et al., 1988), which indicated that it had an efficient lignin degrading system. MnP and LiP activities were implicated in the decolorization of partially biodepurated paper waste waters by *P. flavidoalba* FPL 106507, but LiP activity seemed to play a more important role (Perez et al., 1997). Besides, LiP of *P. chrysosporium* led to high levels of olive mill waste water decolorization (Catchesida & Ralph, 1999). Decolorization of synthetic dyes was found to increase linearly with ligninase concentration (Kadam & Drew, 1986).

Molecular analysis of LiP gene have been carried out in *P. chrysosporium* (Stewart et al., 1992), *Phlebia radiata* (Saloheimo et al., 1989), *Coriolus versicolor* (Jonsson & Nyman, 1992) and *Bjerkandera adusta* (Kumuora et al., 1991). The presence of LiP gene in a fungus was the only reliable proof for the presence of LiP so far. LiP and MnP genes from *P. chrysosporium* were expressed in *Aspergillus niger*

whereby the enzymes were secreted into the extracellular medium but the proteins were not found active (Conesa et al., 2000).

Ligninase production by *P. chrysosporium* was increased substantially through improvement of strains and culture parameters. Originally the activities measured in nitrogen starved stationary cultures were low (~ 5 U/L) based on veratryl alcohol oxidation (Tien & Kirk, 1984). Buswell et al. (1984) later measured over 400 U/L in nitrogen sufficient stationary cultures of a new strain, INA-12, grown on glycerol. Ligninase titre was increased by adding lignins or related low-molecular weight aromatics including veratryl alcohol to cultures (Faison & Kirk, 1985; Leisola et al., 1985). Linko et al. (1986) achieved continuous and repeated batch production of upto 245 U/L of ligninase with wild type mycelium immobilized in agar or agarose gels. Further improvements in ligninase production have been obtained with agitated submerged cultures. Gold et al. (1984) first reported ligninase production in such cultures by a mutant strain and Leisola et al. (1985) later demonstrated production of 60 U/L in wild type cultures to which veratryl alcohol had been added. Activities of upto 670 U/L (36 U/mg protein) were obtained in carbon-limited cultures with concentrated mycelial pellets. Jager et al. (1985) found that detergent in the medium permitted wild type strains to produce ligninase and to degrade lignin to CO_2 in agitated submerged cultures while Asther et al. (1987) measured activity of over 1250 U/L with strain INA-12 in a glycerol medium to which oleic acid emulsified with Tween 80 had been added.

1.2.1.1.2. LiP – laccase group: Two fungi, which apparently did not produce MnP activity but readily secreted LiP in their extracellular fluid have been described (Vares et al., 1992, 1993). Under the same cultivation conditions, compared with *Phlebia radiata* 79, L12-41 and *P. tremellosa* 2845, *P. ochraceofulva* produced higher LiP activities and

reached the maximal activity relatively early. LiPs from *P. ochraceofulva* showed multiple forms, five of which were characterized partially (Sarkanen et al., 1991). In addition, the fungus produced laccase while MnP was not produced even in elevated Mn(II) concentrations. *Junghuhnia separabilima* produced 3 laccases, 3 LiPs and a novel heme containing enzyme (Vares et al., 1992).

1.2.1.1.2. MnP-laccase group: Several fungi which are efficient lignin degraders in nature and especially suitable for selective lignin degradation apparently did not produce LiP. This had led to re-evaluation of the role and significance of LiP in lignin biodegradation (Sarkanen et al., 1991). However, there might be many reasons for difficulties in demonstrating LiP activity in a certain fungus, such as the use of unsuitable medium or cultivation conditions. Inductors or elicitors, e.g. veratryl alcohol and other aromatic compounds, strongly regulated the production and profile of different enzymes (Rogalski et al., 1991). The ratio of LiP and MnP production depended in *P. chrysosporium* and in many other fungi on the Mn(II) concentration in the medium (Bonnarme & Jeffries, 1990; Perie & Gold, 1991). An excess of carbon source repressed lignin degradation to $^{14}CO_2$ in *Coriolus versicolor* and in *Pleurotus ostreatus* (Johansson & Nyman, 1987). *Panus tigrinus* (Syn. *Lentinus tigrinus*)(Matlseva et al., 1991), *Dichomitus squalens* (Syn. *Polyporus anceps*) (Perie & Gold, 1991) and *Rigidoporus lignosus* (Galliano et al., 1991) produced MnP and laccase but not LiP. *Lentinula edodes* which readily produced MnP on oak-wood substrate (Forrester et al., 1990) was shown to produce LiP in synthetic medium under low Mn(II) concentrations (Bonnarme & Jeffries, 1990). However, under these conditions, it only degraded 1.5% of [^{14}C] DHP to $^{14}CO_2$ but when supplemented with Mn(II) it mineralized 25% of the lignin (Leatham, 1986). The fungus produced two laccases and MnP or LiP like

peroxidases (Mishra & Leatham, 1990). Veratryl alcohol oxidase (VAO) and other aryl alcohol oxidases (AAOs) were purified from *Pleurotus ostreatus* (Roy-Arcand & Archibald, 1991), *P. eryngii* (Guillen et al., 1992) and *B. adusta* (Muheim et al., 1990) but their role in lignin degradation was not known. A new extracellular peroxidase was characterized in *Bjerkandera* sp. BOS55 (deJong et al., 1992). Peroxidase activities which were not LiP or MnP have been found in *B. adusta*, *D. squalens* and *Pycnoporus cinnabarinus* (Waldner et al., 1988; Nerud et al., 1991).

H_2O_2 producing enzymes: Peroxisome like structures just beneath the cell walls in hyphae might be the in vivo site of the enzyme activity (Fomey et al., 1982). Kelley and Reddy (1986) isolated an intracellular glucose-1-oxidase from *P. chrysosporium* and suggested that the H_2O_2 produced by this enzyme diffused to the extracellular environment. Later, glyoxal oxidase, able to utilize a broad range of substrates such as glyoxal, formaldehyde, glycoaldehyde and methyl glyoxal were discovered. It was also reported that the two substrates that provided the greatest activity, methyl glyoxal and glyoxal were found in the extracellular fluid of cultures of *P. chrysosporium* grown on glucose (Kersten & Kirk, 1987). Thus it appeared that the fungus produced substrates for this enzyme from glucose even when it was nutrient limited.

The most often adopted method to determine lignin degrading ability was to measure $^{14}CO_2$ evolution from [^{14}C] DHP (dehydrogenation polymer of coniferyl alcohol or other lignin precursor alcohol). Apparently, high evolution of $^{14}CO_2$ from ^{14}C-[Ring]-labeled DHP was in good correlation with the production of LiP in cultures of *J. separabilima* and *P. ochraceofulva*. These two fungi which did not

secrete MnP appeared to be very good degraders of DHP (Hatakka et al., 1993). The role of laccase was not known.

It had been argued that LiP was not a prerequisite for lignin degradation in vivo, but it was suggested that LiP might accelerate the conversion of lignin derived preparation to CO_2 (Sarkanen et al., 1991). Perez and Jeffries (1992) concluded that in *P. chrysosporium*, MnP seemed to have a role in initial depolymerization of lignin, mediated by Mn(II)-Mn(III)-organic acid complex, and subsequently more extensive degradation of the lignin oligomers was achieved by LiP. The comparison of enzyme profiles of other white rot fungi supported the concepts. Moreover data also indicated that fungi which readily produced both LiP and MnP were very efficient lignin degraders.

The LiP-laccase group fungi seemed to possess the most inefficient system for lignin biodegradation. *J. separabilima* failed to degrade ^{14}C-[Ring]-labeled DHP to $^{14}CO_2$ unless 50 fold normal level Mn(II) together with a chelator, 10 mM malonate, was supplemented to the medium. Then ~9% was evolved as $^{14}CO_2$ in 36 days (Hatakka et al., 1993). Thus the participation of MnP or other Mn activated enzyme could not be ruled out.

The production of ligninolytic enzymes by selected white rot fungi are given in Table 1.

1.2.1.2. Soft rot fungal lignin degraders: Kadam et al. (1986) isolated the ascomycete *Aspergillus fumigatus* and studied their lignin degradation abilities. It was capable of liberating 38% of the initial radioactivity as $^{14}CO_2$ in comparison to only 6% for *Coriolus versicolor*. *Petriellidium boydii*, *Phialophora mutabilis* and *Chaetomium globosum* removed methoxyl groups as formaldehyde from vanillic and ferulic acids (Ander et al., 1988). Softwood *Pinus radiata* was degraded by the ascomycete *Chrysonilia sitophila* during three months with a lignin loss

of 25% (Ferraz & Duran, 1995). By using *Aspergillus niger* 180, a 69% removal of color from molasses wastewater was obtained after 3 to 4 days in batch process (Miranda et al., 1996). Removal of phenol compounds from olive mill wastewater using *A. niger* and *A. terreus* was reported by Garcia et al. (2000). Uptake of reactive dyes was over 95% within 48 hours of growth of *A. foetidus* (Sumathi & Manju, 2000). Dekkeri and Barbosa (2001) reported of an extracellular laccase constitutively produced by *Botryosphaeria* sp. MAMB-5 when the fungus was grown in basal medium containing veratryl alcohol.

1.2.1.2. Brown rot fungal lignin degraders: A brown rot fungus, *Polyporus ostreiformis*, produced MnP and LiP with peak activity on the sixth day of incubation. It removed 18.6% lignin compared to 54.6% by *P. chrysosporium* from rice straw in three weeks but achieved 99% decolorization of congo red in nine days (Dey et al., 1994). Demethoxylation of lignin model compounds with enzyme extracts from *Gloeophilum trabeum* was reported by Lopretti et al. (1998). Wheat straw cultures of G. striatum and G. trabeum degraded upto 54% of 2,4 - dichlorophenol and 27% of pentachlorophenol on a defined culture medium which lacked carbon, nitrogen and phosphorus sources (Fahr et al., 1999). Biodegradation of Pinus radiata softwood by brown rot fungi, *Wolfiporia cocos* and *Laetiporus sulphoreus*, were compared with that of white rot fungi where the latter were found to be better degraders (Ferraz et al., 2001).

1.2.2. Lignin degrading marine microbes: A number of marine fungi belonged to ascomycetes and deuteromycetes, while basidiomycetes were relatively rarely reported from such marine environments. Studies on lignin degrading enzymes among these fungi are sparse (Cuomo et al., 1987; Rihrmann & Molitoris, 1992). Varying

levels of laccase activities were present in most of the marine fungi isolated from different biotopes. LiPs and MnPs were not so readily produced by these fungi (Quazim, 1999; Raghukumar et al., 1994). Recently Raghukumar et al. (1999) was able to isolate a basidiomycete, *Flavodon flavus*, from coastal marine environment, capable of producing LiP, MnP and laccase; able to mineralize 24% of ^{14}C-labeled synthetic lignin to $^{14}CO_2$ in 24 days and also showed effective degradation of various dye pollutants. The maximum LiP activity reported was 50 U/L, MnP – 600 U/L and laccase – 507 nKatals/L.

1.2.3. Bacterial lignin degraders: Neither rapid nor extensive bacterial degradation, even under highly aerobic conditions, has been reported. Bacterial lignin degradation has been most extensively studied in actinomycetes, particularly *Streptomyces* sp. *S. viridosporus* and *S. setonii* caused losses of 32-44% of the lignin in spruce and maple lignocelluloses (Antai & Crawford, 1981). Fourteen *Streptomyces* strains known to degrade lignocelluloses were able to decolorize three anthrone-type dyes (Pasti & Crawford, 1991) while Perez et al. (1997) reported that *S. viridosporus* strain T7A degraded lignosulphonates to the extent of 10% and 25% respectively for higher and shorter molecular weights. *Nocardia, Pseudomonas* and *Corynebacterium* were able to grow on lignin related phenols, but none was able to degrade lignin (Janshekar & Fiechter, 1982). So till date, no study has shown that lignin is mineralized rapidly or extensively by aerobic bacteria. As in the case of anaerobes, a limiting factor might be the size of the lignin polymer.

Table 1. Production of ligninolytic enzymes by selected white rot fungi

Sl.No.	Fungus	Enzymes produced
1	*Phanerochaete chrysosporium*	LiP, MnP
2	*Phanerochaete flavidoalba*	LiP, MnP
3	*Phanerochaete magnoliae*	LiP, MnP
4	*Phellinus pini*	LiP, MnP
5	*Phlebia subserialis*	MnP
6	*Phlebia tremellosa* 2845	LiP, MnP, Laccase
7	*Phlebia tremellosa* 77-51	LiP, MnP, Laccase
8	*Phlebia ochraceofulva*	LiP, Laccase
9	*Ceriporiopsis subvermispora*	MnP, Laccase
10	*Phlebia brevispora*	MnP, LiP, Laccase
11	*Phlebia radiata* 79	MnP, LiP, Laccase
12	*Phlebia radiata* L12-41	MnP, LiP, Laccase
13	*Phlebia radiata* MJL-1198-Sp.	LiP, MnP
14	*Bjerkandera adusta*	LiP, MnP, Laccase
15	*Panús tigrinus*	MnP, Laccase
16	*Dichomitus squalens*	MnP, Laccase
17	*Coriolus versicolor*	LiP, MnP, Laccase
18	*Lentinula edodes*	MnP, Laccase
19	*Pleurotus ostreatus*	MnP, Laccase
20	*Pleurotus eryngii*	Laccase
21	*Pleurotus sajor-caju*	MnP, Laccase
22	*Rigidoporus lignosus*	MnP, Laccase
23	*Junghuhnia separabilima*	LiP, Laccase
24	*Trametes trogii*	LiP, MnP, Laccase
25	*Flavodon flavus*	LiP, MnP, Laccase

1.2.4. Studies on applications of bioligninolytic systems: The environmental pollutants which could be acted upon by lignin degrading fungi are vast, some of which are given below.

1.2.4.1. Dyes: Glenn and Gold (1983) first reported on the decolorization of several polymeric dyes by *P. chrysosporium*. Experiments with dyes Poly B-411, Poly R-481 and Poly Y-606 indicated that the dyes were substrates for lignin degrading enzymes. Decolorization of Remazol Brilliant Blue R was reported by Ulmer et al. (1984). The decolorization of the dye Poly B-411 (poly-(vinylamine sulfonate)-anthraquinone) and the correlation of color loss with lignin degradation by fungi were investigated by others (Chet et al., 1985). Good correlations between the mineralization of ^{14}C-labeled lignin and decolorization of dyes were established. Decolorization of Poly R-478 was used for screening 170 strains of white, brown-, soft-rot and xylophilous fungi for their peroxidase and phenol oxidase activity. The authors concluded that such a method was not sensitive enough and that no simple relation existed between the presence of MnP or LiP and decolorization of the dyes (Freitag & Morrell, 1992). However, others have shown good correlations between dye decolorization and peroxidase production. The Poly R decolorization activity of three of the 67 new fungal strain isolates were significantly higher than that of *P. chrysosporium* (deJong et al., 1992). Poly R-478 decolorization in packed bed reactor by *P. chrysosporium* was reported by Rodriguez et al. (2000). *P. chrysosporium* decolorized triphenylmethane dyes, including crystal violet, pararosaniline, cresol red, bromophenol blue, ethyl violet, malachite green and brilliant green (Bumpus & Brock, 1988). Cripps et al. (1990) added an entirely new family of dyes, azo dyes, to the long list of organic compounds attacked by *P. chrysosporium*. Azo dyes, the largest class of commercially produced dyes, were not readily degraded

by microorganisms (Wuhrmann et al., 1980). Sulfo and azo groups were not naturally occurring, thus, sulfonated azo dyes were recalcitrant to biodegradation. The susceptibility of the azo dyes to degradation was increased by attaching guaiacyl substituents similar to structures found in lignin (Paszczynski et al., 1991a). Paszczynski and Crawford (1991) reported that veratryl alcohol was involved in the oxidation of some azo dyes by lignin peroxidase. Ollikka et al. (1993) found the dyes belonging to four different groups – polymeric, azo, heterocyclic, and triphenylmethane – were decolorized by three major lignin peroxidase isoenzymes (H2, H7, H8). Although purified enzymes decolorized all dyes investigated, with isoenzymes, veratryl alcohol was required for decolorization in some cases, but not in others. Spadaro et al. (1992) demonstrated that *P. chrysosporium* was capable of mineralizing a variety of azo dyes without sulfo groups and the azo dye Disperse orange 3 with a nitro group. Pasti-Grigsby et al. (1994b) examined a number of azo dyes for their potential use as substrates for assaying LiP and MnP of white rot fungi. Capalash and Sharma (1992) found that both the ligninases of *P. chryosporium* and absorption biomass were responsible for decolorizing 8 out of 18 commercially used dyes. The degradation of methylene blue by a crude extracellular medium of *P. chrysosporium* (Kling & Neto, 1991) and the aerobic biodegradation of the exotic dye Rose Bengal (Gogna et al., 1991) further demonstrated the ability of these enzymes to bring about dye decolorization. Partial decolorization of Orange-G and amaranth, besides complete decolorization of bromophenol blue and malachite green was achieved by *Pycnoporus sanguineus* cultures in submerged liquid culture producing laccase as the sole phenol oxidase (Pointing & Vrijmoed, 2000).

1.2.4.2. Chlorinated organic compounds: 3,4,3',4'-tetrachlorobiphenyl, DDT, 3,4,3',4' - tetrachlorobiphenyl; 2,4,5,2'4',5' -

hexachlorobiphenyl, 2,3,7,8 - tetrachlorodibenzo-p-dioxin, lindane, 3,4 - dichloroaniline and dieldrin could be degraded by *P. chrysosporium* (Bumpus & Aust, 1987a; Morgan et al., 1991). During typical 30 days incubation, mineralization of these compounds to CO_2 was reported to vary in amount from 1% to 15%. In every case, a majority of the starting compound, if not mineralized, was at least transformed. Oxidative dechlorination of aromatic rings catalyzed by lignin peroxidase was shown by Hammel and Tardone (1988), who performed several experiments at pH 3.0, where chlorinated phenols were insoluble but ligninases were active, by emulsifying the substrates with Tween 80. Mileski et al. (1988) used purified ligninase to convert pentachlorophenol (PCP) into 2, 3, 5, 6-tetrachloro-2,5-cyclohexadiene-1,4-dione (TCHD). A silicon membrane biofilm reactor was used to study fungal lignin peroxidase production and PCP degradation (Venkatadri, 1992). The degradation of PCP and pentachloroanisole (PCA) in soil have also been examined (Lamar et al., 1989). Valli and Gold (1991) observing that *P. chrysosporium* degraded 2,4-dichlorophenol by cycles of oxy reduction and methylation, suggested a pathway involving the oxidative dechlorination of 2,4-dichlorophenol to yield 1,2,3,4-tetrahydroxy-benzene, which was then cleaved to produce malonic acid. Batch cultures of *P. chrysosporium* showed complete removal of phenol (500 x 10^{-3} kg/m^3) in 30 hours at 30°C (Manimekalai and Swaminathan, 2000).

Mineralization of polychlorinated biphenyls by *P. chrysosporium* was first reported by Eaton (1985), who investigated the degradation of Aroclor 1254, a recalcitrant mixture of biphenyls chlorinated upto 54% (w/w) and containing 4-7 chlorines per biphenyl molecule. *P. chrsyosporium* mineralized 2,4'-dichlorophenoxyacetic acid (2,4-D) but this might have limited application since pure cultures of bacteria were known to mineralize this faster (Yadav & Reddy, 1993b).

1.2.4.3. Simple and Polycyclic aromatic hydrocarbons: Degradation of benzene, toluene, ethyl benzene, and xylenes (BTEX), a group of common organopollutants derived from gasoline and aviation fuels was observed in work with *P. chrysosporium*. Benzene was reduced by 18%, toluene by 41%, ethyl benzene by 99%, o-xylene by 49% and m-xylene and p-xylene by 67% (Yadav & Reddy, 1993a). Degradation experiments with the polycyclic aromatic hydrocarbon pyrene showed that pyrene-1,6-dione and pyrene-1,8-dione were major products of pyrene oxidation by *P. chrysosporium* (Hammel et al., 1986). At the same time, Hammerli et al. (1986) showed that ligninase was able to oxidize benzopyrene to 1,6-, 3,6- and 6,12-quinones. The oxidation of pyrene was enhanced in the presence of 3,4-dimethoxybenzyl alcohol (Cancel et al., 1993). 22 major components from anthracene oil were degraded by 70-100% during 27 days of incubation (Hammel et al., 1986). An initial pathway for the degradation of phenanthrene in ligninolytic cultures was proposed. The fungus oxidized phenanthrene and phenanthrene-9,10-quinone at their C-9 and C-10 positions to give the ring fission product 2,2'- diphenic acid (Hammel et al., 1992). To determine whether *P. chrysosporium* could effectively operate in an actual field sample of oil contaminated soil, the following study was performed by Brodkorb and Legge (1992). Under aerobic conditions, the native soil flora mineralized 20% of [^{14}C] phenanthrene, but the addition of *P. chrysosporium* enhanced mineralization to 38% in 21 days of incubation. The authors concluded that with further refinement, this system could prove effective for the bioremediation of soil contaminated with polyaromatic hydrocarbon (PAH) compounds. The percentage catabolism of PAH in the soil with *P. chrysosporium* was enhanced to 86% (w/w) when 0.5% Tween 80 washwater was used (Zheng & Obbard, 2000). Biodegradation of PAH by native microflora and combinations of white

rot fungi in a coal tar contaminated soil was reported by Canet et al. (2001).

1.2.4.4. Lignocellulosic materials : Pulps produced by kraft pulping were bleached in part to remove residual lignin. The degradability of toxic chlorinated kraft bleach mill effluents containing high molecular weight chlorolignin and low molecular weight chlorinated organic compounds was investigated with white-rot fungi. Bleach plant effluent from the first alkali extraction stage after chlorine bleaching was decolorized by *P. chrysosporium*. Ligninolytic culture conditions were required for the degradation of bleach plant products (Sundman, 1980). Different reactor designs have been developed, including the mycelial color removal (MyCoR) and related processes. These have been assessed using *P. chryosporium* as the biocatalyst (Messner et al., 1990). Effluent color reduction of up to 80% have been observed in 2 days of operation (Chang et al., 1983). Ferrer et al. (1991) immobilized a type III lignin peroxidase from *Chrysonilia sitophila* and used it to remove color from kraft effluent. The fungal enzyme was only slightly more active than immobilized horse radish peroxidase and about half as active as whole fungal cultures. Only about 30% of the color was removed in 72 hours by the immobilized system. This system appeared to be significantly less promising than similar *Phanerochaete* processes. Bajpai and Bajpai (1994) have compared the ability of different micro-organisms to decolorize pulp and paper waste water. A new pulp biobleaching system involving MnP immobilized in a silica support with controlled pore sizes was developed by Sasaki et al. (2001).

1.2.4.5. Nitro-substituted compounds: The biodegradation of 2,4,6-trinitrotoluene (TNT) by *P. chrysosporium* was first reported by Fernando et al. (1990). TNT was first reduced by the fungal mycelium to amino - dinitrotoluenes and diamino - nitrotoluenes. In the second phase,

reduced TNT metabolites were oxidatively transformed and mineralized (Aken et al., 2001). When a low concentration of TNT (1.3 mg/l) was used, approximately 35% of [^{14}C] TNT was mineralized in liquid culture. Only about 7% was mineralized in soil supplemented with fungal corn cobs after 18 days of incubation. When higher concentrations of TNT were used, approximately 85% of TNT was removed. Plasma membrane redox system might be involved in the reduction of TNT. Toxicity was inversely related to the amount of fungal hyphae present (Stahl & Aust, 1993b). Other research showed that biotreatment of pink water, another waste stream associated with munitions processing, with the white-rot fungus might be a cost effective alternative to carbon absorption. An experiment was described in which *P. chrysosporium* immobilized on a rotating biological contractor was able to remove 99.5% of TNT at 120-175 ppm and RDX at 25 ppm from contaminated water (Sublette et al., 1992). TNT (50 mg/l) removal was 100% for *P. chrysosporium* IFO 31249, *Pycnoporus coccineus*, *Schizophyllum commune* (Kim & Bong, 2000). Immobilized *P. chrysosporium* was able to maintain its viability and transformation ability over a long period of time, with a requirement of a carbon source for optimal transformation (Rho et al., 2001).

1.2.4.6. Modified polymers: Arjmand and Sandermann (1985) demonstrated that *P. chrysosporium* not only degraded pure lignin but also simultaneously degraded chloroaniline bound to lignin. The metabolites formed from wheat lignin containing covalently bound 4-chloroaniline and 3,4-dichloroaniline were mineralized as readily as chloroaniline free lignins. White rot basidiomycetes were able to degrade LPS (lignin-polystyrene) graft copolymers. *P. chrysosporium* degraded 40% of the polystyrene and 80% of the lignin from LPS containing 32% lignin. Production of LiP and MnP was also observed (Milstein, 1992). Incubation of a water soluble fraction of bituminous or

lignite coal with a partly purified preparation of ligninase and MnP resulted in substantial depolymerization of the coal (Wondrack et al., 1989).

1.2.4.7. Other compounds: In a report on the metabolism of cyanide, Shah et al. (1991) showed that *P. chrysosporium* was able to mineralize [^{14}C] KCN to $^{14}CO_2$ and that lignin peroxidase oxidized cyanide to cyanoradical in the presence of H_2O_2. Spores were found to be more sensitive than mycelial cells. Oxidation of halides by lignin peroxidases and their subsequent reduction by EDTA or H_2O_2 was demonstrated (Shah & Aust, 1993).

The importance of lignin degrading fungi and the enzymes involved could be understood from the above reports. In view of this the present study on the lignin degrading enzyme producing marine microbes gains added attention.

1.3. OBJECTIVES OF THE PRESENT WORK

1. Isolation and screening of dye decolorizing micro-organisms from mangrove area.
2. Screening of the selected microorganisms for the production of lignin degrading enzymes.
3. Identification of the potent micro-organisms.
4. Characterization of the crude enzyme, lignin peroxidase, of the selected fungi -- *Aspergillus* sp. SIP 11 and *Penicillium* sp. SIP 10. This included the determination of the optimum pH, temperature, veratryl alcohol and H_2O_2 concentration. Besides the stability of crude LiP at different pHs and temperatures were studied.

5. Submerged fermentation of the selected fungus, *Aspergillus* sp. SIP 11 and the optimization of cultural and nutritional parameters for maximum production of lignin peroxidase.
6. Solid state fermentation of *Aspergillus* sp. SIP 11 and determining the effect of growth conditions on lignin peroxidase synthesis.
7. Purification of lignin peroxidase of *Aspergillus* sp. SIP 11.
8. Determination of the molecular weight of purified lignin peroxidase.
9. Zymogram analysis of the purified lignin peroxidase.
10. Determination of the optimum pH and temperature along with the stability of the purified LiP at different pHs and temperatures.
11. Kinetic studies of the purified LiP by determining the K_m and V_{max} using H_2O_2 and veratryl alcohol as substrates.
12. Dye decolorization studies using the purified lignin peroxidase.

1.4. SCOPE OF THE WORK

1. The immense applications, particularly in bioremediation, to which the lignin degrading micro-organisms could be used make this study important.
2. The ascomycetes and deuteromycetes fungi, especially from the marine environment were least studied with respect to their ligninolytic enzyme system making this study an initial step in unraveling the vast hidden potential of these microbes in bioremediation.
3. The marine microbes are halophilic in nature which make them better suited to cope with the high salinity of industrial effluents thereby giving them added advantage in the field of bioremediation.

Chapter 2

Isolation and screening of lignin degrading enzymes producing micro-organisms from mangrove area

2.1. INTRODUCTION

Mangroves are specialized marine environments which are widely distributed along the Indian coastline. Mangrove leaves consist of about 50% lignocellulosic structural polymers and 50% soluble organics which include tannins and phenolics. They are the major contributors of lignocelluloses in the highly productive mangrove swamp. Marine fungi are important in the degradation of mangrove leaves, wood, seagrasses and other lignocellulosic materials by the production of extracellular enzymes, resulting in the formation of detritus (Benner & Hodson, 1985). There are only a few reports on low level degradation of [^{14}C] labeled lignocellulose by marine fungi (Bergbauer & Newell, 1992; Raghukumar et al., 1996). Moreover, the presence of MnPs, LiPs and laccases in these fungi have been little investigated (Raghukumar et al., 1999, 1994; Pointing et al., 1988). The halophilic nature of these fungi make them better suited for various biotechnological applications. In view of all these, the present study on lignin degrading enzyme producing marine microbes and their enzymatic studies were undertaken.

2.2. MATERIALS AND METHODS

Isolation and screening of lignin degrading enzymes producing microbes were carried out in three stages using methyl orange agar medium, Poly R-478 medium and carbon limited liquid medium.

2.2.1. Primary screening by plate assay: Decayed leaves mixed with soil from mangrove area of Kochi, Kerala State was taken as microbial sources and the samples were suspended in sea water of salinities 15, 25 and 35 g/kg. Suspensions, after serial dilution were spread onto methyl orange dye containing agar plates with different salinities – 15, 25 and 35 g/kg. For bacterial isolation, the composition of the media was (g %) : glucose - 2.0, peptone - 0.5, methyl orange - 0.5, agar - 2.0 at pH 5.0, 7.0 and 10.0. For the isolation of fungal strains a different media of composition (g %): potato extract - 20, glucose - 2.5, methyl orange - 0.5, Penicillin - 10,000 units, Ampicillin - 0.05 at pH 5.0 was used. Suitable pHs were adjusted with the addition of 1N HCl or 1N NaOH. The plates were incubated for two weeks at 30°C (Lynx Lawrence Mayo incubator).

2.2.2. Secondary screening: The cultures selected by primary screening were subjected to secondary screening which was done in two stages as given below.

2.2.2.1. Plate assay: The isolated micro-organisms were spread onto Poly R-478 dye (Sigma Chemical Co.) containing medium, the decolorization of which was associated with ligninolytic activity (Field, 1993; Rodriguez et al., 2000). The media composition used was the same as in the primary screening except that methyl orange was replaced by the same amount of Poly R-478 dye. For bacteria, the pHs used were 5.0 and 7.0 while for fungi the pH was 5.0 at salinities 25 and 35 g/kg.

2.2.2.2. Submerged culture : The selected cultures from the plate assay were grown in Poly R-478 liquid medium of the same composition as used in plate assay. Erlenmeyer flasks (250 ml) containing 100 ml medium of salinity 25 g/kg was agitated at 120 rev. min^{-1} at 30 $\pm$ 2°C for two weeks. In

the case of bacterial isolates, 5% inoculum of 18 hours old culture was used and for fungi, spore suspension giving a spore count of 3 x 10^6 spores/ml was the inoculum used. Uninoculated flasks were kept as control. Samples were taken regularly and biomass estimation was done. The samples were then centrifuged at 10,000 g for 10' (Hettich Zentrifugen EBA 12).

2.2.3. Analytical Methods: The culture supernatant obtained after centrifugation was used for the following analyses.

2.2.3.1. pH : The pH of the culture filtrate was measured using Cyberscan 1000 pH meter.

2.2.3.2. Protein estimation: Protein was determined by Lowry's method (Lowry et al., 1951). The basis of the method was the biuret reaction of proteins with copper under alkaline conditions and the Folin Ciocalteau phosphomolybdic phosphotungstic acid reduction to heteromolybdenum blue by the copper catalyzed oxidation of aminoacids. The protein standard was prepared with Bovine serum albumin.

2.2.3.3. Reducing sugar estimation: This was done by the Dinitrosalicylic acid (DNS) method (Miller, 1959). Reducing sugar was estimated using a standard graph prepared with glucose. On reaction with glucose 3,5 - DNS gets reduced to 3 - amino 5 - nitro salicylic acid while the sugar gets oxidized to gluconic acid.

2.2.3.4. Poly R-478 decolorization: Culture supernatant (1 ml) was diluted with 2 ml of distilled water and absorbance taken at the λ_{max} of Poly R-478 at 520 nm. The dye adsorbed onto the biomass was extracted with 50% methanol and also read at 520 nm. The decolorization was determined as the decrease in absorbance of the sample to the control.

2.2.3.5. Biomass estimation: This was done before centrifugation of the samples. Bacterial growth was estimated by determining the absorbance of the sample at 600 nm. In the case of fungi, the sample was filtered through a filter paper and the paper with the wet biomass was dried in the oven at 101°C for three hours when a constant weight was obtained.

2.2.4. Screening of micro-organisms for the production of enzymes:

The selected isolates were further screened for extracellular enzymes -- lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase -- in carbon limited liquid medium in seawater of salinity 25 g/kg which contained (g %): glucose - 0.3, KH_2PO_4 - 0.5, NH_4NO_3 - 12.5 mM, $MgSO_4.7H_2O$ - 0.1, tween 20 - 0.02, veratryl alcohol - 1mM at pH 5.0.

For the determination of MnP activity, the basal medium was supplemented with $MnSO_4$ (0.05%). In the case of bacterial isolates, 5% inoculum of 18 hours old culture was used and for fungi, spore suspension giving a spore count of 3×10^6 spores/ml was used as inoculum. Growth medium (25 ml) was taken in 100 ml Erlenmeyer flasks with rubber stoppers pierced with glass tubes for aeration. One-third of the flasks were agitated at 100 rev min^{-1} and also aerated for one hour daily. Another one-third were agitated without providing aeration, while the rest were kept stationary. Samples were removed at regular intervals and analyzed for pH and biomass. The cells were separated by centrifugation at 10,000 g, 10', 4°C (HITACHI CR 20B2). The supernatant was used as the source for crude enzymes and for the estimation of reducing sugar and soluble protein.

2.2.4.1. Enzyme assays: Culture supernatants were used for the assay of the following enzymes which was done as pooled duplicates.

2.2.4.1.1. Lignin peroxidase (EC 1.11.1.14) : LiP activity was assayed by measuring the rate of H_2O_2 – dependent oxidation of veratryl alcohol to veratraldehyde (Tien & Kirk, 1988) spectrophotometrically. The standard reaction mixture (2.05 ml) contained 0.8 mM veratryl alcohol in 0.1 M sodium tartrate buffer (pH 3.0) and 1 ml of culture supernatant. The reaction was started by the addition of 150 mM H_2O_2 and the linear increase in absorbance at 310 nm was monitored for one minute at 30°C. One unit of LiP was defined as 1 μmol of veratraldehyde formed per minute and was expressed as U/ml ($\varepsilon = 9300\ M^{-1}\ cm^{-1}$).

$$1\ \text{Unit} = \frac{\Delta E/\Delta t \times V}{\varepsilon \times v \times t}$$

where $\Delta E/\Delta t$ = Increase in absorbance per minute

V = Volume of assay solution in the cuvette

ε = Extinction coefficient

v = Volume of sample

t = time in minutes

2.2.4.1.2. Manganese peroxidase (1.11.1.13): Determination of MnP activity was by the method of Paszczynski et al. (1988) by monitoring the oxidation of Mn^{2+} to Mn^{3+}. The assay solution (3.06 ml) contained 0.1 mM guaiacol and 0.1 mM $MnSO_4$ in 0.1 M sodium tartrate buffer (pH 5.0) with 1 ml of culture filtrate. The reaction was started by 0.1 mM H_2O_2 addition. One unit of enzyme activity was defined as the increase in absorbance at 465 nm per minute ($\varepsilon = 12.1 \times 10^3\ M^{-1}\ cm^{-1}$).

2.2.4.1.3. Laccase (1.10.3.2) : The laccase activity was determined by monitoring the oxidation of 500 μM ABTS (2,2' - azino - di - [3 - ethyl benzothiazoline - 6 - sulphonic acid] Boehringer) buffered with 50 mM sodium succinate buffer (pH 4.5) at 436 nm (Niku-Paavola et al., 1990). The reaction mixture (3 ml) contained 1 ml of culture filtrate. One unit was defined as 1 μM of ABTS oxidized per minute ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$).

2.2.5. Identification of the fungal isolates: Slide cultures of the fungi at different hours of growth were prepared and stained with lactophenol cotton blue. The slides were then examined under high power of microscope (Nikon Alphaphot -2 YS2). Measurements were taken using micrometre and the fungi identified by the criteria of Smith's Introduction to Industrial Mycology (Onions et al., 1981).

2.2.6. Characterization of crude lignin peroxidase: The cell free extract of the fungus was taken as the crude LiP preparation. While studying each parameter, the other reaction conditions were kept constant.

2.2.6.1. Effect of veratryl alcohol concentration on LiP activity: The enzyme activity was assayed at different veratryl alcohol concentrations ranging from 0.2 mM to 1 mM at pH 3.0, temperature 30°C and H_2O_2 concentration of 150 mM.

2.2.6.2. Effect of H_2O_2 concentration on LiP activity: This was analyzed by taking different concentrations of H_2O_2 ranging from 0.3 mM to 300 mM with veratryl alcohol concentration of 0.8 mM at pH 3.0 and temperature 30°C.

2.2.6.3. Effect of pH on LiP activity: Different reaction pHs 2.0 to 5.5 at temperature 30°C were used to determine the optimum pH for activity.

2.2.6.4. Effect of temperature on LiP activity: The assay was done at different reaction temperatures of 30°C to 45°C at pH 3.0.

2.2.6.5. Stability studies: The stability of the crude enzyme at different pH and temperature were studied as given below.

2.2.6.5.1. pH: In order to study the effect of pH on the stability of LiP, the enzyme was incubated in 0.1 M sodium tartrate buffer (pH 2.0 to 4.0), 0.1 M sodium acetate buffer (pH 5.0) and citrate phosphate buffer (pH 6.0 & 7.0) at 30°C for two hours. At specified intervals aliquots of the enzyme was taken and LiP activity was determined.

2.2.6.5.2. Temperature: For determining the stability of LiP at different temperatures, the crude enzyme was incubated at temperatures between 10°C and 60°C at pH 3.0 for two hours. Residual activity was determined at regular intervals as in the case of pH.

2.3. RESULTS AND DISCUSSION

2.3.1. Primary Screening of micro-organisms: During primary screening using methyl orange agar plates, 75 bacteria and 26 fungi which showed varying levels of decolorization of methyl orange were isolated at pH 5.0 and 7.0 for bacteria and at pH 5.0 for fungi. These micro-organisms showed good growth at salinities 25 g/kg and 35 g/kg. Besides the decolorization of methyl orange was best under these salinities compared with that at 15 g/kg salinity where there was little decolorization. By primary screening, 22

bacteria and 5 fungi which showed decolorization of methyl orange at pH 5.0 and salinities 25 g/kg and 35 g/kg were selected for secondary screening.

2.3.2. Secondary screening of micro-organisms:

2.3.2.1. Plate assay: Of the micro-organisms subjected to secondary screening in Poly R-478 agar medium two fungal strains, SIP 11 and SIP 10, showed decolorization to some extent. SAP 9 was the only bacterium to decolorize the dye but not to the same extent as that of the fungi. So these micro-organisms were selected for submerged culture and enzymatic studies. The decolorization was also comparatively better at salinity 25 g/kg, which was used in further studies. The components of seawater of salinity 25 g/kg are given in Table 2a & 2b.

2.3.2.2. Submerged culture: When the selected micro-organisms - SAP 9, SIP 10 and SIP 11 - were cultured under submerged condition with the addition of Poly R-478 dye, the bacterial isolate SAP 9 showed 8% decolorization after 4 days whereas the fungal strains, SIP 10 and SIP 11, showed 28% and 39% decolorization respectively after 8 days (Fig. 5). Further period of incubation did not bring about increased decolorization. These three microbes were then screened for the presence of extracellular ligninolytic enzymes by culturing in carbon limited liquid medium.

The decolorization of methyl orange agar plates, Poly R-478 agar plates and Poly R-478 liquid culture by the fungal isolates, SIP 11 and SIP 10, are given in Plates III, IV and V respectively.

2.3.3. Screening of micro-organisms for enzyme production: MnP and laccase were not detected in the culture supernatant even after 10 days of incubation. In the case of LiP, there was little or no LiP activity during the initial growth phase, while peak enzyme productivity was obtained after 5

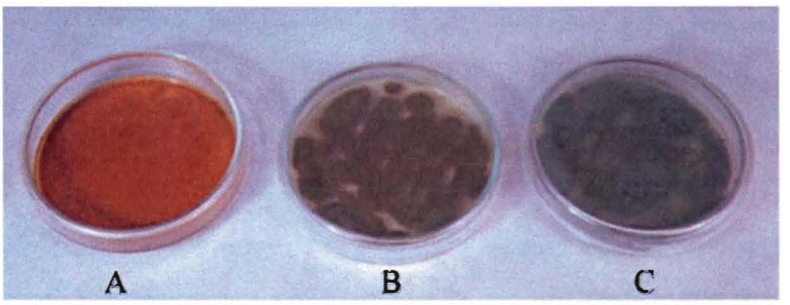


Plate III Decolorization of methyl orange agar plates

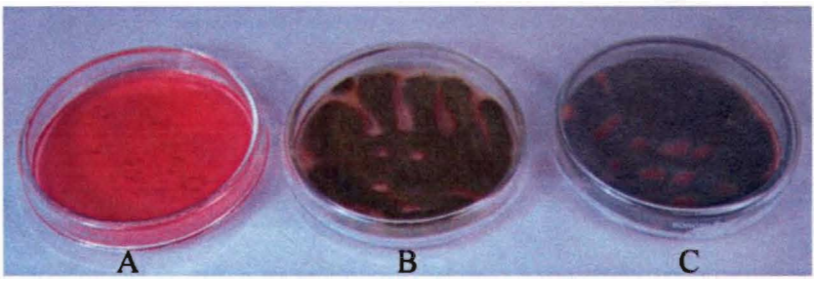


Plate IV Decolorization of Poly R-478 agar plates

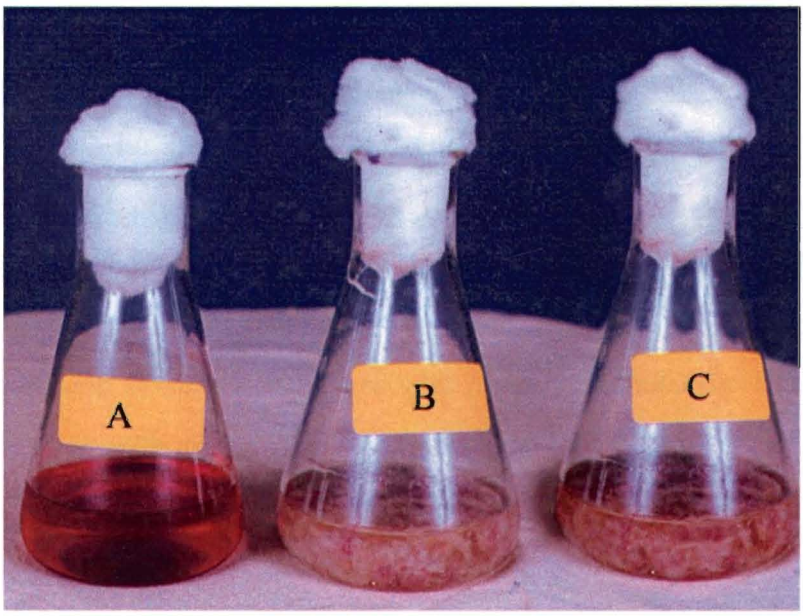


Plate V Decolorization of Poly R-478 in submerged culture

A - Control B - SIP 11 C - SIP 10

days for SAP 9; 8 and 10 days respectively for SIP 10 and SIP 11. Only 47%, 57% and 66% of the activity obtained in the aerated and agitated condition were produced when only agitation was provided to the culture medium. Comparatively low activity was obtained under stationary condition thereby necessitating an aerated and agitated atmosphere for LiP production by these microbes. The screening results of the LiP producing microbes is given in Table 3. Kirk et al. (1978) had reported of the requirement of oxygen for enhanced lignin degradation. Though the bacterium, SAP 9 gave LiP activity in 5 days, the enzyme production was much lower compared to the fungi.

Of the two fungi, SIP 10 produced only 37% of the activity as by SIP 11, though two days in advance. SIP 11 gave 67 U/ml activity on the tenth day of incubation compared to 21 U/ml by SIP 10 as shown in Fig. 6. It was noticed that low amounts of extracellular LiP occurred during the logarithmic phase of growth, while maximum activity was shown during the idiophase, on the tenth day of incubation, following which there was a decrease in activity. According to Cancel et al. (1993) the decrease in ligninase activity was correlated with the appearance of extracellular protease activity; the degradation of lignin and appearance of ligninase activity were both associated with nutrient limitation in the media leading to secondary (idiophase) metabolism in the agitated cultures.

LiP activity was correlated with pH change, growth, reducing sugar and soluble protein which showed a similar pattern for both the fungi (Fig. 7). Maximum growth of the fungi was reached on the second day itself showing the faster growth potential of the isolates. Soluble protein levels exhibited an initial increase and became low when peak enzyme activity was obtained, giving thereby a high specific activity for the enzyme (268 U/mg

protein for SIP 11). The reducing sugar was exhausted within three days while the pH of the culture medium showed an initial decline but later increased to nearly the initial pH of 5.0. In the absence of 1 mM veratryl alcohol in the medium, the peak LiP activity was delayed to the eleventh day and it was only 15% of the activity produced with added veratryl alcohol (Fig. 8). So a seven fold increase in activity resulted when 1 mM veratryl alcohol was added to the medium. It was reported earlier that a five fold stimulation was expressed in cultures following the addition of veratryl alcohol, which is a secondary metabolite produced by *P. chrysosporium* and related fungi in response to nitrogen limitation. Besides it was a substrate for ligninase (Faison & Kirk, 1985; Leisola et al., 1984). Two possible mechanisms were suggested by which veratryl alcohol induced an increase in enzyme activity. First, a protective effect might stabilize the enzyme against inactivation or proteolytic decay. These compounds would thus counteract with the rapid decline in ligninase activity observed in aging cultures. Such a protective effect would be independent of protein synthesis. Second, a true increase in ligninase activity may be caused by an effect on the amount or type of ligninase(s) produced and this mechanism would require de novo protein synthesis (Faison et al., 1986).

2.3.4. Identification of fungal isolates – SIP 10 and SIP 11: SIP 11 colonies were olive green, the mycelium was septate and 4 μm in diameter. Conidiophores arose from specialized thick walled enlarged mycelial cells (foot cells), was non-septate and enlarged towards the apex terminating in a club shaped vesicle. The vesicle bore from the upper part phialides and conidia were produced from the tip of the phialides forming chains. The

conidia were globose with diameter of 4 μm. The fungus was identified as belonging to the genus *Aspergillus* (Plate VI).

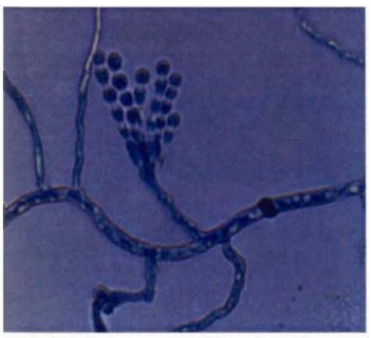
Vegetative mycelium of SIP 10 was dark green, septate and 4 μm in diameter. Conidiophores terminated in a broom like whorl of branches (the penicillus) and was terverticillate. The conidia were globose and green. It was identified as *Penicillium* sp. (Plate VII).

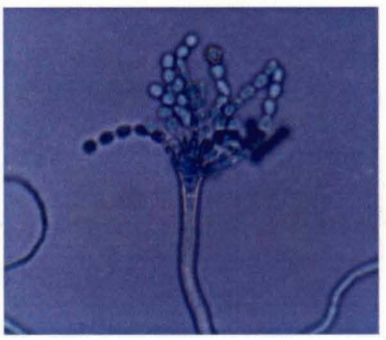
Both these fungi belonged to Class Ascomycetes, causing soft rot of wood, which was the predominant class of fungi in mangrove areas.

2.3.5. Characterization of LiPs from *Aspergillus* sp. SIP 11 and *Penicillium* sp. SIP 10:

2.3.5.1. Effect of veratryl alcohol concentration on LiP: The enzyme activity showed a linear increase with veratryl alcohol concentration upto 0.8 mM for *Aspergillus* sp. SIP 11 and 1 mM for *Penicillium* sp. SIP 10 (Fig. 9). According to Tien and Kirk (1988) 1 mM veratryl alcohol was found to be the saturation level for LiP from *P. chrysosporium* which was similar to that obtained by *Penicillium* sp. SIP 10 but higher than for *Aspergillus* sp. SIP 11.

2.3.5.2. Effect of H_2O_2 concentration on LiP: No activity was shown for lower H_2O_2 doses of 0.3 mM, 1 mM, 6 mM and 40 mM H_2O_2, the enzyme being active at a H_2O_2 dose of 150 mM (Fig. 10). Earlier reports indicated that excess hydrogen peroxide resulted in the conversion of the active enzyme into the inactive compound III with subsequent loss of catalytic activity (Cai et al., 1989). It was found that very high doses (> 200 mM) of H_2O_2 resulted in inactivation of LiP from both the fungal strains.





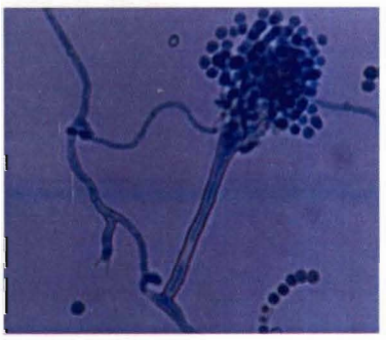


Plate VI Light micrographs of *Aspergillus* sp. SIP 11 (40 X x 10 X)

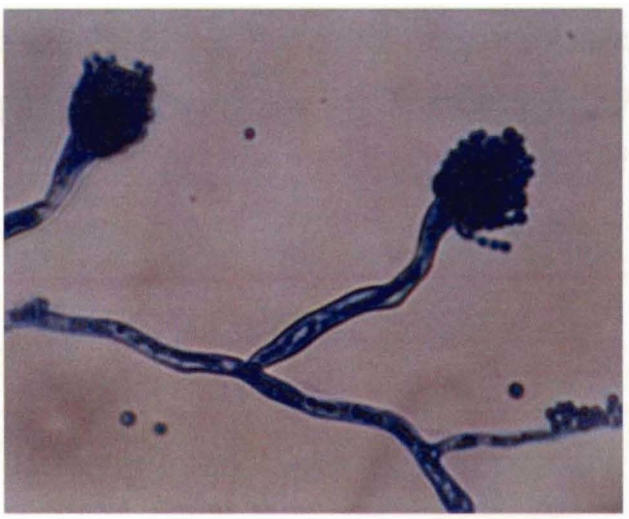


Plate VII Light micrograph of *Penicillium* sp. SIP 10 (40 X x 10 X)

2.3.5.3. Effect of pH on LiP : Optimum lignin peroxidase activity was obtained at pH 3.0. With increase in pH, there was a decrease in activity with complete inactivation at pH 5.0 (Fig. 11). One of the unique features of this enzyme was its very low pH optima. Increase in pH resulted in the disruption of the hydrogen bond formed between the heme propionate and aspartic acid residue in the active site of the enzyme, thereby inactivating it (Edwards et al., 1993). Studies by Raghukumar et al. (1999) and Tien & Kirk (1984) on *Flavodon flavus* and *P. chrysosporium* respectively have confirmed the acidic pH range of LiP.

2.3.5.4. Effect of temperature on LiP : LiP had an optimum activity at the assay temperature of 30°C. At 35°C, 76% of the activity was retained while further increase in temperature resulted in drastic decrease in activity (Fig. 12). This showed that higher temperatures resulted in gradual inactivation of LiP.

2.3.5.6. Stability studies with crude LiP:

2.3.5.6.1. pH: LiP was stable for two hours at pH 2.0 to 5.0. At pH 6.0, there was a gradual loss in activity with time and after one hour no residual activity was detected. At pH 7.0, the activity was lost immediately (Fig. 13). Earlier reports on LiP stabilities at different pHs from *P. chrysosporium* suggested that though higher enzyme activities were expressed at low pHs, stability at this pH was low and it increased as the pH was also increased (Tuisel et al., 1990). But the LiP from the fungal isolates was stable at low pH when maximum enzyme activity was expressed.

2.3.5.6.2. Temperature: The enzyme was stable for two hours over a wide range of temperatures, 20°C to 60°C. At 70°C, there was a gradual loss in activity with complete loss in two hours time (Fig. 14). Tuisel et al. (1990) had shown that the LiP of *P. chrysosporium* retained 80% of its initial activity upto 50°C for 48 hours, and at 60°C activity was lost after 6 hours.

The LiPs produced by both the ascomycetes, *Aspergillus* sp. SIP 11 and *Penicillium* sp. SIP 10, showed similar characteristics in all respects except in the concentration of veratryl alcohol required in the assay reaction mixture. *Aspergillus* sp. SIP 11, which was best with regard to the LiP production of 67 U/ml on the tenth day of incubation was, therefore selected for further studies.

Table 4a. Major components of seawater (25g/kg) in the medium

Component	Concentration (mg/kg)
Calcium	294
Magnesium	924
Nitrogen	11
Potassium	285
Silicon	2
Sodium	7685
Strontium	6
Bicarbonate	104
Boron	3
Bromide	48
Chloride	13821
Fluoride	1
Sulphate	1937

Table 4b. Few minor components of seawater (25g/kg)

Component	Concentration (µg/kg)
Iron	1.4
Zinc	2.3
Copper	~0.2
Manganese	~0.2
Nickel	~0.2
Cobalt	~0.02

Table 5. Screening results of LiP producing microbes

Microbe	Period of maximum enzyme activity (day)	Growth characteristics and enzyme production of cultures at the day of maximum enzyme activity					
		pH	Dry weight in mg/ml	Soluble protein in mg/ml	LiP activity (U/ml)		
					Aer. & Agi.	Agi.	St.
SAP 9	5	4	2	0.5	6	4	2
SIP 10	8	4	6	0.3	21	12	8
SIP 11	10	5	5	0.2	67	32	10

Aer. & Agi. --- Aeration and agitation

Agi. --- Agitation only

Aer. --- Aeration only

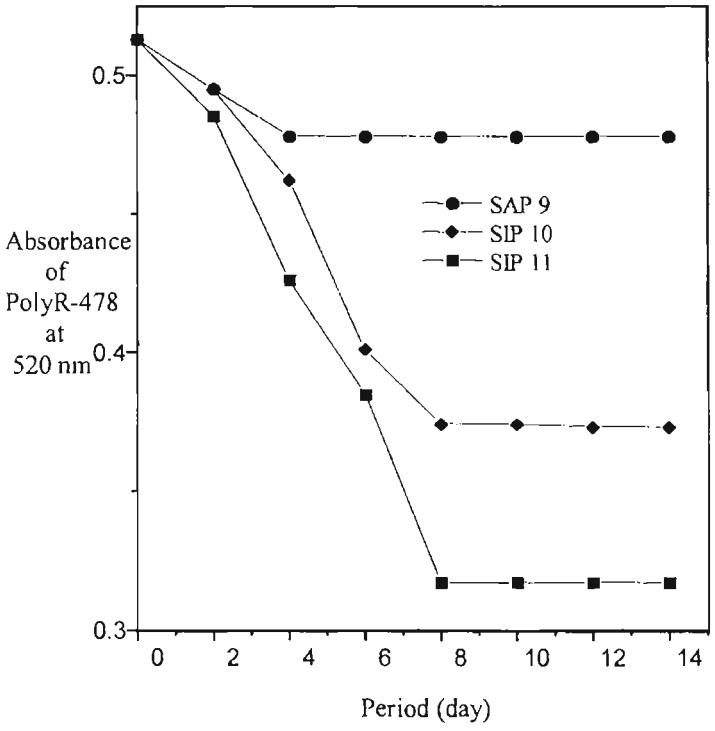


Fig. 5 Decolorization of Poly R-478 dye by the microbial isolates - SAP 9 (bacterium) and SIP 10 and SIP 11 (fungi) under submerged fermentation

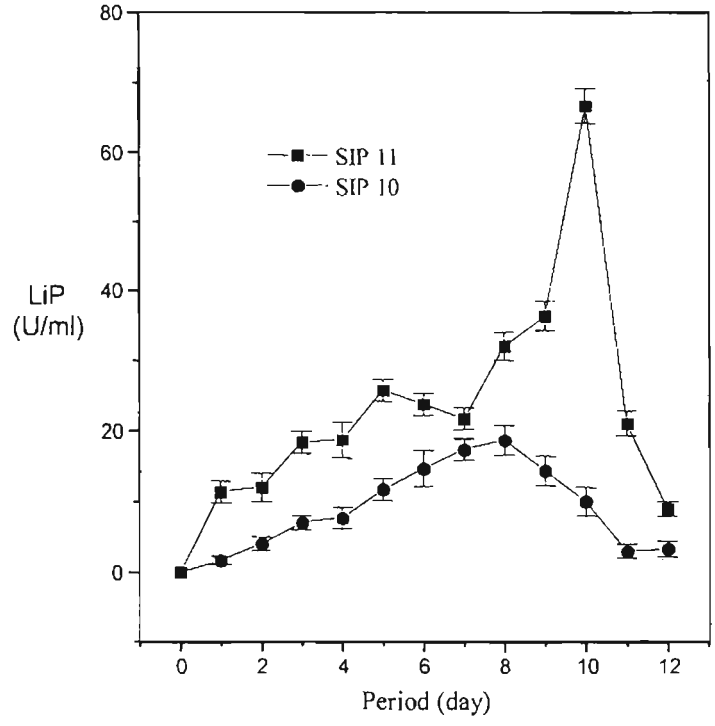


Fig. 6 LiP production by the fungal isolates -- SIP 10 and SIP 11 -- in carbon limited liquid medium with 1mM veratryl alcohol

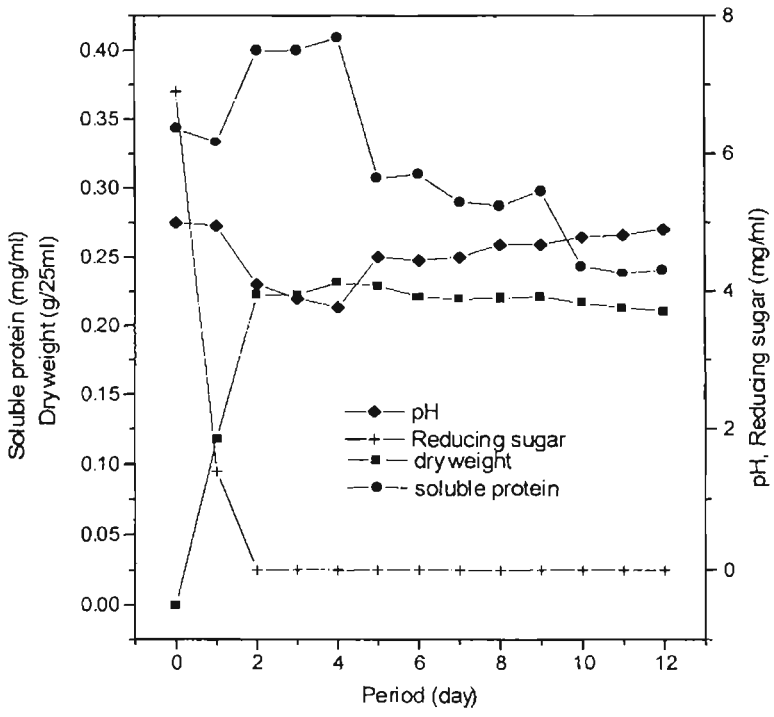


Fig. 7 Growth profile of the fungal isolates -- SIP 10 and SIP 11 -- under submerged fermentation in carbon limited medium

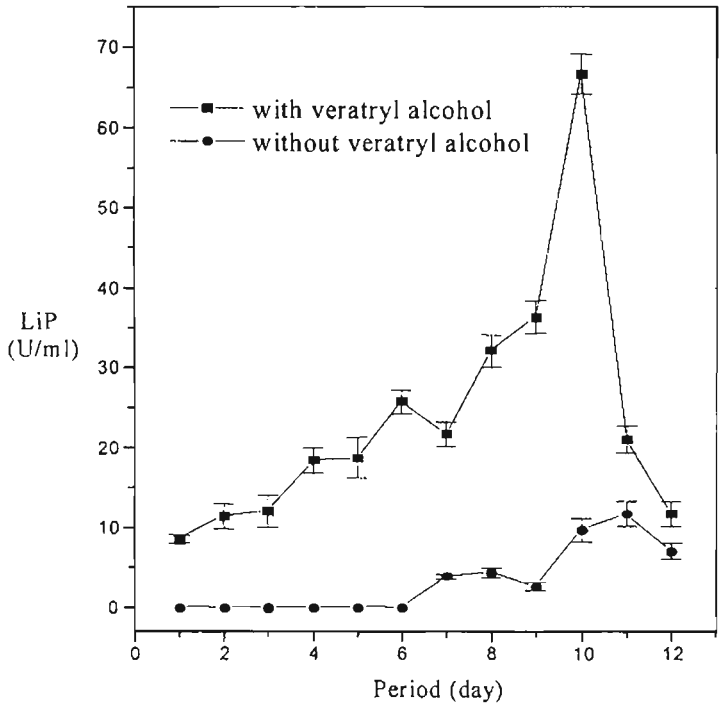


Fig. 8 Influence of veratryl alcohol on LiP production by *Aspergillus* sp. SIP 10

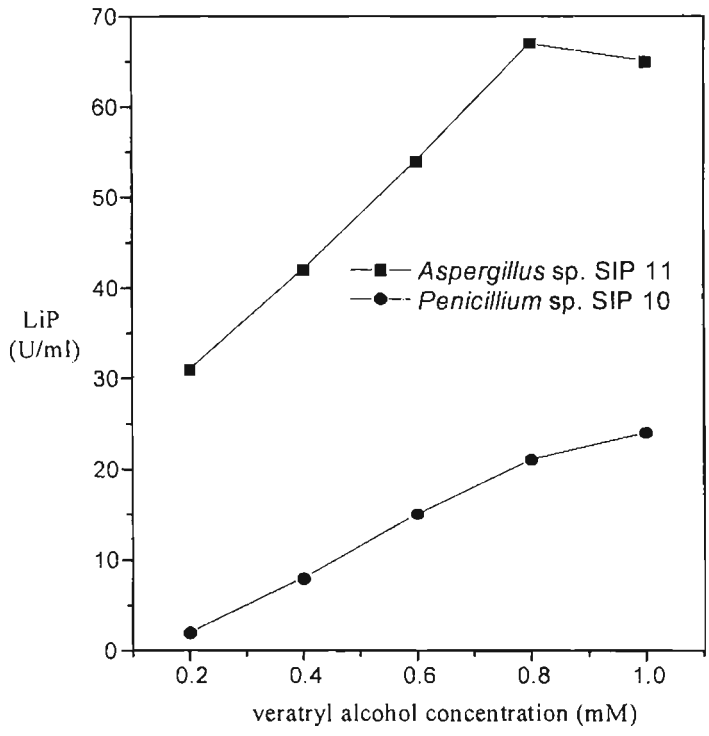


Fig. 9 Effect of veratryl alcohol concentration on LiP activity of *Aspergillus* sp. SIP 11 and *Penicillium* sp. SIP 10

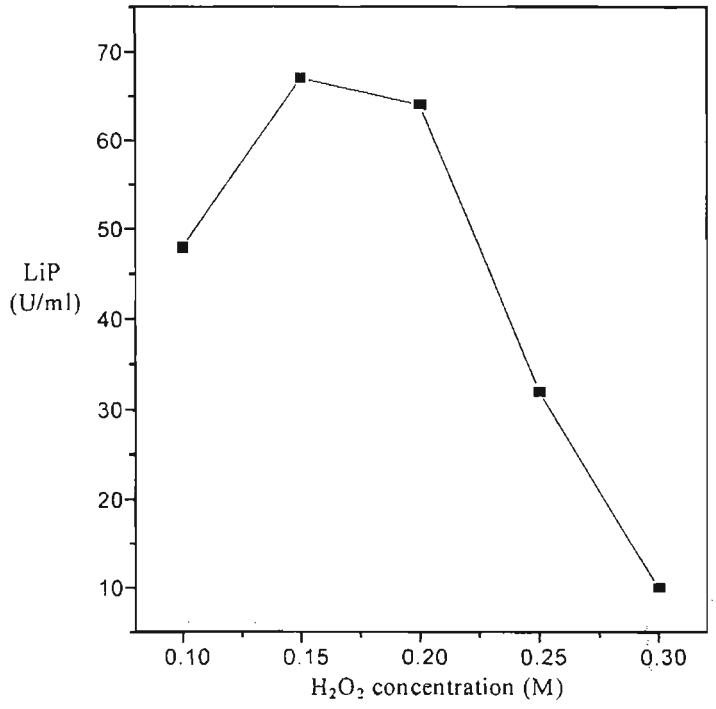


Fig. 10 Effect of H_2O_2 concentration on LiP activity of *Aspergillus* sp. SIP 11 and *Penicillium* sp. SIP 10

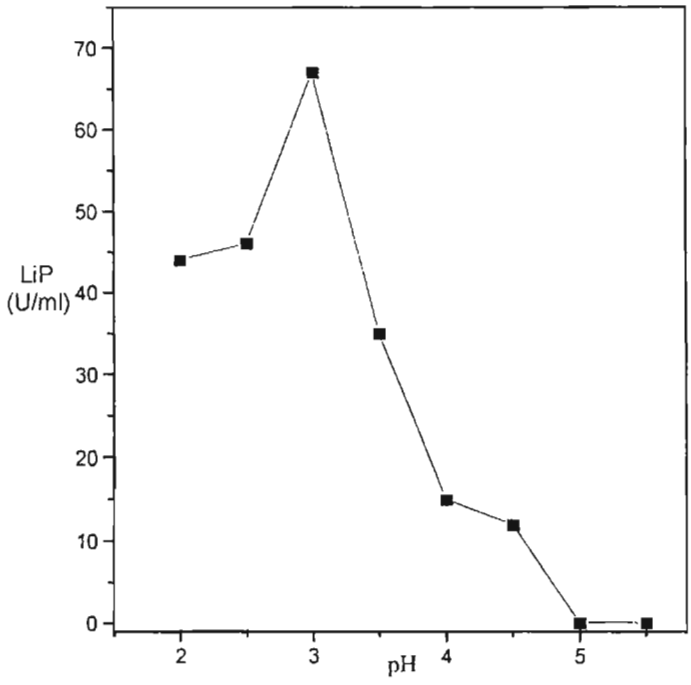


Fig. 11 Effect of pH on LiP activity of *Aspergillus* sp. SIP 11 and *Penicillium* sp. SIP 10

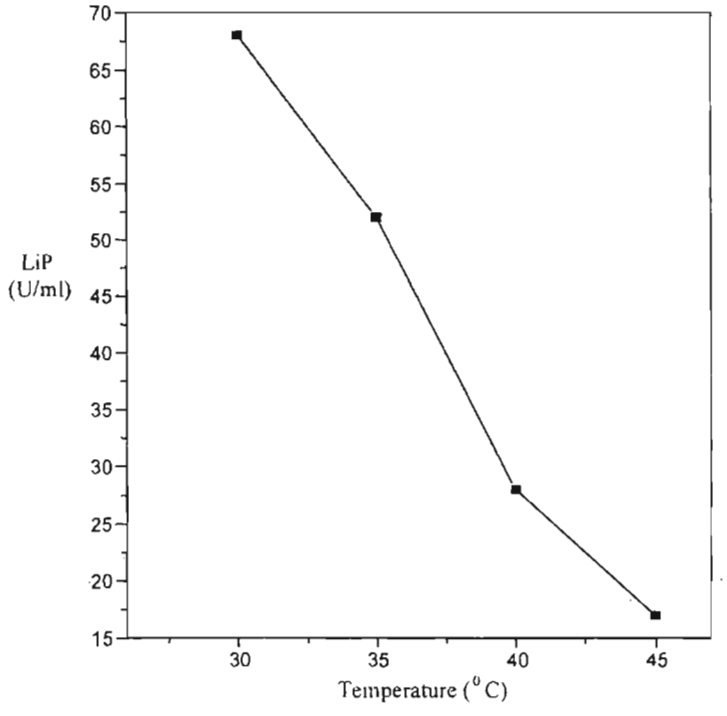


Fig. 12 Effect of temperature on LiP activity of *Aspergillus* sp. SIP 11 and *Penicillium* sp. SIP 10

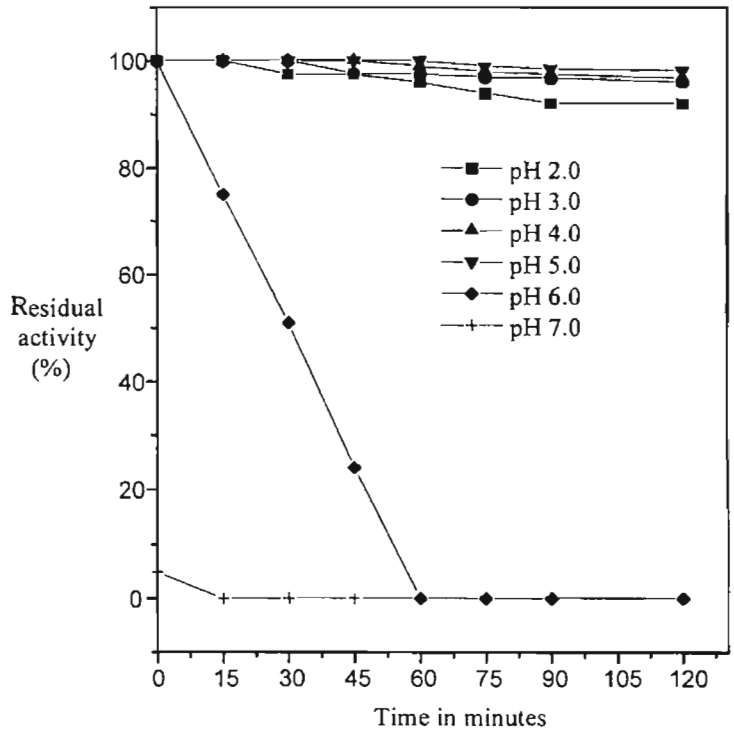


Fig. 13 Stability of LiP at different pHs

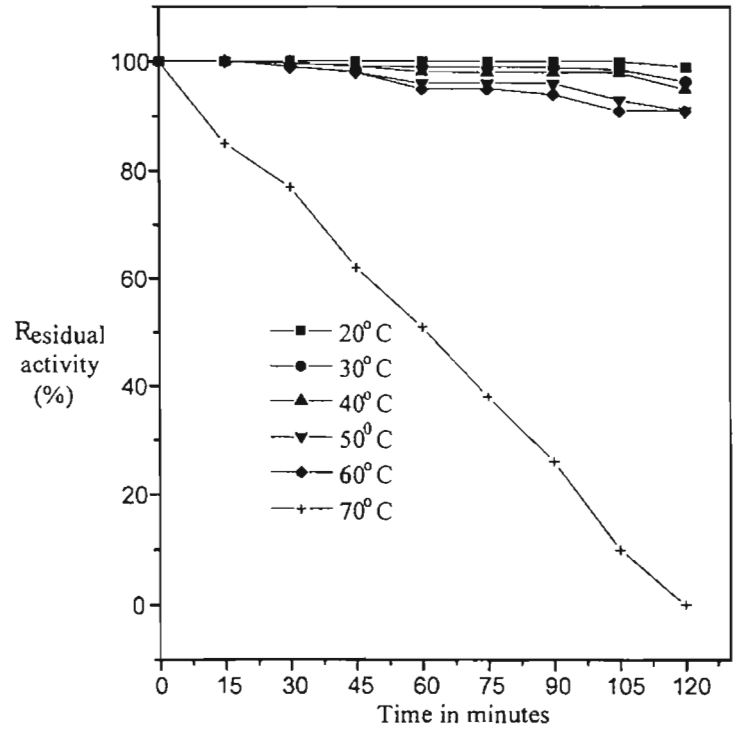


Fig. 14 Stability of LiP at different temperatures

Chapter 3

Influence of culture parameters and media nutrients on LiP production in submerged fermentation by *Aspergillus* sp. SIP 11

3.1. INTRODUCTION

During the 1980's LiP production was mainly studied using complicated synthetic media based on the composition originally designed by Kirk et al. (1978) for lignin degradation studies by the white rot fungus *P. chrysosporium* (Linko, 1992). The enzyme was excreted as a secondary metabolite typically under carbon or nitrogen limitation. The role of phosphorus was not yet clear, except that LiP production did not seem to be triggered by phosphorus limitation (Jeffries et al., 1981). Because of the dependence of the ligninolytic system on nutrient limitation, most studies on the production of lignin degrading enzymes by *P. chrysosporium* were performed in unbalanced media, usually with nitrogen limitation (Faison & Kirk, 1985; Dosoretz & Grethlein, 1991). The metal salt concentration, vitamin and trace element composition of the media were varied quite considerably. Mainly glucose or glycerol was used as carbon source (Linko, 1992). Already in their earlier work Kirk et al. (1978) emphasized the necessity of pH control in lignin degradation. In addition to the importance of environmental conditions such as pH, temperature, activators, agitation and aeration, nutritional aspects played a key role in LiP production by *P. chrysosporium*. Certain trace elements and cations, and their relative ratios, were shown to strongly affect ligninolytic activity (Kirk et al., 1986; Leatham, 1986). The expression of ligninolytic enzymes by *P. chrysosporium* was highly dependent on culture conditions and medium composition (Dosoretz & Grethlein, 1991; Kirk & Farrell, 1987).

A balanced fermentation medium was critical for good growth and enzyme yields. The carbon limited medium used for the initial production of LiP by *Aspergillus* sp. SIP 11 has to be modified for enhanced production

of LiP. So the present study was undertaken for the optimization of cultural and nutritional parameters for LiP production from *Aspergillus* sp. SIP 11 in submerged fermentation.

3.2. MATERIALS AND METHODS

The effect of different parameters on LiP production was studied individually, by varying one factor at a time. At each step, the selected factor was included in the control medium for getting a set of conditions that enabled maximum production of LiP from *Aspergillus* sp. SIP 11. In all cases, samples were taken regularly and analyzed for pH, protein, reducing sugar, biomass and LiP activity as described in Section **2.2.3.** and **2.2.4.1.**

3.2.1. Effect of temperature on LiP activity: *Aspergillus* sp. SIP 11 was grown in carbon limited medium at varying temperatures ranging from 25°C to 40°C to ascertain its influence on LiP production.

3.2.2. Effect of pH on LiP activity: In order to study the effect of initial pH of the culture medium on LiP production, the fungus was grown in the basal medium with initial pH varied between 3.0 and 5.5.

3.2.3. Effect of agitation and aeration on LiP production: The culture medium inoculated with the fungus was agitated at different speeds of 0 to 200 rev. min^{-1} to determine the optimum agitation required for LiP production. Due to the requirement of aeration by this ascomycete, the aeration period per day was varied between 0 and 5 hours to find out the optimum aeration for maximum LiP activity.

3.2.4. Effect of inducers on LiP activity: Various compounds which were known to stimulate LiP production was added to the culture medium individually by replacing veratryl alcohol at the time of inoculation . These were alkali extracted lignin from coir (0.02%), humic acid (0.02%), veratryl alcohol (0 to 1.2 mM) and tryptophan (0 to 1.2 mM).

3.2.5. Effect of carbon sources on LiP activity: Glucose (0.3%) in the basal medium was replaced by various carbon sources like fructose, glycerol, sucrose and starch.

Glucose, which was found to be the best carbon source for LiP production, was studied at different levels (0.1% to 1.5%) for understanding the optimum concentration required for maximum LiP activity.

3.2.6. Effect of lignocellulosic substrates on LiP activity : Various lignocellulosic wastes like wheat bran, coir pith, rice bran, sawdust, bagasse, pigeon pea hull and rice straw were taken at 1% level (w/v) and incorporated into the medium in the absence of 1 mM veratryl alcohol.

As coir pith was found to be the best lignocellulosic substrate, different concentrations (0.25 to 3%) were added to the medium for maximization of LiP production.

3.2.7. Effect of nitrogen sources on LiP activity: Ammonium nitrate (12.5 mM), which was used in the basal medium, was replaced by various inorganic nitrogen sources - $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, NH_4Cl and ammonium tartrate - and organic nitrogen sources at 1% (w/v) - peptone and yeast extract.

$(NH_4)_2SO_4$ which was found to be the best nitrogen source was tried at different levels (2 mM to 20 mM) in the medium to ascertain the optimum level for maximum activity.

3.2.8. Effect of phosphorus sources on LiP activity: Different phosphorus sources - NaH_2PO_4, K_2HPO_4 and Na_2HPO_4 - at 0.5% level replaced KH_2PO_4 in the basal medium.

As KH_2PO_4 was found to be the best phosphorus source, varying concentrations (0.05 to 0.5%) was added to the medium to understand its effect on LiP production.

3.2.9. Effect of salinity and metal ions on LiP activity: As *Aspergillus* sp. SIP 11 was a halophilic fungus, the influence of salinity of the medium on LiP production was determined by preparing the medium in seawater of salinities 5 g/kg to 35 g/kg. Besides the influence of individual metal ions on LiP production was also studied by adding 1 mM of different metal ions (KCl, $MnCl_2$, $CaCl_2$, $CuCl_2$, $FeCl_3$, $ZnCl_2$, $CoCl_2$, $NiCl_2$) to the medium.

To determine whether the metal ion Mg^{2+} as $MgSO_4.7H_2O$ already present in the basal medium was absolutely necessary for enhanced LiP production, varying concentrations of this salt (0.05 to 0.15%) was added to the medium and its effect studied.

3.2.10. Effect of vitamins on LiP activity: The necessity of vitamins for *Aspergillus* sp. SIP 11 was determined by adding 0.2 mg/l of thiamine hydrochloride and biotin individually and in combination.

3.3. RESULTS AND DISCUSSION

3.3.1. Effect of temperature on LiP activity: The optimum culture temperature was found to be 30°C. When the temperature was increased to 35°C, the activity was reduced by 18% while further increase of 5°C led to 66% loss in activity (Fig. 15). The optimum growth temperature for *P. chrysosporium* was 39°C (Tien & Kirk, 1988), for *Phlebia radiata* it was 28°C (Lundell et al., 1990) and for immobilized *Bjerkandera adusta*, 30°C (Nakamura et al., 1999). The slow growth of the fungus at low temperatures and the inactivation of the enzyme at higher temperatures were likely to be the reasons for the increased LiP titres at the optimum temperature of 30°C.

3.3.2. Effect of pH on LiP activity : The initial pH of the culture medium was found to have a profound influence on the LiP activity of *Aspergillus* sp. SIP 11. With decrease in pH, early and higher production of LiP was noticed. At the optimum pH of 4.0, 94 U/ml of LiP was obtained on the seventh day of incubation. This was 40% higher than at pH 5.0, when LiP peak was reached only on the tenth day (Fig. 16). Below a pH of 4.0 and above 5.5, no LiP activity was detected in the extracellular medium. This was in accordance with the results obtained by Kirk et al. (1978) using *P. chrysosporium* and Nakamura et al. (1999) with *Bjerkandera adusta.*

3.3.3. Effect of agitation and aeration on LiP activity: When the agitation speed was lowered from 100 rev. min^{-1} to 80 rev. min^{-1}, 18% increase in activity to 111 U/ml was observed. Further decrease in agitation led to drastic reduction in activity which might be due to the non-availability of oxygen. This decrease was to the tune of 63% at 50 $rev.min^{-1}$ (Fig. 17). Leisola and Fiechter (1985) reported that in the agitated system ligninase

activity was twice and the absolute amount 15 times higher than in non-agitated system with 1 mM veratryl alcohol addition. The presence of the detergent, Tween 20 in the culture medium for the growth of *Aspergillus* sp. SIP 11 might have helped in enhanced LiP production under agitated condition as reported by Jager et al. (1985) in *P. chrysosporium*. Tween 20 could probably supply fatty acid (lauric acid) to the cultures but the basis for its beneficial effect have apparently not been clarified. Enhancement of polyaromatic hydrocarbon elimination by *P. chrysosporium* was noted in the presence of Tween 80 surfactant due to increased bioavailability (Zheng & Obbard, 2001). But too high agitational speed lead to substantial decrease in activity. At 200 rev. min^{-1}, the LiP activity obtained was less than that for stationary cultures. This might be due to the shear stress suffered by the mycelia which was detrimental to the growth and LiP production by the fungus.

Oxygen was necessary for LiP production and the period of aeration per day, with an oxygen content of 21%, resulted in increase in LiP titre with the maximum obtained with four hours of aeration (122 U/ml). Higher period of aeration resulted in a slight decrease in activity due to the high oxygen tension caused by increased aeration (Fig.18). Lignin decomposition and LiP production began after the initial growth period at 21% and 100% oxygen and not under 5% oxygen according to Kirk et al. (1978). According to Bar-Lev & Kirk (1981), increasing the oxygen level in the medium had a multiple effect. It led to an increase in the titre of the ligninolytic system and the H_2O_2 producing system. Moreover, it increased the activity of the existing lignin-degrading system, evidently by increasing the supply of oxygen for degradative reactions and for H_2O_2 production.

Aeration of cultures of *Botryosphaeria* sp. MAMB-5 led to 4- to 5- fold increase in laccase activity as reported by Dekkeri and Barbosa (2001).

3.3.4. Effect of inducers on LiP activity: With the addition of lignin and humic acid, no enhancement of LiP production occurred. Only 10 U/ml of LiP activity was obtained, as in the case when veratryl alcohol was not added to the medium. Keyser et al. (1978) reported that the ligninolytic system was induced in the absence of lignin while later workers (Ulmer et al., 1984) have suggested an apparent induction of the total ligninolytic system after incubation with 0.02% of dioxane-HCl lignin from wheat straw. An increase in ligninolytic enzymes, specifically laccase was reported during the decolorization of coal derived humic acid by *Trametes versicolor* (Fakoussa & Frost, 1999). In the case of veratryl alcohol as inducer, a concentration of 1 mM which was used in the basal medium was found to be the optimum level. Even in the presence of 1 mM tryptophan only 15 U/ml of LiP activity was obtained which was not much better than in the absence of veratryl alcohol. Higher concentration did not result in any further increase in activity (Fig. 19). The endogenous levels of veratryl alcohol were generally highly correlated with LiP titres. LiP isozymes were known to be rapidly inactivated by physiological levels of H_2O_2 and veratryl alcohol was known to protect LiP from this kind of inactivation (Cancel et al., 1993; Valli et al., 1990). Collins et al. (1997) reported of the large stimulatory effect on LiP activity levels by supplementation of various cultures of white rot fungi with tryptophan. This enhancement was greater than that observed in the presence of veratryl alcohol and was due to its protective effect against H_2O_2 inactivation. But in the case of *Aspergillus* sp. SIP 11, tryptophan was found to have no effect on LiP activity.

3.3.5. Effect of carbon sources on LiP activity: An exogenous carbon source was an absolute requirement for ligninolytic activity. Glucose, which was used in the basal medium, was found to be the best carbon source among those studied for *Aspergillus* sp. SIP 11. This was reported to be the preferred energy source for LiP production by *P. chrysosporium* (Tien & Kirk, 1988; Manimekalai & Swaminathan, 1999). Glycerol, a slowly metabolizable substrate, which also gave high LiP titres when used instead of glucose as carbon source in the case of *P. chrysosporium* INA-12 (Buswell et al., 1984), produced 69% less activity than with glucose in the case of *Aspergillus* sp. SIP 11 (Fig. 20). The polysaccharide, starch served as a poor carbon source, while fructose and sucrose were utilized only to a limited extent. When the glucose concentration was increased to 1%, LiP activity increased by 14% necessitating a carbon sufficient medium for LiP production by this fungus. Further increase in glucose concentration inhibited LiP synthesis. This might be due to the delay in the onset of secondary metabolism after nutrient depletion, an essential condition for LiP production (Fig. 21). In the absence of the energy source, glucose, little growth and enzyme activity was shown.

3.3.6. Effect of lignocellulosic substrates on LiP activity: Among the various lignocellulosic wastes added to the medium at 1% concentration, it was found that coir pith gave an earlier and much higher LiP titres than in its absence. This was when 1 mM veratryl alcohol was not added to the medium. A 96% increase in activity (275 U/ml) on the second day of incubation was obtained (Fig. 22). The decrease in enzyme activity after the second day might be due to the appearance of extracellular protease activity (Cancel et al., 1993). In all earlier reports on LiP production, peak activity

was obtained only on the sixth or seventh day of incubation. Coir pith contained approximately 43% lignin and other micronutrients in addition to the high water holding capacity, which might be the reasons for the induction of enzyme activity on the second day itself (Natarajan, 1995). Stimulation of LiP when the medium was supplemented with lignocellulosic wastes and mineral salts was reported by Arora and Gill (2001). Enhancement of laccase and MnP activity was shown by the addition of 1% bagasse in the medium for the growth of *Pleurotus ostreatus* (Sen et al., 2001). In the presence of coir pith, it was noticed that the fungus showed a diffused uniform growth instead of forming pellets. Besides, it was observed that growth reached the maximum on the second day itself thereby creating a nutrient depleted condition for the onset of secondary metabolism and LiP production. All the other substrates produced LiP only on the seventh day. Wheat bran had almost the same effect as addition of 1 mM veratryl alcohol while other substrates produced low LiP titres with pigeon pea hull producing almost negligible LiP.

With increasing concentrations of coir pith, increasing LiP activity was obtained. At 3% level, there was a 19% increase in LiP activity to 227 U/ml (Fig. 23). As further increase in coir pith level led to a solid substrate fermentation condition, 3% level was taken as the optimum concentration of coir pith in all further studies.

3.3.7. Effect of nitrogen sources on LiP activity: Of the organic and inorganic nitrogen sources studied, $(NH_4)_2SO_4$ gave 5% increase (340 U/ml) in LiP titres over NH_4NO_3 which was used in the basal medium. The stabilizing effect of $(NH_4)_2SO_4$ on the enzyme, thereby preventing its inactivation might be one of the reasons for the slight increase in activity in

its presence. When NH_4Cl was the nitrogen source, a 4% decrease in activity was shown compared to that of NH_4NO_3 (Fig. 24). This demonstrated a preference for NH_4^+- N by *Aspergillus* sp. SIP 11 as in the case of *P. chrysosporium* (Tien & Kirk, 1988; Buswell et al., 1984). Nitrogen supplementation in the form of nitrates or as ammonium tartrate either produced negligible or no LiP activity. Of the organic nitrogen sources, peptone completely inhibited LiP production while yeast extract produced only a meager activity. Slow growth of the fungus was recorded when organic nitrogen sources were used in the medium. Have et al. (1997) reported that L-tyrosine present in peptone and yeast extract inhibited oxidation of veratryl alcohol to veratraldehyde by LiP from both *P. chrysosporium* and *Bjerkandera* sp. strain BOS55. A secondary consequence was the inactivation of LiP by H_2O_2 resulting in the underestimation of the LiP activity. The low or absence of LiP activity in the presence of peptone, yeast extract, KNO_3, $NaNO_3$ and ammonium tartrate might be due to the high culture pH during the growth phase, when the pH of the medium increased to 5.0 on the first day itself and remained so throughout. In the case of other nitrogen sources, the pH was reduced to 3.0 by the first day. This was in accordance with the reports of Haapala and Linko (1993) on *P. chrysosporium* where higher culture pH resulted in an inhibition of LiP activity.

When nitrogen limited or excess nitrogen conditions were provided, LiP activity showed a decrease, whereby the optimum concentration of $(NH_4)_2SO_4$ required was found to be 12.5 mM (Fig. 25). Here LiP synthesis under sufficient nitrogen conditions occurred in response to exhaustion of glucose on the second day of incubation. The importance of nitrogen

stimulation in nature is not clear, because the nitrogen content of most wood is very low. Cowling and Merrill (1966) suggested that nitrogen from in situ nitrogen fixation by nitrogen fixing bacteria associated with major decay fungi might supplement the existing nitrogen resource in wood.

LiP was usually suppressed and delayed by high concentrations of nitrogen (Keyser et al., 1978; Kirk et al., 1978; Faison & Kirk, 1985) and a low nitrogen medium was effective for the efficient production of LiP (Nakamura et al., 1999). But it was demonstrated in *Bjerkandera* sp. strain BOS55 that endogenous production of veratryl alcohol was much higher in nitrogen sufficient medium than in nitrogen limited medium. The nitrogen supplements could have a role in imitating the conditions that the fungus encountered during carbon limitation. Overproduction of LiP in the presence of sufficient nitrogen level and excess nitrogen levels seemed to occur as a response to carbon starvation after rapid glucose depletion. (Dosoretz et al., 1993; Kaal et al., 1993). The increase in LiP titres under nitrogen sufficient conditions by *Aspergillus* sp. SIP 11 could be attributed to the above.

3.3.8. Effect of phosphorus sources: Though there are practically few studies on the influence of phosphorus sources on LiP production, in order to ascertain its correlation with LiP activity of *Aspergillus* sp. SIP 11, various sources of phosphorus were studied. It was found that dihydrogen phosphates were preferred to monohydrogen phosphates, KH_2PO_4 and NaH_2PO_4 being better phosphorus sources than K_2HPO_4 and NaH_2PO_4. KH_2PO_4 and NaH_2PO_4 gave comparable LiP activity, only 5% more activity in the case of KH_2PO_4 than NaH_2PO_4. 94% and 81% of the activity using KH_2PO_4 was obtained with K_2HPO_4 and Na_2HPO_4 as phosphorus sources (Fig. 26). When varying levels of KH_2PO_4 was added to the medium, low

concentration (0.1%) was found to be the optimum for LiP activity, for *Aspergillus* sp. SIP 11 required only a limited amount of phosphorus for growth and metabolism (Fig. 27). As can be seen, high concentrations produced only a meager decrease in activity. From this it was clear that the type of phosphorus sources did not have much influence on LiP activity as in the case of nitrogen or carbon sources. This was in accordance with earlier reports of the surprisingly little effect of phosphorus on LiP production, though highest LiP activity was obtained with low phosphorus concentrations of 0.05% to 0.2% of KH_2PO_4 which was the commonly used phosphorus source (Haapala & Linko et al., 1993).

3.3.9. Effect of salinity and metal ions on LiP production: *Aspergillus* sp. SIP 11 grew well and produced equivalent amount of LiP (345 U/ml) in salinities of 25 g/kg and 35 g/kg. Only 75% of the activity was obtained when the salinity was decreased to 15 g/kg with a reduction of 86% of the activity at 5 g/kg (Fig. 28). In distilled water (0 g/kg), the fungus showed scant growth with no LiP production. The halophilic nature of *Aspergillus* sp. SIP 11 could be deduced from the above results.

Of the different metal ions added to the medium at 1 mM concentration, it was found that all the metal ions inhibited LiP synthesis to varying levels, the highest being for Cu^{2+} (86%) while the least was for K^+ (6%) (Fig. 29). This was surprising in view of the fact that a balance of trace elements was important for LiP activity (Tien & Kirk, 1988). Haapala and Linko (1993) studied about the influence of Cu^{2+}, Mn^{2+} and Zn^{2+} on LiP titre. Early lignin degradation studies have also suggested that a balance of Ca^{2+}, Mg^{2+} and Mn^{2+} were critical for LiP activity (Jeffries et al., 1981). According to Kirk et al. (1986), Cu^{2+} and Mn^{2+} stimulated LiP production in

P. chrysosporium with a doubling of activity when a seven fold increase in their basal trace element concentration was provided. On the other hand, according to Janshekar and Fiechter (1988), the addition of trace elements became unnecessary by replacing distilled water with tap water in medium preparation.

The ligninolytic machinery was shown to be strongly inhibited by Mn^{2+} (Perez & Jeffries, 1992). With the addition of Mn^{2+} to the medium the endogenous level of veratryl alcohol was found to decrease suggesting that it regulated enzymes involved in either the degradation, recycling or biosynthesis of veratryl alcohol, which in turn affected LiP synthesis (Mester et al., 1995). The inhibitory effect of Mn^{2+} on LiP activity of *Aspergillus* sp. SIP 11 might be due to the above reason. The presence of all necessary metal ions in adequate concentrations in seawater of salinities 25 g/kg and 35 g/kg might be the reason for the inhibition caused by all other metal ions.

Only Mg^{2+} which was present in the basal medium as $MgSO_4.7H_2O$ (0.1%) in the medium had a strong influence on LiP, for when its concentration was lowered to 0.05%, a reduction of 16% in LiP activity was noted. But higher concentrations had an inhibitory effect - 30% inhibition of LiP activity at 0.2% concentration (Fig. 30). The strong influence of Mg^{2+} on growth and lignin degradation was substantiated by Jeffries et al. (1981).

3.3.10. Effect of vitamins: Thiamin-HCl and biotin did not have any influence on the growth and LiP production by *Aspergillus* sp. SIP 11. Kirk et al. (1978) reported the necessity of thiamine-HCl (0.2 mg/l) for growth

and lignin metabolism by *P. chrysosporium*, and considered it as good as the complete vitamin mixture, though the complete mixture was somewhat better for growth.

The growth profile of *Aspergillus* sp. SIP 11 under optimized conditions is shown in Fig. 31. The reduction in the pH of the medium to 3.0 by the first day might be due to the production of acid metabolites by the fungus on utilization of glucose. Maximum growth was reached by the second day itself, when glucose became exhausted, leading to nutrient limitation and the appearance of secondary metabolism. The soluble protein showed an increase with peak enzyme activity.

When the growth conditions were optimized, *Aspergillus* sp. SIP 11 produced an LiP activity of 345 U/ml (862.5 U/mg protein) on the second day of incubation. So there was a five fold increase in activity under optimized conditions when compared to the non-optimized media with peak LiP titres occurring much earlier in comparison to the tenth day of incubation before optimization (Fig. 32). The only other marine fungus capable of LiP production was *Flavodon flavus*, a basidiomycete, which gave an LiP activity of 50 U/L on the seventh day of incubation with added veratryl alcohol in the medium (Raghukumar et al., 1999).

A carbon and nitrogen sufficient medium was found to give higher LiP titres by *Aspergillus* sp. SIP 11. Further, there was no requirement for expensive veratryl alcohol in the growth medium, which was an absolute necessity for white rot and other lignin degrading fungi for LiP production. Addition of coir pith, a lignocellulosic waste, to the medium enhanced LiP production with maximum synthesis on the second day of incubation

compared to an LiP peak obtained only on the sixth or seventh day in other fungi. The degradation products of coir pith probably replaced veratryl alcohol besides increasing LiP production. High LiP titres even at high saline conditions makes this species a halophilic one. These unique features of *Aspergillus* sp. SIP 11 give it added advantage over other ligninolytic fungi for various biotechnological applications.

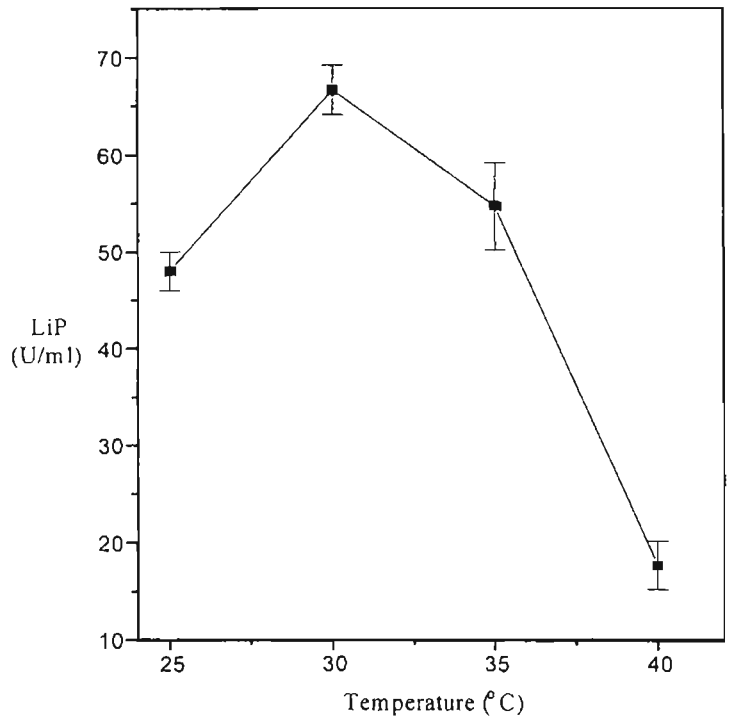


Fig. 15 LiP activity at different temperatures

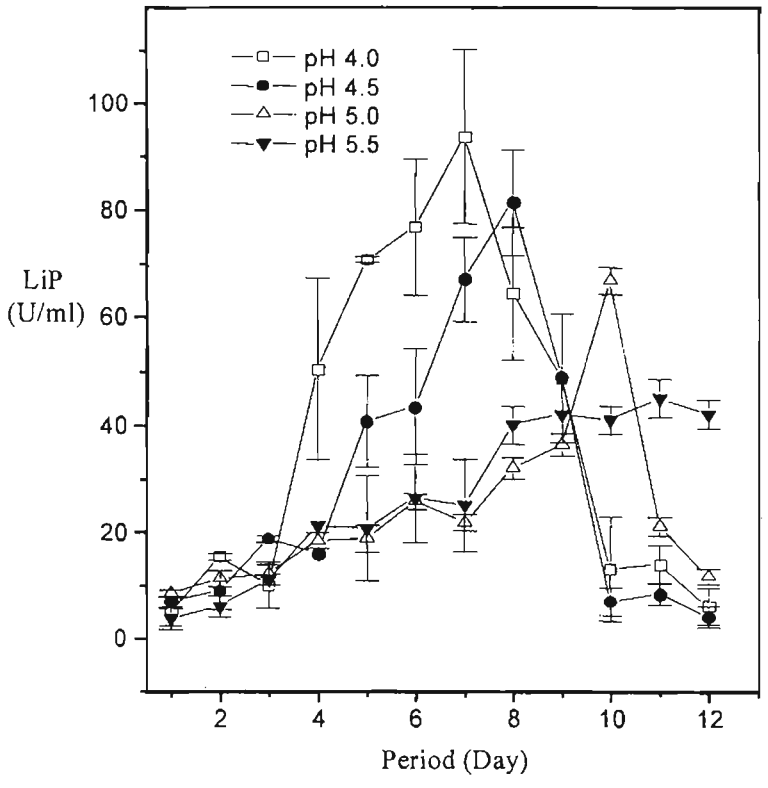


Fig. 16 Effect of LiP activity at different pHs

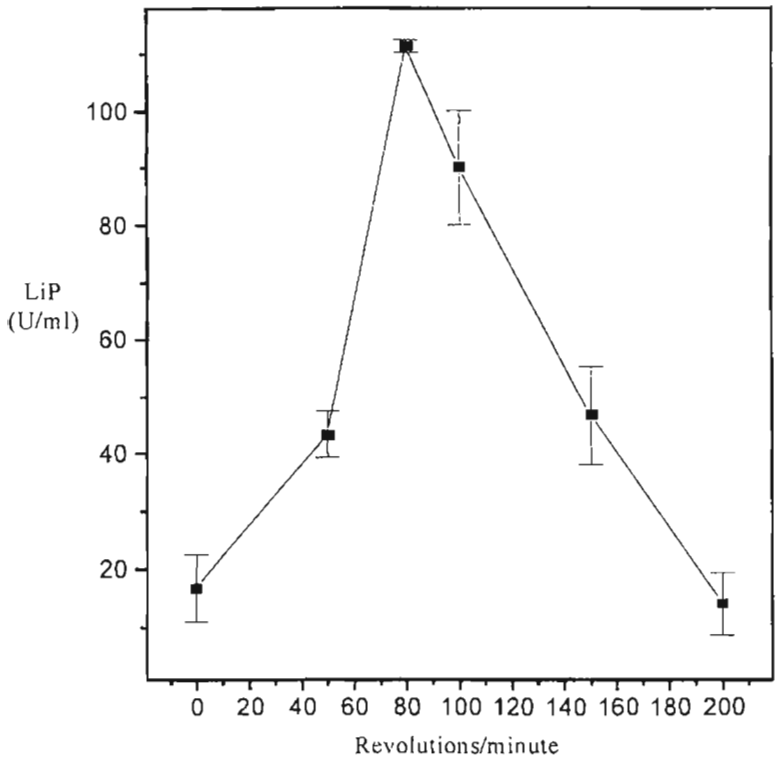


Fig. 17 Effect of agitation on LiP activity

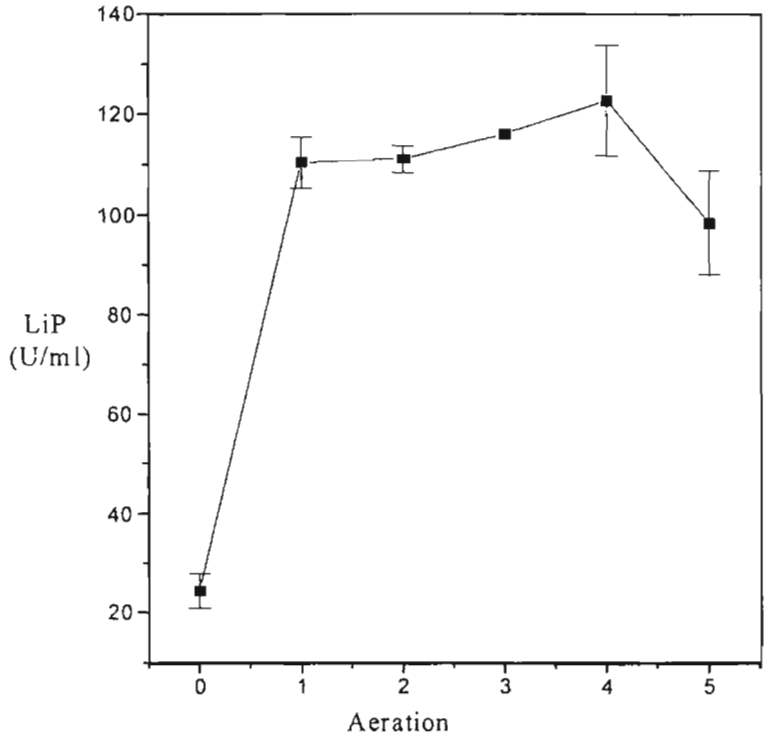


Fig. 18 Effect of aeration on LiP activity

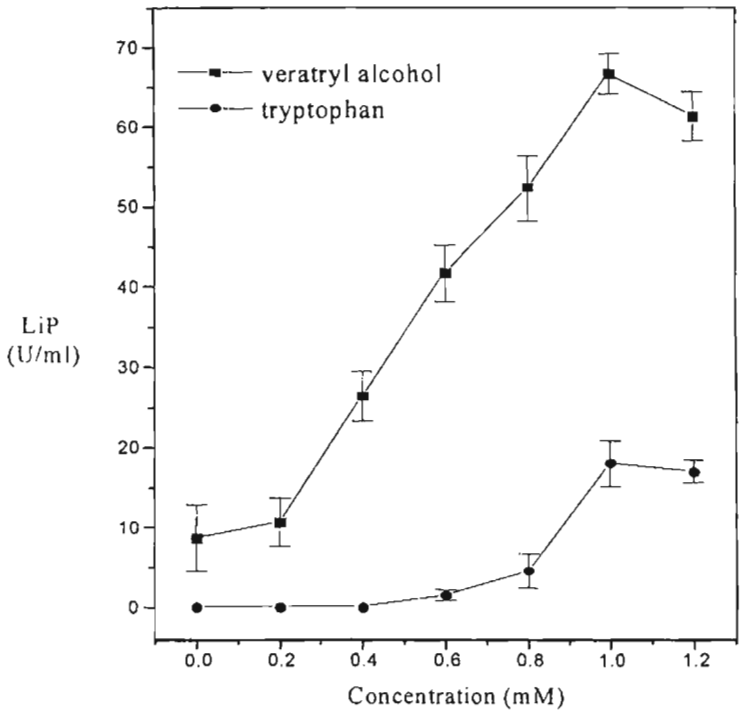


Fig. 19 Effect of inducers on LiP activity

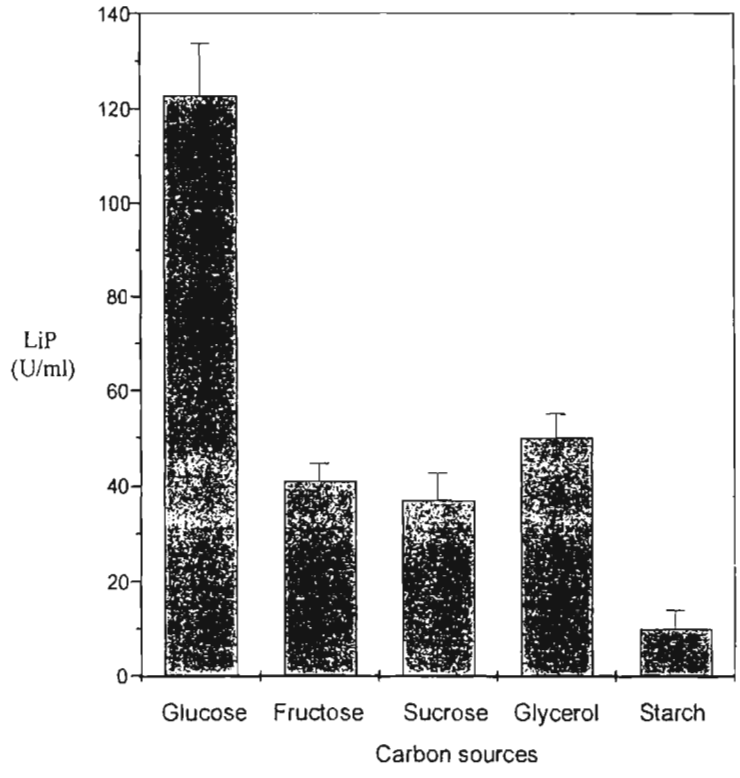


Fig. 20 Effect of different carbon sources on LiP activity

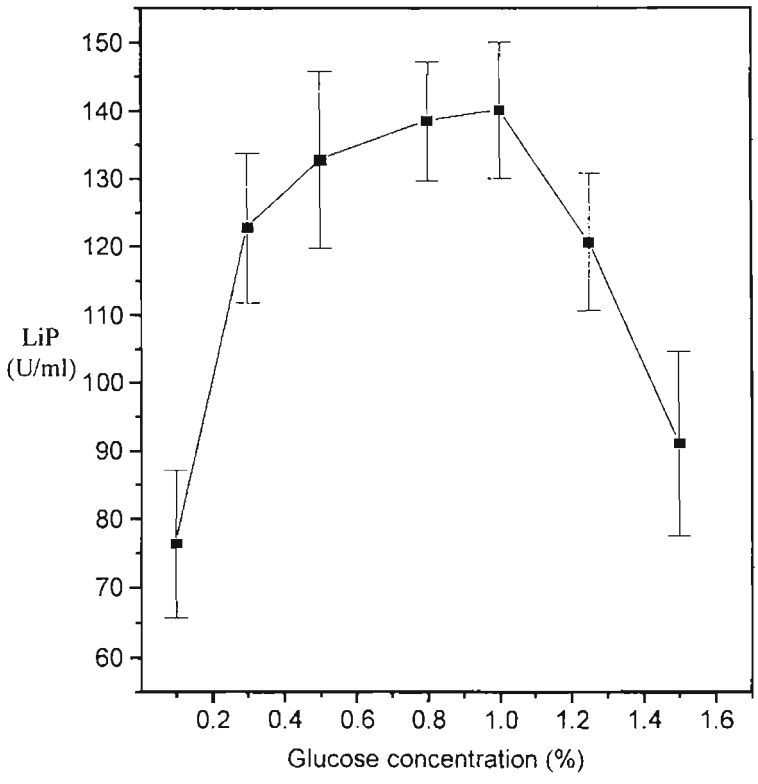


Fig. 21 Effect of various concentrations of glucose on LiP activity

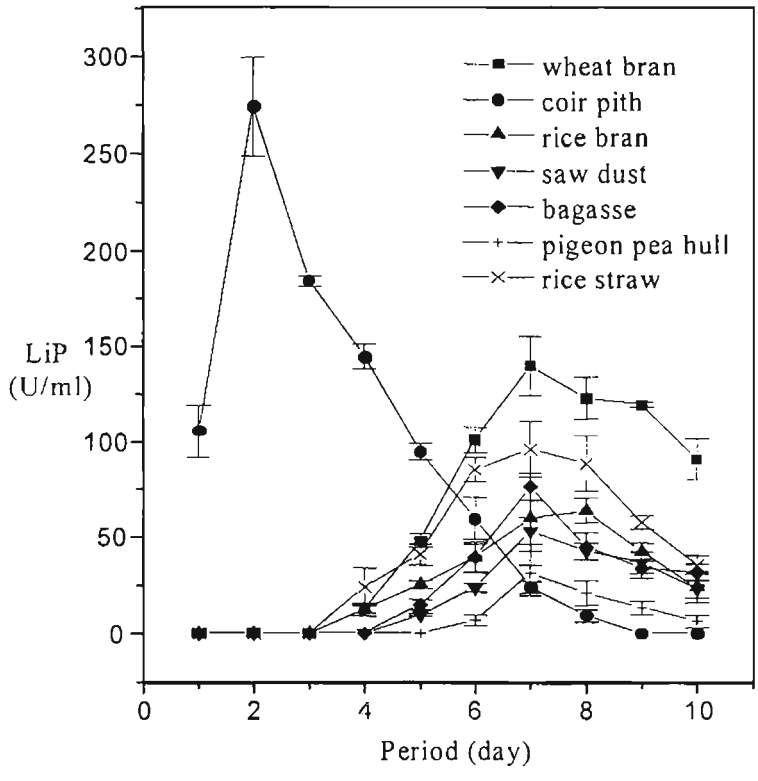


Fig. 22 Effect of lignocellulosic substrates on LiP activity

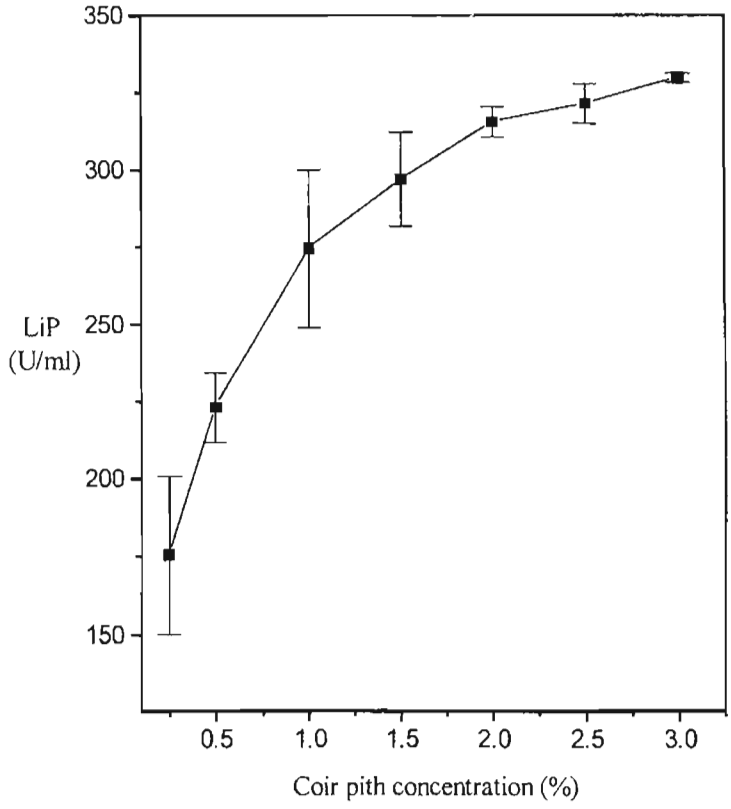


Fig. 23 Effect of different concentrations of coir pith on LiP activity

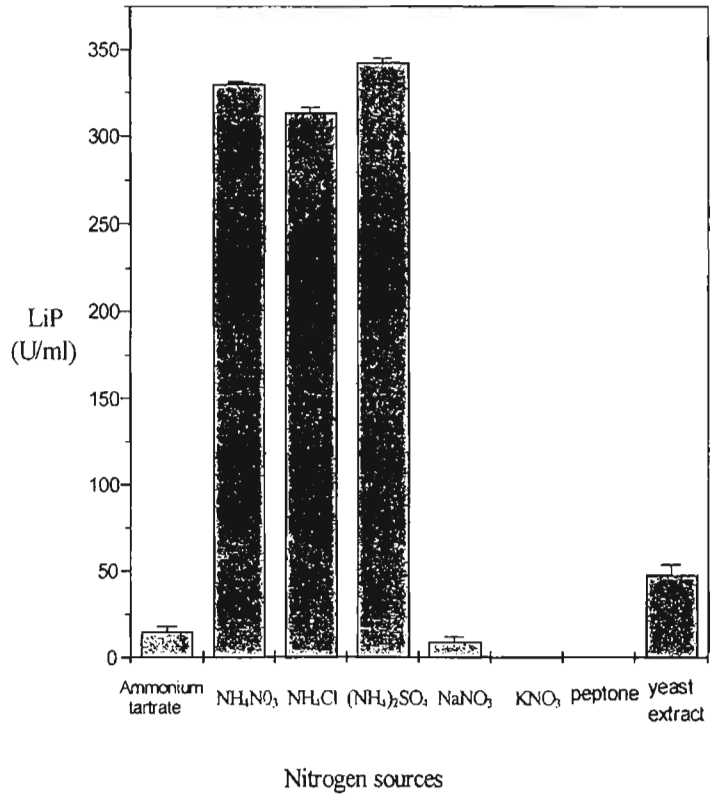


Fig. 24 Effect of different nitrogen sources on LiP activity

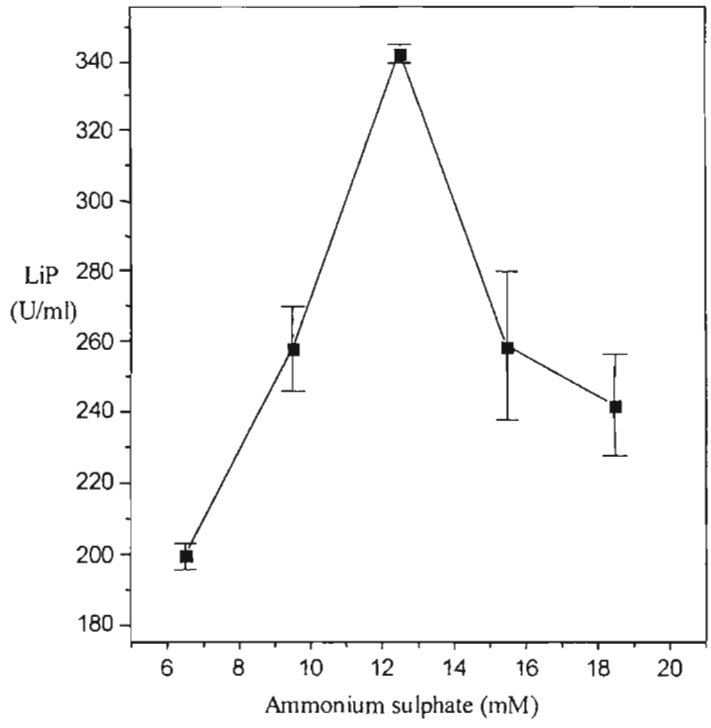


Fig. 25 Effect of ammonium sulphate concentrations on LiP activity

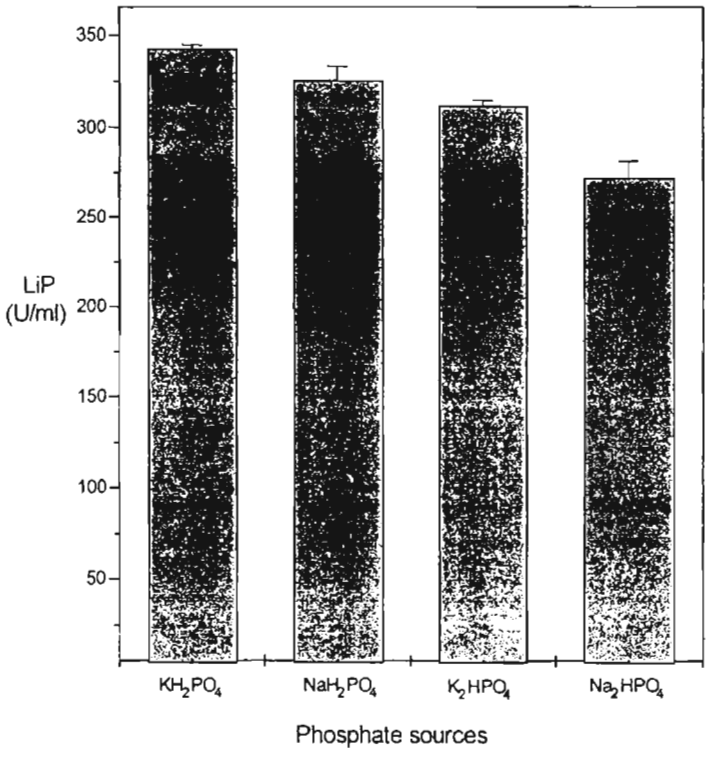


Fig. 26 Effect of different phosphorus sources on LiP activity

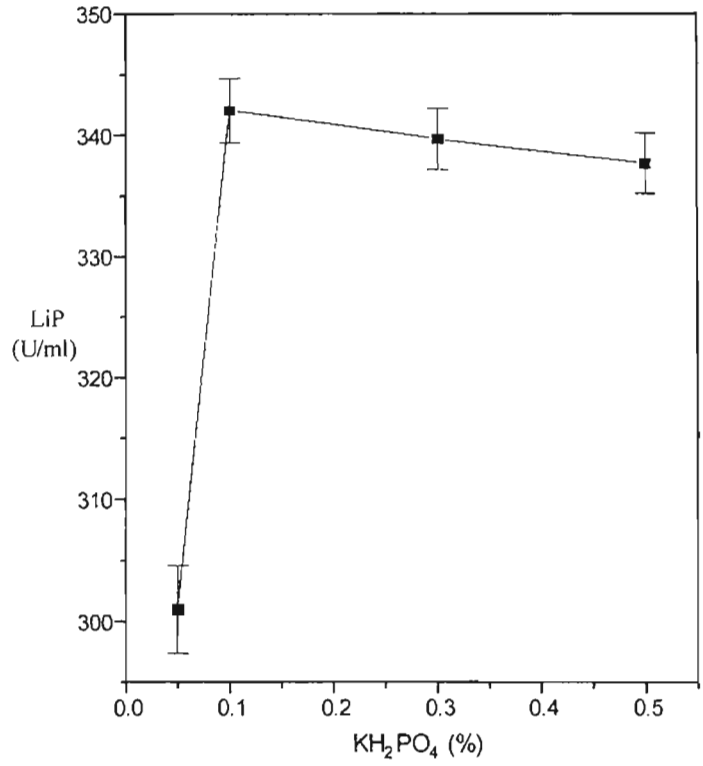


Fig. 27 Effect of various concentrations of KH_2PO_4 on LiP activity

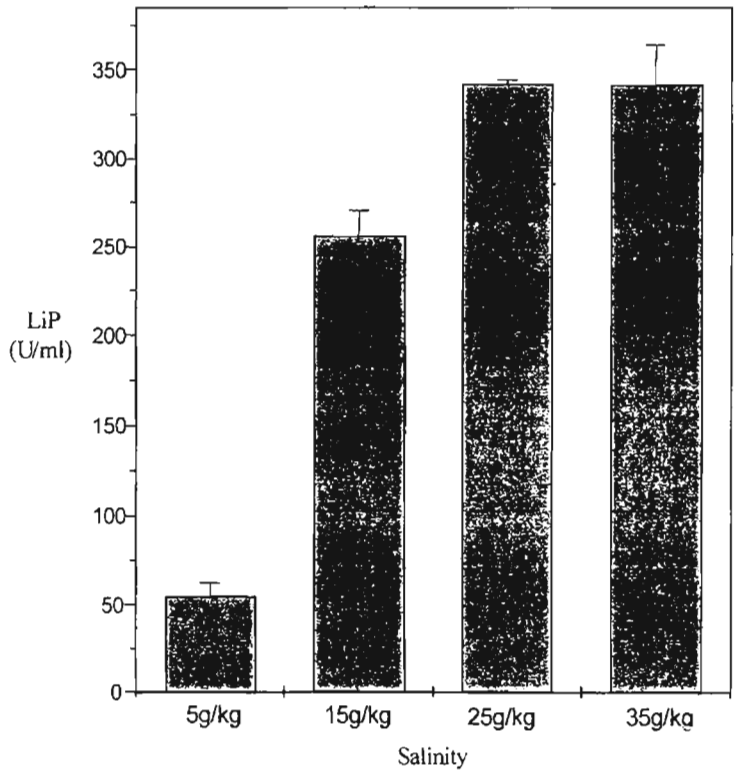


Fig. 28 Effect of different salinities on LiP activity

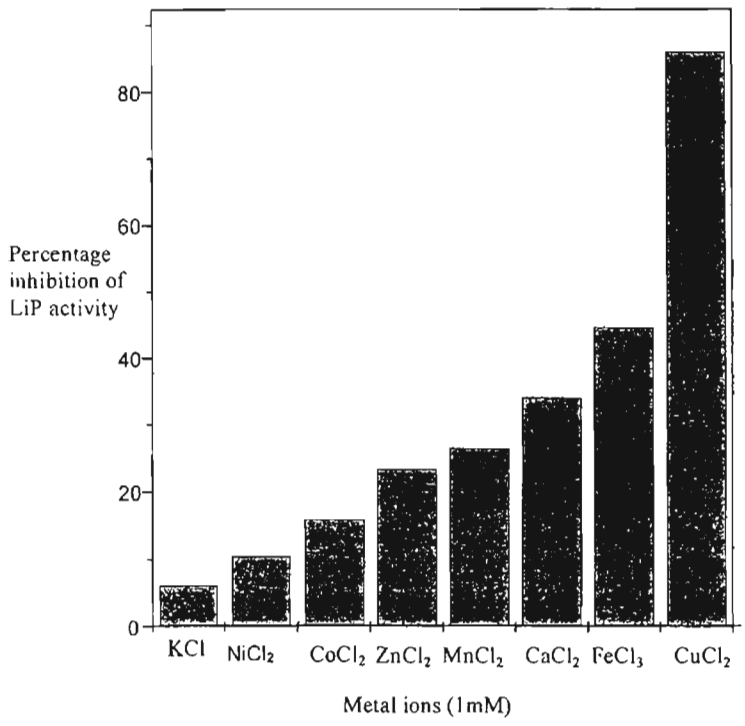


Fig. 29 Effect of metal ions on LiP activity

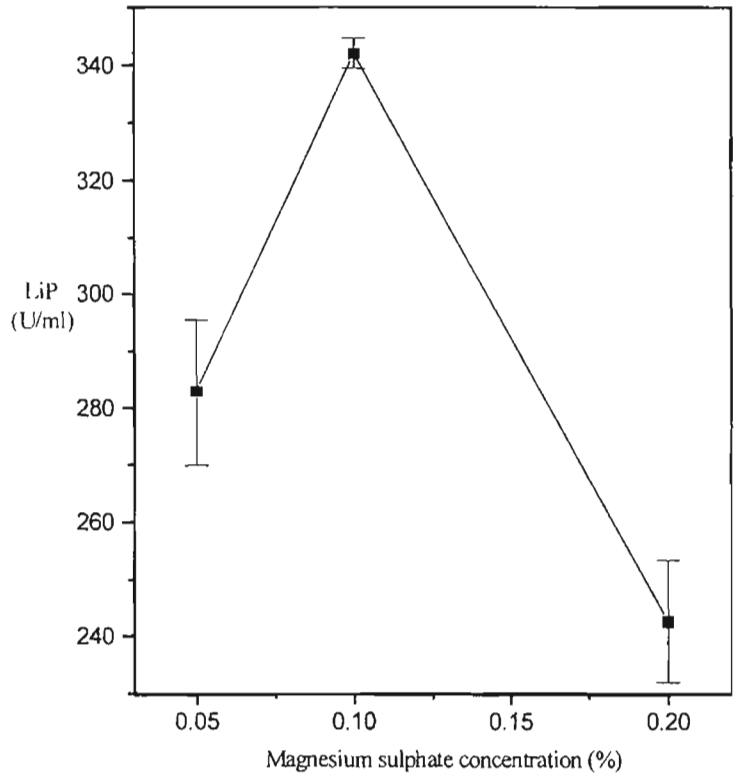


Fig. 30 Effect of magnesium sulphate concentrations on LiP activity

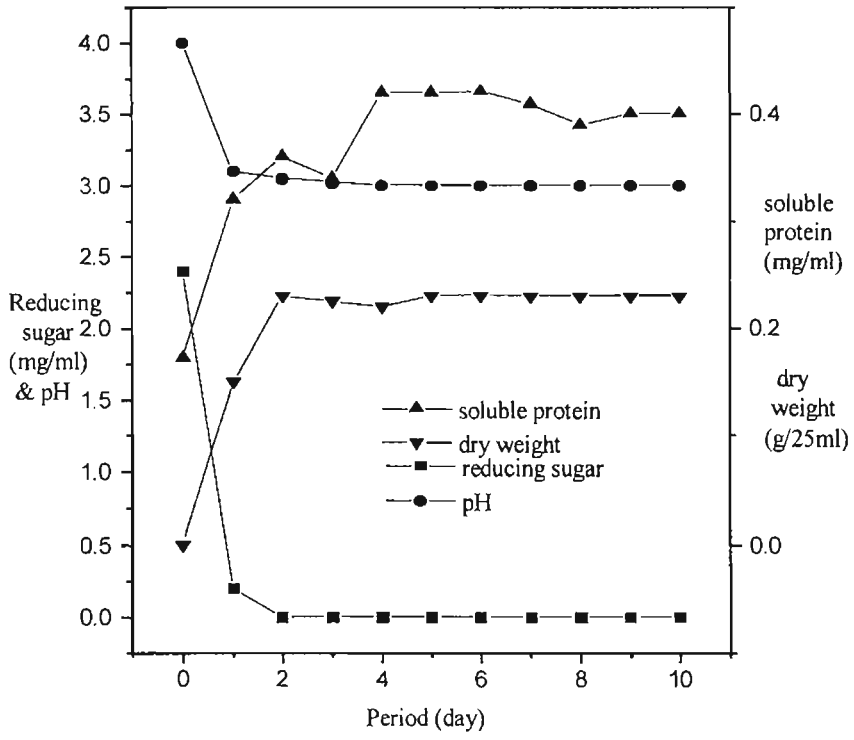


Fig. 31 Growth profile of *Aspergillus* sp. SIP 11 under optimized conditions

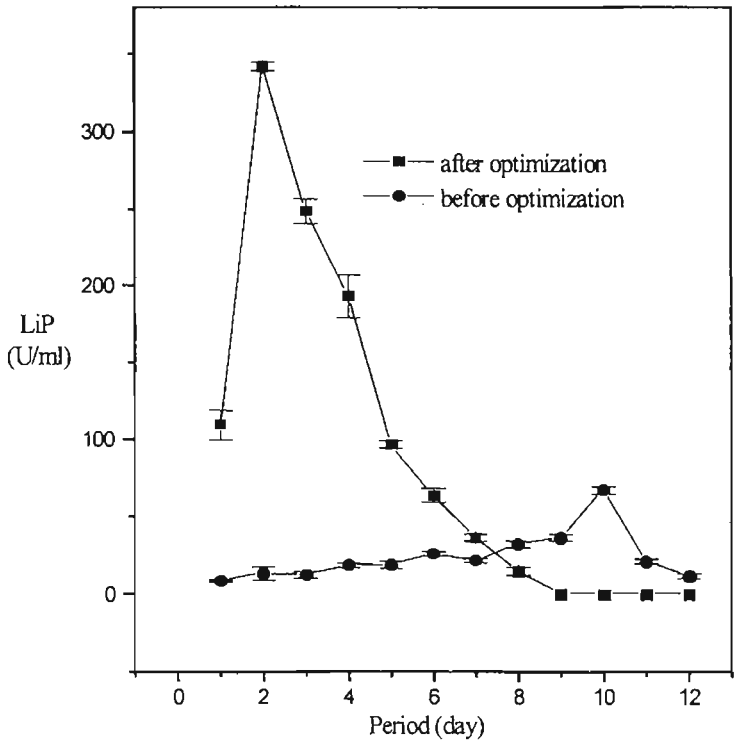


Fig. 32 Comparison of LiP activity in optimized and non-optimized medium

Chapter 4

Optimization of growth conditions during solid substrate production of LiP by *Aspergillus* sp. SIP 11

4.1. INTRODUCTION

Solid substrate fermentation (SSF) is a technique in which moist, water insoluble solid substrate is fermented by micro-organisms in the absence or near absence of free water. The substrate not only supplied the nutrients to the microbial culture but also served as an anchorage for the cells. Although this technique had been in practice since ancient times for fermented foods, cheese making and in composting, little progress has been made in this field as compared to SmF. More recently a number of modern applications of SSF technology have been developed including the production of enzymes, organic acids, antibiotics, alkaloids etc and also for the disposal of organic toxic wastes. SSF system is particularly suitable for lignocellulolytic enzyme production for various agrobiotechnological applications (Lonsane, 1994).

SSF provides many advantages over SmF which include higher product titres, lower capital as well as recurring expenditure, lower wastewater output, reduced energy requirement, absence of foam problem, simplicity, elimination of the need for solubilization of nutrients, lesser fermentation space, less complex plant as well as machinery, economical to use at small scale, easier induction or suppression of sporulation, lower cost of downstream processing, amenability of dried fermented solids for storage over a long period and easier control of bacterial contamination. However, this process suffers from certain problems such as difficulties in scale up, metabolic heat generation and heat transfer limitations, requirement for continuous monitoring of moisture, oxygen, pH and CO_2 level, difficulty in biomass estimation, undesired product formation, solid waste disposal

problems and very little engineering data available on the design of reactors. (Mitchell, 1998).

The use of water at arbitrarily selected level might be the major cause for adverse effects on the productivity and performance of the otherwise well optimized SmF processes. This fact alone might also have a vital role in imparting all the generally applicable and specific advantages to the SSF system, as requirement of water was always optimized in SSF processes (Ramesh & Lonsane, 1990).

Most previous studies of the ligninolytic enzymes of white rot fungi were carried out with liquid media (Hatakka, 1994; Waldner et al., 1988), with only a few studies on the production and action of these enzymes during SSF. Production of LiP during SSF have been observed only during growth of *Phlebia radiata*, *P. chrysosporium* and *Trametes versicolor* (Datta et al., 1991; Vares et al., 1995; Gupte et al., 1998). In this study, the LiP producing fungus, *Aspergillus* sp. SIP 11, was grown under SSF conditions and different growth parameters were optimized for maximum production of enzyme so as to determine its effectiveness over SmF.

4.2. MATERIALS AND METHODS

4.2.1. Enzyme production in SSF: Erlenmeyer flasks (250ml) each containing 10 g of wheat bran of particle size 300 μ - 420 μ was mixed thoroughly with 10 ml of the optimized medium of SmF (g/l : glucose - 10, KH_2PO_4 - 1.0, $(NH_4)_2SO_4$ - 12.5 mM, $MgSO_4.7H_2O$ - 1.0, Tween 20 - 0.2) in seawater of salinity 35 g/kg, to give a moisture level of 50% at pH 4.0. These were then autoclaved at 121°C, 15 lb psi for 30 minutes, cooled and inoculated with 3 x 10^6 spores/g substrate of *Aspergillus* sp. SIP 11,

incubated at 30°C. The flasks were periodically removed and the contents were mixed by gentle tapping. At regular intervals, 100 ml distilled water was added to each flask along with a few glass beads. After agitation at 120 rev. min^{-1} for one hour, these samples were filtered through nylon cloth and centrifuged at 10,000 g, 20' at 4°C. The supernatant was used as the source of crude enzyme.

4.2.2. Analytical methods: The crude enzyme obtained by centrifugation was used for the following analyses.

4.2.2.1. pH, protein, LiP activity and reducing sugar were estimated as described in Section **2.2.3.** and **2.2.4.1.**

4.2.2.2. Moisture level : One gram of the sample was weighed out and dried in a hot air oven (Beston) at 110°C overnight when a constant weight was obtained. The moisture level was calculated as follows:

$$\frac{\text{Initial weight} - \text{final weight}}{\text{Initial weight}} \times 100$$

4.2.3. Optimization studies: There are several important factors which affect SSF processes. Among these, substrate and selection of process parameters are crucial. The effect of different parameters on LiP production was studied individually by varying one factor at a time. At each step, the selected parameter was included in the control medium.

4.2.3.1. Effect of moisture level on LiP activity : The influence of moisture level on enzyme titre was evaluated by varying the ratio of wheat bran to the salt solution. Different moisture levels ranging from 40% to

80% moisture levels were provided and its influence on LiP activity was studied.

4.2.3.2. Effect of particle size of wheat bran on LiP activity: Varying particle sizes of wheat bran, <300 μ, 300 μ - 420 μ, 420 μ - 500 μ and >500 μ , at 50% moisture level were studied to determine the optimum size for maximum LiP activity.

4.2.3.3. Effect of pH and temperature on LiP activity: The influence of pH of the medium on LiP activity was determined at the incubation temperature of 30°C by varying the initial pH from 2.0 to 5.0.

Similarly at pH 4.0, the temperature of incubation was varied between 25°C and 40°C to ascertain its effect on LiP titre.

4.2.3.4. Effect of lignocellulosic substrates on LiP activity: *Aspergillus* sp. SIP 11 was grown on different lignocellulosic substrates - wheat bran, coir pith, rice bran, bagasse, green gram hull, pigeon pea hull and rice straw - at 50% moisture level with the objective of determining the best substrate for LiP production.

4.2.3.5. Effect of inducers on LiP activity: Certain substances which were believed to induce LiP production were added individually to the medium at the time of inoculation. These were lignin (0.02%), humic acid (0.02%), veratryl alcohol (1 mM) and tryptophan (1 mM).

4.3. RESULTS AND DISCUSSION

4.3.1. Effect of moisture level on LiP activity : The *Aspergillus* sp. SIP 11 grown in wheat bran with moisture level 50% showed an LiP activity of 180 U/gm dry wt. on the third day of incubation. Only 28% of the activity at 50% moisture level was produced when the moisture level was raised to 60%. At 80% moisture level the LiP activity was almost absent (Fig. 33). High moisture resulted in decreased substrate porosity which in turn prevented oxygen penetration, leading to bacterial contamination. On the other hand, low moisture content led to poor accessibility to nutrients resulting in poor microbial growth (Kumar & Lonsane, 1990). The moisture content of the substrate influences the fungal growth, enzyme activity and regulates product formation. Furthermore, moisture content in the wet fibrous crop residues influence the mass transfer rate of O_2 and CO_2, and rate of heat dissipation (Puniya & Singh, 1998). The optimum moisture content for growth and substrate utilization for microbes was between 40% and 70% but depended upon the organism and the substrate used for cultivation. In general, bacteria require higher values for growth than fungi, thereby enabling fungi to compete more successfully at the moisture level encountered in SSF processes. Zadrazil and Brunnert (1981) reported that SSF of wheat straw with various fungi was optimum at 75% moisture content.

4.3.2. Effect of particle size on LiP activity: The moisture level at which free moisture becomes apparent varies depending on the particle size of the substrate. Generally, smaller substrate particles would provide larger surface area for microbial attack and thus should be considered as a

desirable factor. However, too small substrate particles resulted in substrate agglomeration in most of the cases, which might interfere with microbial respiration / aeration efficiency resulting in poor growth. At the same time larger particles provided better respiration / aeration but limited surface for microbial attack (Pandey et al., 2000). Lignin degradation largely occurred under oxidative conditions. Puniya et al. (1994) had reported that gaseous phase was one of the controlling factor in the ecology and physiology of *P. chrysosporium* during SSF. Aeration rate was found to be the most influential factor in SSF of *P. chrysosporium* grown on steam exploded wheat straw (Chen et al., 2002). The maximum LiP activity of 180 U/g dry wt. obtained with a particle size of 420 μ - 500 μ was found to be the optimum size. A particle size of >500 μ and 300 μ - 420μ particle size produced only 33% and 12% of the activity respectively of that of optimum size. Particle size of less than 300 μ gave negligible activity (Fig. 34).

4.3.3. Effect of pH and temperature on LiP activity: Maximum activity was obtained at a pH of 4.0, while at pH 5.0 the activity was reduced to 50%. At pH 3.0 only 11% activity was retained (Fig. 35). The extent of lignin degradation depended on the pH of the substrate. In general, lignin degradation by fungi occurred at acidic pH (Reid, 1985).

The optimum temperature was found to be 30°C, a higher or lower level inhibited LiP activity. Less than half of the activity at 30°C was retained under these temperatures (Fig. 36). High temperature inhibited fungal growth with increased metabolism and more heat generation while at lower temperature there was slow growth and reduced metabolism. Most lignin degrading fungi grow best at temperatures between 20°C and 30°C. Elevated tempertures had adverse effects on progress of fermentation and

led to less lignin degradation and decreased digestibility (Zadrazil and Brunnert, 1982). A temperature of 35°C was found to be optimum for SSF of wheat straw by *Sporotrichum pulverulentum* (Parveen et al., 1986).

4.3.4. Effect of different lignocellulosic substrates on LiP activity: Wheat bran, among the lignocellulosic substrates tried, gave the maximum activity of 180 U/g dry wt. on the third day of incubation. Wheat bran was considered the prime among all substrates for SSF (Pandey et al., 2000). Coir pith, which was found to be effective in SmF, produced only 39% of the maximum activity which was comparable to that obtained using pigeon pea hull. The other substrates gave only 8 to 11% of the activity obtained using wheat bran (Fig. 37). The moisture level at which free water became apparent varied considerably between substrates and was dependent upon their water binding characteristics (Oriol et al., 1988a). At 50% moisture level, it might be that only for wheat bran the availability of free water was sufficient for fungal growth and enzyme production while for the other substrates it might be less (coir pith) or high (green gram hull).

4.3.5. Effect of inducers on LiP activity: When lignin was added to the substrate, on the fifth day of incubation a maximum LiP activity of 230 U/g dry wt. was obtained. The other inducers, veratryl alcohol and tryptophan, delayed peak LiP titre, producing 140 U and 110 U/g dry wt. respectively. Humic acid had an inhibitory effect on LiP production (Fig. 38). Lignin being a substrate for LiP action might be the reason for high LiP activity on addition of lignin.

The growth profile of *Aspergillus* sp. SIP 11 in wheat bran under optimized conditions is given in Fig. 39. The pH reached the initial pH of

4.0 after 10 days of incubation. Reducing sugar was completely exhausted by the fifth day while soluble protein showed only little variation.

Only a few reports are available on LiP production in SSF by white rot fungi. Vares et al. (1995) purified the enzyme from SSF cultures of *Phlebia radiata*, which produced LiP, MnP and laccase during degradation of wheat straw. Correlation of brightening with cumulative enzyme activity related to lignin biodegradation during biobleaching of kraft pulp by *P. chrysosporium* and *Trametes versicolor* was determined in SSF system (Katagiri et al., 1995). LiP was not detected in the extracellular enzyme extracts of *Coriolopsis polyzona* and *P. chrysosporium* grown on wheat straw while *Trametes versicolor* secreted LiP only after 10 days of growth (Vyas et al., 1994). *Pleurotus ostreatus* grown on cotton stalks did not produce LiP when studied in SSF (Kerem et al., 1992). Only low levels of LiP was produced by the basidiomycetes fungus, *Nematoloma frowardii* during SSF of wheat straw (Hofrichter et al., 1999). When *T. versicolor* was grown on wheat straw and indulin combination, peak LiP production was obtained on the 33rd day of incubation (16 U/ml/minute) (Gupte et al., 1998). With added veratryl alcohol, LiP activity of 356 U/L was obtained for *P. chrysosporium* in solid state bioreactor, which was capable of degrading Poly R-478 dye (Rivela et al., 2000). Much higher activities of LiP (2600 U/L) and MnP (1375 U/L) was obtained for *P. chrysosporium* in SSF than SmF on using steam exploded wheat straw which was found to replace veratryl alcohol required in SmF (Xu et al., 2001).

From the above it is clear that LiP, which played an important role in lignin degradation, was produced in much greater quantity and in a shorter incubation period by *Aspergillus* sp. SIP 11 in SSF compared to other fungi reported, though it produced higher LiP titres in SmF (Fig. 40). In addition,

the fungus was halophilic in nature. These characteristics make this species important in lignin degradation by SSF and also for other biotechnological applications.

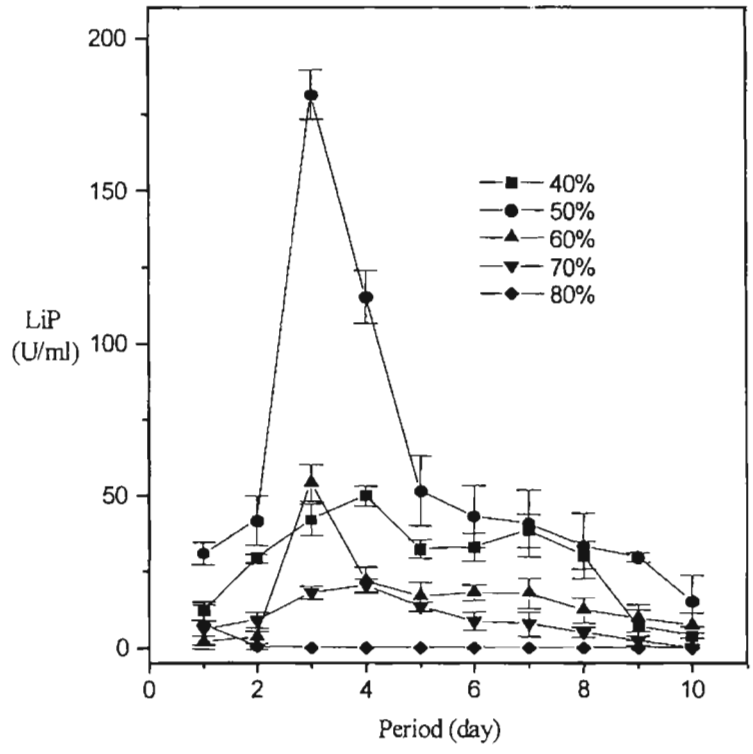


Fig. 33 Effect of moisture level on LiP activity

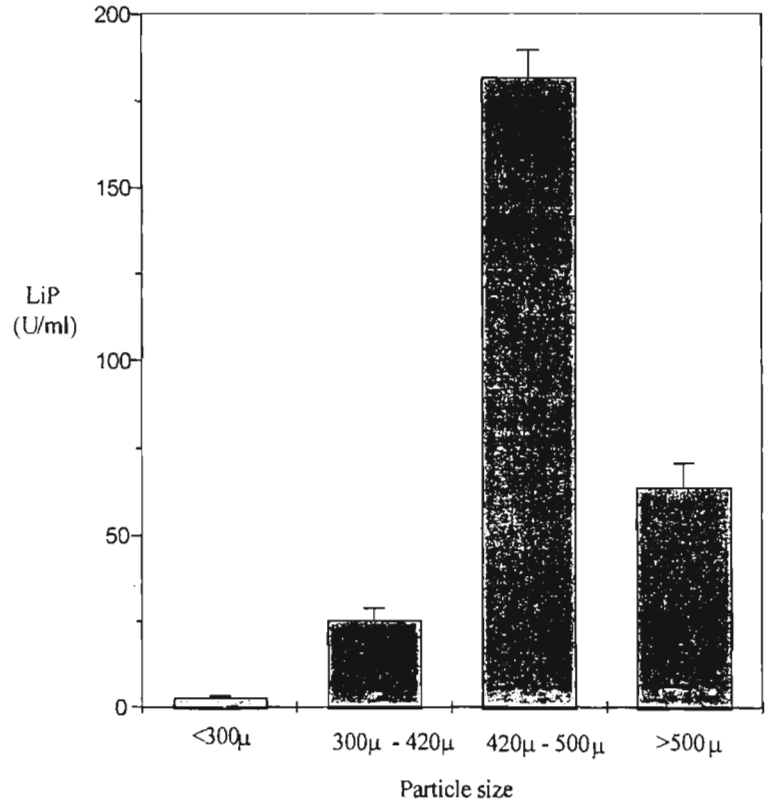


Fig. 34 Effect of particle size of wheat bran on LiP activity

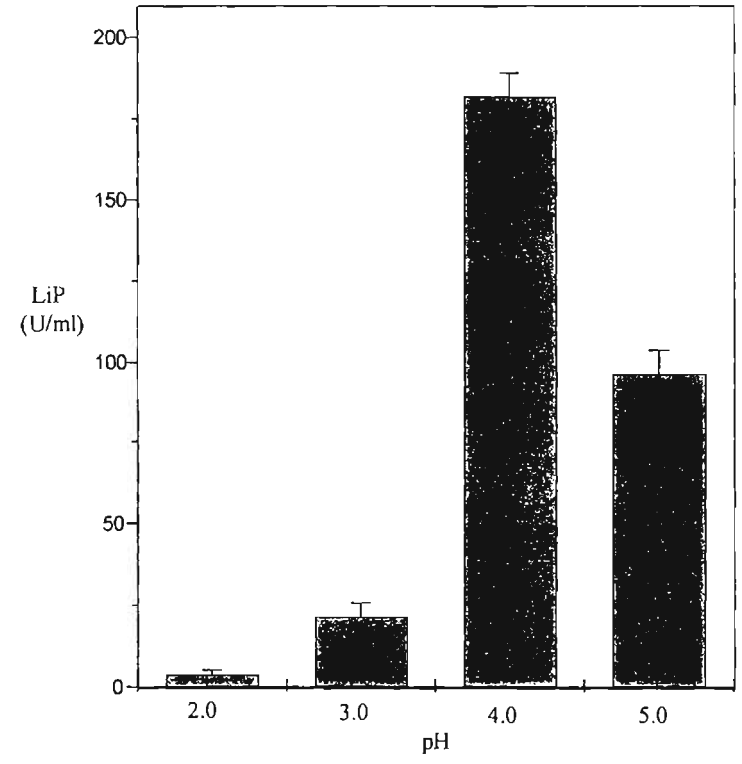


Fig. 35 Effect of pH on LiP activity

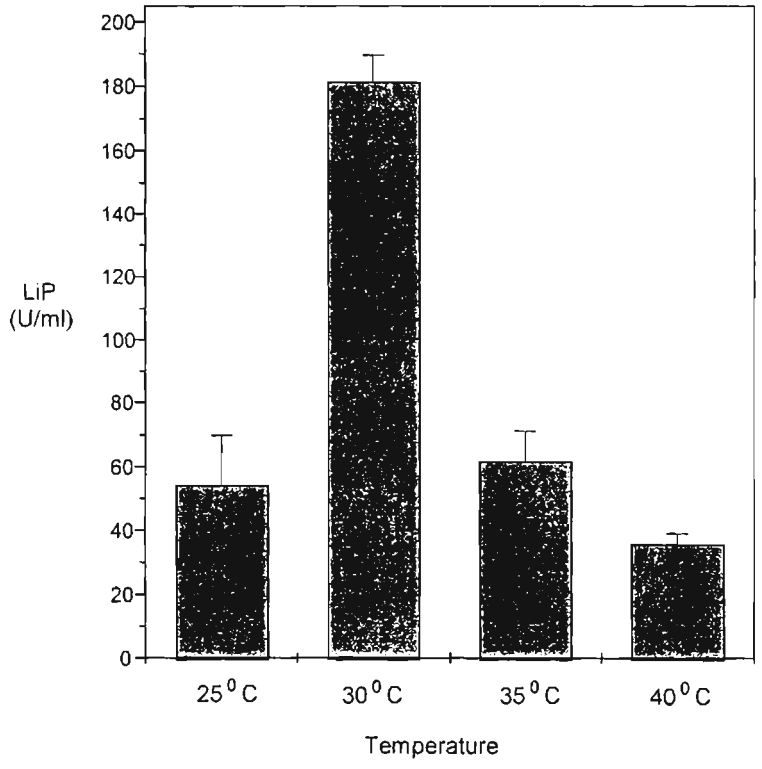


Fig. 36 Effect of temperature on LiP activity

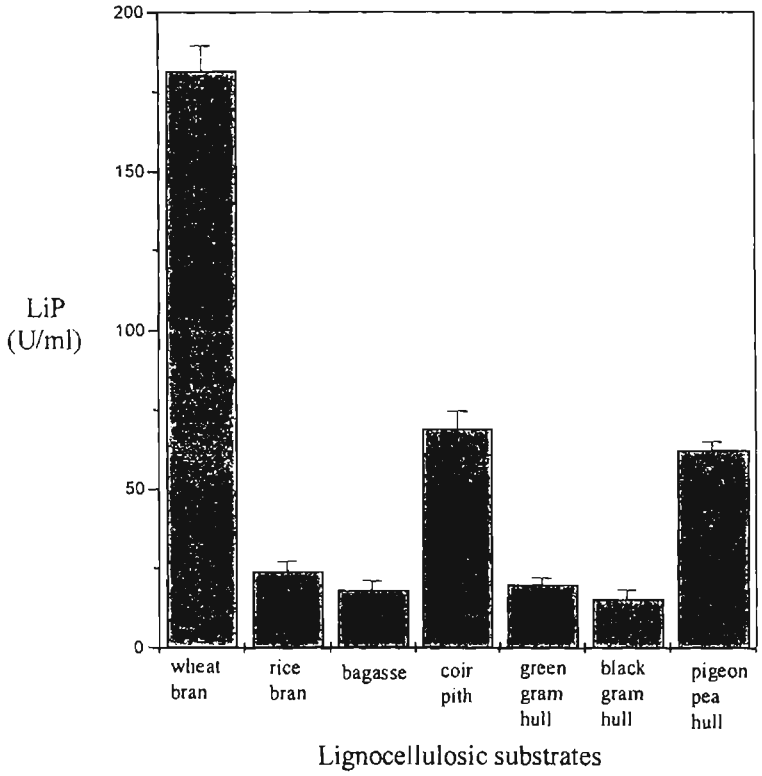


Fig. 37 Effect of lignocellulosic substrates on LiP activity

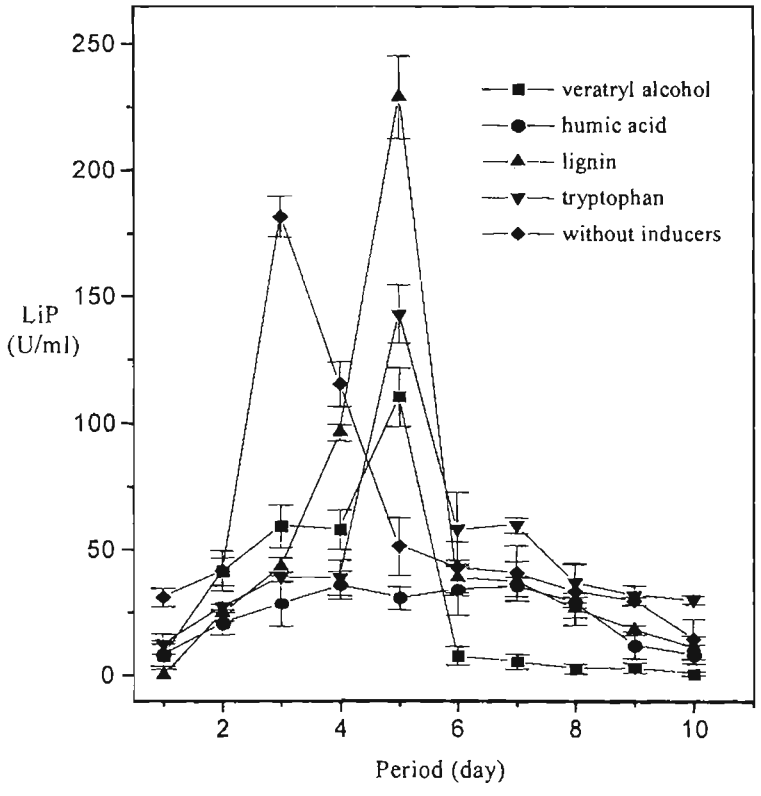


Fig. 38 Effect of inducers on LiP activity

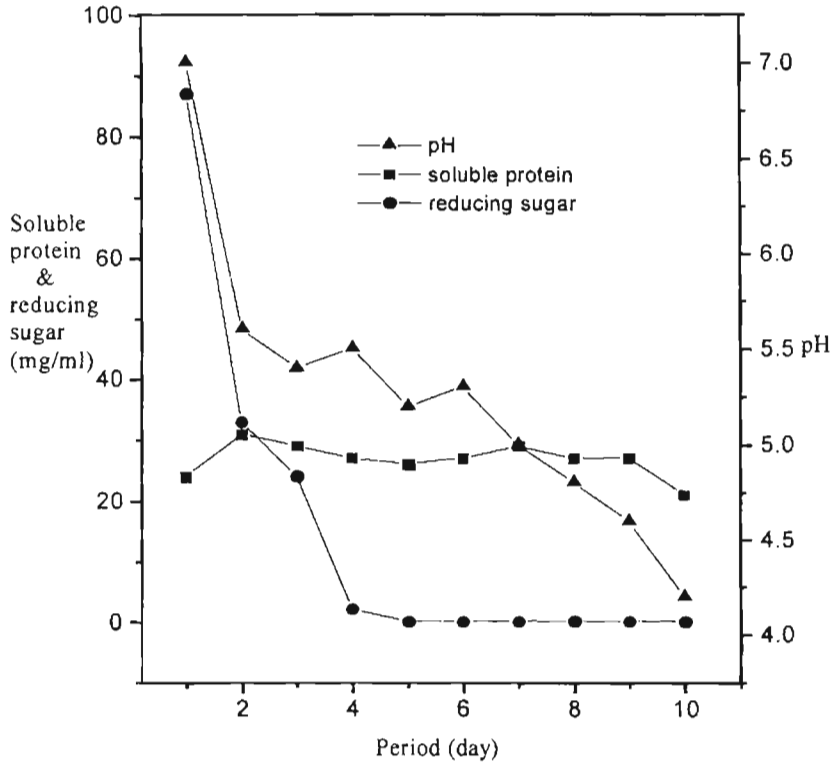


Fig. 39 Growth profile of *Aspergillus* sp. SIP 11 under optimized conditions

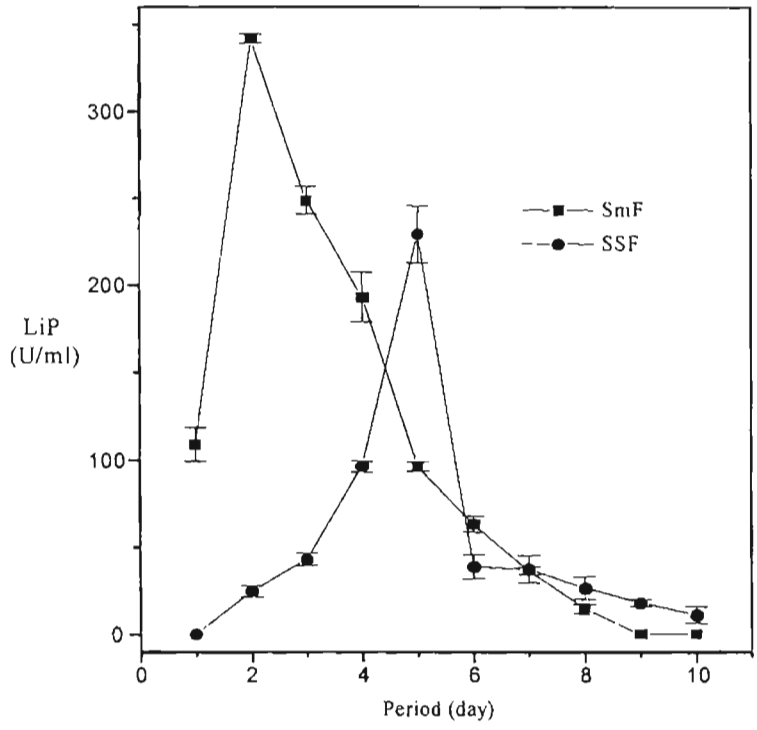


Fig. 40 Comparison of LiP activities in SSF and SmF

Chapter 5

Lignin peroxidase from *Aspergillus* sp. SIP 11 – Purification, characterization and decolorization of synthetic dyes

5.1. INTRODUCTION

Lignin peroxidase (LiP) is a generic name for a group of isozymes that catalyzed the oxidative depolymerization of lignin. The number of LiP isozymes produced by the white rot fungus *Phanerochaete chrysosporium* was reported to vary from 2 to 15, based on the strain, culture conditions, and separation efficiency (Kirk et al., 1986; Tien & Kirk, 1988). LiP isozymes exhibited a high degree of homology. They are all heme containing glycoproteins and all cross reacted with a polyclonal antibody raised to the predominant LiP (Kirk et al., 1986). All of them oxidize veratryl alcohol to veratraldehyde but exhibit considerable differences in isoelectric point, molecular mass, sugar content, spectral characteristics, substrate specificity and stability (Faison et al., 1986; Kirk et al., 1986). Farrell et al. (1989) purified ten hemeproteins, designated H1 - H10 from *P. chrysosporium* BKM-1767 grown under nitrogen limited conditions. Six of them, H1, H2, H6, H7, H8 and H10 were LiPs while the others showed MnP activity. The major isozyme, H8, was extensively characterized and was the protein initially isolated by Tien & Kirk (1984). The lignin peroxidases had different isoelectric points, between pI 4.7 and 3.3, and molecular weights of 38 kDa to 43 kDa.

With the goal of more clearly defining the origin(s) of the numerous LiP isozymes which were initially thought to be post-translational variants of the same gene product, the isolation and characterization of LiP genes and cDNA copies were undertaken by several groups (Alic & Gold, 1991; Reiser et al., 1989). Several LiP cDNAs and genes were cloned and sequenced. Their structural analyses revealed that the different LiP isozymes were encoded by distinct genes, that the different LiP genes

exhibited high levels of nucleotide homology to each other, and there were atleast three subfamilies of LiP genes within the LiP gene family of *P. chrysosporium* (Reddy, 1993). The LiP cDNA and genomic sequences from other white rot fungi including *Bjerkandera adusta* (Kumoura et al., 1991), *Phlebia radiata* (Saloheimo et al., 1989) and *Trametes versicolor* (Black & Reddy, 1991) have also been analyzed. Taken together, the results indicated that the diversity of LiP isozymes must be due, in part, to the genomic multiplicity of the LiP sequences. The LiP transcripts appeared under conditions of carbon or nitrogen limitation and that the levels of specific transcripts were affected by culture conditions (Holzbaur & Tien, 1988; James et al., 1992).

LiP, MnP and laccase produced by white rot fungi were capable of oxidizing various recalcitrant xenobiotics in industrial effluents especially synthetic dyes used extensively for textile dyeing, paper printing, color photography and as additives in petroleum products (Bumpus, 1989). Most experiments on degradation of dyestuffs by white rot fungi have been carried out using either whole cultures or crude enzyme preparations containing most or all extracellular enzymes of the ligninolytic system of the fungus (Bumpus & Brock, 1988; Cripps et al., 1990; Spadaro et al., 1992). In this work, the LiP from *Aspergillus* sp. SIP 11 produced in SmF was purified and this was used in dye decolorization studies. Besides, the characterization and kinetic studies of the purified LiP together with the effect of metal ions and inhibitors on purified LiP was studied. As in vitro stability of the enzyme was an important factor in determining the feasibility of application for industrial uses, the stability studies of the purified LiP was also done.

5.2. MATERIALS AND METHODS

5.2.1. Purification of LiP: The purification procedures followed by other workers are given in Table 4. The purification protocol followed here was as follows:

5.2.1.1. Crude enzyme preparation: *Aspergillus* sp. SIP 11, under optimized conditions, produced maximum LiP activity of 345 U/ml on the second day of incubation in SmF. 800 ml of the culture medium was harvested on the second day of incubation, filtered through glass wool to remove the mycelial fragments and coir pith. The culture filtrate was centrifuged (10,000 g, 4°C, 20') to obtain an yellow supernatant which was used for the determination of LiP activity and protein as described in Section **2.2.4.1.** and **2.2.3.2.** respectively. The supernatant served as the crude enzyme which was used for ammonium sulphate precipitation.

5.2.1.2. Ammonium sulphate precipitation: The protein concentration of the crude enzyme was found to be only 0.4 mg/ml. For the successful precipitation of the protein using ammonium sulphate, bovine serum albumin was added to give a final protein concentration of 1 mg/ml. Finely powdered solid ammonium sulphate was slowly added to the crude enzyme with mild stirring at 4°C. After incubating for one hour, the mixture was centrifuged and the precipitate was dissolved in double volume of buffer (20 mM of sodium acetate buffer, pH 5.0). After removing the precipitate of fraction 1, the supernatant was again subjected to further precipitation to the next level by adding the required amount of ammonium sulphate. This process was repeated upto 100% fraction with gradual increase between the

consecutive fractions. LiP estimations of the different fractions revealed maximum activity in the range of 40% to 80%.

For a 40% saturation, to the 800 ml of crude enzyme 154.8 g of ammonium sulphate was added slowly with stirring at 4°C. After the last bit of salt was dissolved, stirring was continued for 30 minutes to allow complete equilibration between dissolved and aggregated proteins. Then the solution was centrifuged (10,000 g, 4°C, 20'). The supernatant was decanted and the pellet was discarded. 179.8 g of ammonium sulphate was then added slowly to the supernatant for 80% saturation. When the salt was completely dissolved the solution was kept at 4°C overnight. It was then centrifuged, the supernatant was discarded and pellet dissolved in 50 ml of 20 mM sodium acetate buffer, pH 5.0. This was then dialyzed against 5 litres of the same buffer with four changes. The volume increased to 99 ml after dialysis which was then lyophilized.

5.2.1.3. Chromatographic separation

5.2.1.3.1. Ion exchange chromatography: As LiP was known to be an anion with isoelectric points of 3.3 to 4.7, a weak anion exchange matrix, DEAE Sepharose CL-6B which had positively charged diethyl amino ethyl group (DEAE) was selected. To determine the appropriate pH for ion exchange chromatography, test tubes containing 1ml of the ion exchanger was equilibrated with buffers of different pHs of 2.0 to 6.0 containing 10 mM NaCl. Crude enzyme (100 µl) was added to 1ml of the buffer that covered the matrix in each tube. The best conditions were those under which the protein was bound, but at a pH not far from one at which the protein dissociated. Elution of the LiP was tried using 0 - 1 M NaCl, whereby the appropriate pH for ion exchange was found to be 5.0. Sodium

acetate buffer (pKa - 4.8) at 20 mM concentration and pH 5.0 was, therefore selected for chromatographic separation of LiP.

DEAE Sepharose CL-6B was swollen in the buffer. The material was degassed and packed carefully in a column of dimension 8 cm x 2.5 cm evenly without air bubbles. For completing the packing process and also for equilibration of the matrix to the buffer, five bed volumes of the buffer was run through the column. Flow rate was adjusted to 40 ml/hour by using a Pharmacia LKB peristaltic pump. The lyophilized LiP sample was dissolved in 30 ml of the buffer and loaded onto the column. Two bed volumes of the buffer was run to wash down all unbound enzyme. Then the protein was eluted by applying a linear gradient of 0 - 1 M NaCl prepared in the buffer. Fractions of 3 ml each were collected. For each fraction, protein was determined at 280 nm, heme at 409 nm and LiP activity at 310 nm. The fractions showing peak LiP activity were pooled together and used for further purification by Gel Permeation Chromatography.

5.2.1.3.2. Gel Permeation Chromatography (GPC): As the reported molecular weight of LiP was in the range of 38 kDa to 43 kDa, the gel filtration matrix, Biogel P-100 with a fractionation range of 5 - 100 kDa was selected. Biogel P-100 was swollen in buffer, degassed and packed into a glass column (Pharmacia, dimension 70 cm x 2.5 cm). The matrix was equilibrated with 3 bed volumes of the buffer to which 100 mM NaCl was added to block non-specific ionic interactions. Flow rate was adjusted to 30 ml/hour. The void volume (V_o) was found out using blue dextran (Sigma Chemical Co., 2000 kDa). Blue dextran (2 ml) solution of concentration of 1 mg/ml was loaded onto the column. The volume of the buffer collected from the point of injection of sample to the peak maximum of blue dextran

was the void volume. The total bed volume was determined by loading potassium dichromate solution. The column was washed with one bed volume of buffer and a standard protein sample was loaded. Bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa) [Sigma Chemical Co.] were the standard proteins used. 1 mg/ml of the standard proteins were loaded. 1.5 ml fractions were collected. The absorbance of the fractions were taken at 280 nm and the elution volume (V_e) for each protein was found out. The ratios of the elution volumes to the void volume (V_e/V_o) for each protein was calculated and plotted against molecular weights in a semi-logarithmic graph to obtain a standard curve (Andrews, 1965).

The active LiP fractions from ion exchange chromatography were pooled together and 10 ml sample was loaded onto the GPC column. After the void volume, 1.5 ml fractions were collected. For each fraction, protein was determined at 280 nm, heme at 409 nm and LiP at 310 nm. V_e/V_o of LiP was calculated and plotted onto the standard graph whereby its molecular weight was obtained. The active LiP fractions were pooled together and lyophilized.

5.2.1.4. Gel Electrophoresis

5.2.1.4.1. Under denaturing conditions: SDS-PAGE is used to determine the molecular weight of LiP and also its purity according to Laemmli (1970). Mini slab gel apparatus (Genei) was used for electrophoresis of ion exchange and GPC samples. The gel was prepared using 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8). 40 µl of the sample was dissolved in 200 µl sample buffer containing 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue, 25% glycerol, 0.9 ml water and

60 mM Tris HCl buffer (pH 6.8). The gel was run at a constant potential difference of 200V. The sample was run along with molecular weight markers (Genei). The markers added were phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). Coomassie Blue R-250 was used to stain the protein according to Neuhoff et al. (1988).

5.2.1.4.2. Under non-denaturing conditions: The gel was prepared as in the case of the SDS-PAGE. Samples were loaded under non-denaturing conditions and run at 200 V. Crystal violet dye incorporated agarose gel was prepared in 0.1 M sodium tartrate buffer, pH 3.0. The polyacrylamide gel with LiP was placed over the crystal violet agarose gel for 30 minutes after which the gel was removed to get the zymogram.

5.2.2. Characterization of LiP: The purified enzyme was used for the following studies:

5.2.2.1. Effect of pH on activity and stability of LiP: The enzyme was incubated at different pHs, 1.5 to 5.0, using KCl-HCl buffer (pH 1.5), sodium tartrate buffer (pH 2.0 to 3.5) and sodium acetate buffer (pH 4.0 to 5.0). The stability of the enzyme at different pHs ranging from 2.0 to 7.0 was determined for two hours at intervals of 15 minutes. Citrate phosphate buffer was used to get a pH of 6.0 and 7.0.

5.2.2.2. Effect of temperature on activity and stability of LiP : The assay temperature was varied between 30°C and 45°C (SHIMADZU UV-2401PC). The stability of the enzyme at different temperatures were done

by incubating the enzyme at temperatures of 10°C to 60°C and determining the LiP activity at intervals of 15 minutes under standard assay conditions.

5.2.2.3. Effect of substrate concentration on LiP: The Michaelis Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined by using veratryl alcohol and H_2O_2 as substrates with concentrations ranging from 0.4 mM to 0.8 mM veratryl alcohol and 20 mM to 150 mM H_2O_2. The value of K_m and V_{max} were calculated from Lineweaver-Burk plot (Lineweaver & Burk, 1934). K_m value was the substrate concentration at which velocity was half maximum.

5.2.2.4. Effect of metal ions and inhibitors on LiP : Different metal ions ($AgCl_2$, $HgCl_2$, $NiCl_2$, $K_2Cr_2O_7$, $CuCl_2$, $CdCl_2$, NaCl, $CoCl_2$, NH_4MoO_3, $MgCl_2$, KCl, $MnCl_2$, $CaCl_2$, $FeCl_3$) at 1.0 mM and 10 mM concentrations were added to the enzyme and the residual activity was determined after 10 minutes of incubation. Inhibitors like EDTA and sodium azide at 0.1 mM concentration was added to LiP and its effect was similarly studied.

5.2.3. Dye decolorization studies: The dyes studied belonged to five structurally different groups: Anthrapyridone (RBBR - Remazol Brilliant Blue R - λ_{max}, 595 nm), azo (methyl orange - λ_{max}, 500 nm), polymeric (Poly R-478 - λ_{max}, 520 nm), triphenyl methane (Crystal violet - λ_{max}, 590 nm; Bromophenol blue - λ_{max}, 592 & 437 nm) and heterocyclic (Methylene blue - λ_{max}, 667 nm).

To 0.8 mM of veratryl alcohol in 0.1 M sodium tartrate buffer and 50 µM of the dye, 2 units of LiP was added. After incubation for 30 minutes, decolorization of the dye was monitored at the visible absorbance maximum

of each dye (λ_{max}). Crystal violet which was decolorized to a greater extent was selected for further studies.

Crystal violet (50 μM) with 2 units of LiP was incubated under different conditions:

a) 0.8 mM veratryl alcohol and 150 mM H_2O_2.

b) 0.8 mM veratryl alcohol

c) 150 mM H_2O_2.

d) Under varying concentrations of veratryl alcohol of 0.1 mM to 0.8 mM to determine the optimum for dye decolorization.

5.3. RESULTS AND DISCUSSION

5.3.1. Purification of LiP

5.3.1.1. Ammonium sulphate precipitation: The precipitation of proteins can be done by the addition of salts which dehydrated the hydrophobic regions of the protein, leading to their aggregation and precipitation. The most effective salts were those with multiple charged anions like sulphate. Ammonium sulphate was the preferred salt as its solubility varied very little in the range of 0°C to 30°C and was cheaply available in sufficiently pure state besides bringing about stabilization of proteins. The only requirement for efficient precipitation was that the protein concentration should be 1 mg/ml (Scopes, 1994).

It was found that 40% to 80% saturation of ammonium sulphate produced maximum precipitation of the enzyme (Fig. 41), which was in accordance to those reported by earlier workers (Vyas & Molitoris, 1995).

5.3.1.2. Ion exchange chromatography: This is a technique most commonly used for the initial purification of the protein from a crude extract due to its ease of use and scale up, wide applicability and low cost in comparison with other separation methods. Ion exchange of proteins involves their adsorption to the charged groups of a solid support followed by elution with fractionation and/or concentration in an aqueous buffer of higher ionic strength. The net charge of a protein depends on the relative numbers of positive and negative charged groups which varied with pH. Above their pI, the proteins have net negative charge while below it their overall charge is positive (Roe, 1989).

Using DEAE Sepharose CL 6B ion exchange chromatography LiP was eluted at 0.22 M - 0.33 M NaCl concentration (Fig. 42). The red brown appearance of this eluted LiP protein suggested that it might contain a heme group, and this was confirmed by measuring its absorbance at 409 nm.

5.3.1.3. Gel Permeation Chromatography (GPC): This a method where the proteins are separated according to their size. The gel filtration matrix contain pores which allow small proteins to enter while excluding larger ones. So the first to elute are the larger proteins, thereby leading to further purification (Bollag et al., 1996).

In Biogel P-100 gel permeation chromatography, the void volume (V_o) was found to be 80 ml and the total bed volume 374 ml. LiP was eluted in the 9 ml to 30 ml volume of the buffer following V_o, thereby specifying the comparatively high molecular weight of LiP (Fig. 43). V_e/V_o of LiP was found to be 1.21 and from the semi-logarithmic graph the molecular weight of the enzyme was found to be 58 kDa (Fig. 44).

5.3.1.4. Gel Electrophoresis: Proteins are charged at a pH other than their pI and thus will migrate in an electric field in a manner dependent on their charge density. The high resolution capacity of PAGE (polyacrylamide gel electrophoresis) makes this a method of choice for most applications. It has also become an almost mandatory analytical procedure for the characterization of protein purity (Dunn, 1989).

5.3.1.4.1. Under denaturing conditions: SDS (Sodium dodecyl sulphate), an anionic detergent, solubilizes protein and also binds to the hydrophobic portions of a protein, disrupting the folded structure and allowing it to exist stably in solution in an extended conformation. As a result, the length of the SDS-protein complex was proportional to its molecular weight. Subsequent electrophoretic separation is dependent only on the effective molecular radius (M_r), which roughly approximates to molecular size and occurs solely as a result of molecular sieving through the gel matrix. The polyacrylamide gel concentration used determine the effective separation range of SDS-PAGE (Dunn, 1989).

By SDS-PAGE, the molecular weight of LiP was determined as 29 kDa (Plate VIII), which was the lowest for LiP reported till date (Table 5). From this it could be inferred that in GPC the enzyme was eluted as a dimer. The ion exchange sample showed two bands as compared to a single band in GPC which confirmed that the protein was further purified by GPC (Plate IX). The purification fold was 24.07 and the yield was 18.7% (Table 6).

5.2.1.4.2. Under non-denaturing conditions: Native-PAGE separated proteins based on their size and charge properties. This has the advantage of retention of the biological and enzymatic properties of the proteins. While

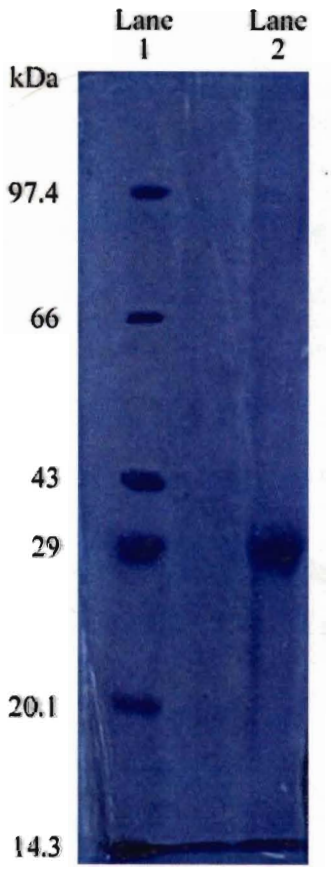


Plate VIII SDS PAGE of LiP

Lane 1 - Molecular weight markers

Lane 2 - GPC Sample

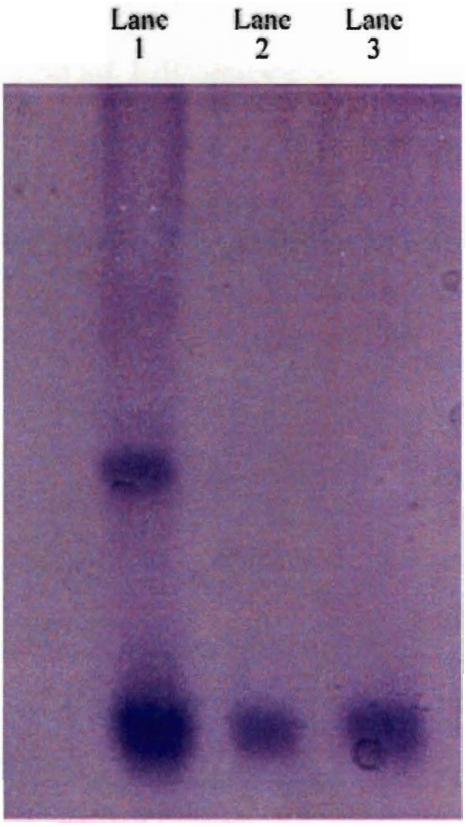
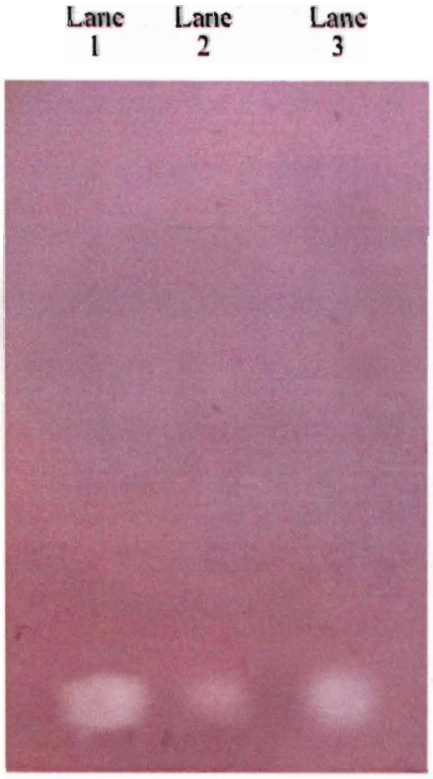


Plate IX Native PAGE of LiP



Lane 1 - Ion exchange sample

Lane 2 & 3 - GPC Sample

Plate X Zymogram of LiP

the acrylamide pore size served to sieve molecules of different sizes, proteins which were more highly charged at the pH of the separating gel had a greater mobility. Native-PAGE was commonly run with high pH buffers (pH 8.8) at which most proteins were negatively charged and migrated towards the anode (Dunn, 1989).

Zymogram using crystal violet dye produced a clear zone in the region of the LiP band confirming the peroxidase activity of the band (Plate X). There were reports regarding the verification of LiP activity of the purified protein using dyes and other substrates (Raghukumar et al. 1999; Waldner et al., 1988).

5.3.2. Characterization of LiP

5.3.2.1. Effect of pH on activity and stability of LiP: The purified LiP had an optimum pH of 2.0 as compared to the crude enzyme which had a pH optimum of 3.0. The purified LiP retained only 10% activity at pH 3.0 compared to that at pH 2.0 (Fig. 45). During purification of LiP from *P. chrysosporium* BKM-F-1767 by Kirkpatrick and Palmer (1989), an anionic polysaccharide containing fraction was also separated which was found to inhibit LiP activity at pH less than pH 3.2, thus resulting in a shift in the pH optimum of the purified isozymes back to a similar nature as obtained for the crude enzyme. This must possibly be the reason for the low pH optimum of 2.0 for purified LiP from *Aspergillus* sp. SIP 11. A similar optimum pH of 2.0 for a purified isozyme of LiP from *P. chrysosporium* was obtained by Tien et al. (1986).

The enzyme was stable for two hours over a range of pHs 2.0 to 6.0, while at pH 7.0 LiP was inactivated within 15 minutes (Fig. 46). Ligninase activity was known to increase with decreasing pH and rapid enzyme inactivation had previously been proposed to occur at a pH near the optimum (Tien et al., 1986; Glumoff et al., 1990). But the LiP from *Aspergillus* sp. SIP 11 was found to be stable at its low optimum pH. It was found that stability of LiP increased with purification of the enzyme, especially at pH 6.0.

5.3.2.2. Effect of temperature on activity and stability of LiP: As compared to the crude enzyme which had a temperature optimum of 30°C, the purified LiP's optimum temperature was found to be 35°C. 96% of the activity was retained at temperature of 40°C while at 30°C the activity obtained was only 83% of that at optimum temperature. Even at 45°C, 93% of the activity was produced (Fig. 47). A slightly higher temperature of 37°C was the optimum assay temperature which was originally proposed for LiP from *P. chrysosporium* (Tien & Kirk, 1984), 23°C for LiP from *P. chrysosporium* by Aitken and Irvine (1989) while for *Phlebia radiata* and *P. tremellosa* it was 25 °C (Hatakka et al., 1991).

LiP was stable over a range of temperatures 10°C to 50°C for two hours, while at 60°C only 25% of the activity was retained after two hours. It was found that on purification the stability had decreased as the crude enzyme was stable at 60°C for two hours and lost its activity only at 70°C (Fig. 48). Aitken and Irvine (1989) reported that the LiP from *P. chrysosporium* began to deviate from the Arrhenius relationship at

approximately 35°C and thermal instability at higher temperatures were apparent.

5.3.2.3. Effect of substrate concentration on purified LiP: K_m value using veratryl alcohol as substrate was 0.34 mM and V_{max} was 0.227 U/mg protein (Fig. 49). For H_2O_2 as substrate the corresponding values for K_m and V_{max} were 23 mM and 0.21 U/mg protein (Fig. 50). The lower the K_m value, the higher the affinity of the enzyme to the substrate. Here, LiP had more affinity towards veratryl alcohol than H_2O_2. Table 6 shows the K_m and V_{max} values of purified LiP from other fungi, from which it was clear that the K_m for veratryl alcohol was almost nearer to the values already reported while for H_2O_2, the K_m was much higher thereby inferring the low affinity of this enzyme to H_2O_2.

5.3.2.4. Effect of metal ions and inhibitors on LiP: 1 mM Cr^{2+} completely inhibited the enzyme which might be due to the fact that $K_2Cr_2O_7$ being a strong oxidizing agent, oxidized the iron in the heme part of the enzyme thereby inactivating it. While Ni^{2+} and Fe^{2+} had no effect on LiP activity, the other ions showed varying levels of inhibition (Table 7). Kirkpatrick and Palmer (1989) reported of the stimulating effect of Ca^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} on LiP activity, but none of these ions were found to have any effect on LiP activity of *Aspergillus* sp. SIP 11. 0.1 mM sodium azide and EDTA completely inhibited the enzyme. Sodium azide was a typical hemeprotein inhibitor while EDTA was a metal chelating agent thereby inactivating the enzyme. The inhibitory nature of these compounds, though at a higher concentration, was reported by Shin et al. (1997).

5.3.3 Dye decolorization studies: Among the various dyes studied at 50 μM concentration, crystal violet which is a triphenylmethane dye, was decolorized to the maximum extent of 41% in 30 minutes by 2 units of the enzyme. Poly R-478 was completely adsorbed while methyl orange, an azo dye, was decolorized to the extent of 36%. Bromophenol blue showed an intermittent disappearance and appearance of blue color on incubation. Methylene blue and RBBR were not decolorized efficiently (Table 8). It had been reported that heterocylic dyes were resistant to enzymatic oxidation (Ollikka et al., 1993). It seemed that LiP from *Aspergillus* sp. SIP 11 had higher affinity for triphenyl methane dyes and azo dyes as substrates which was in accordance with the reports of Shin and Kim, 1998. To exclude the possibility that the decolorization of the dyes were due to a non-biological oxidation, the dyes were incubated with 150 mM H_2O_2 in the absence of the enzyme. None of the dyes showed any change in absorption after 30 minutes of incubation.

The complexity of the dye structure was found to have an influence on the decolorization rate by LiP. The diverse structures of dyes might affect the approach of ligninase cation radicals to dye molecules. Each dye molecule contained a chromophore, and its color disappeared only after the chromophore structure was destroyed, which might need many attacks of LiP radicals. High dye concentrations implied less average attacks of LiP to each dye molecule, and hence slower color removal rate (Young & Yu, 1997).

Of the different conditions under which decolorization was studied, presence of 0.8 mM veratryl alcohol gave maximum decolorization of 41%. On addition of 150 mM H_2O_2, the decolorization was lowered to 31% which

might be due to the inactivation of LiP at high H_2O_2 concentration, which could also be inferred from the low decolorization of 10% when only 150 mM H_2O_2 was added (Fig. 51). An overdose of H_2O_2 would cause a decline in decolorization was confirmed by Young and Yu (1997).

Of the varying concentrations of veratryl alcohol used in dye decolorization, 47% decolorization was obtained at 0.3 mM veratryl alcohol, which was found to be the optimum concentration required for maximum decolorization. Further increase in veratryl alcohol concentration, to above 0.3 mM, had little positive effect (Fig. 52). This concentration was much lower than that reported by earlier workers where 1 mM veratryl alcohol was an absolute requirement for decolorization (Young & Yu, 1997; Ollikka et al., 1993). The need of veratryl alcohol to bring about decolorization was confirmed by the higher decolorization in its presence. Ollikka et al. (1993) reported that the ability of the purified isoenzymes of LiP to decolorize the dyes was greatly decreased when veratryl alcohol was omitted from the reaction mixture, suggesting that it acted as a mediator in the reaction. The low percentage of decolorization brought about even in the absence of veratryl alcohol might be due to the fact that some dyes acted as substrates for LiP compound II, while others were unable to do so. Besides, the LiP isozymes had different specificity for dyes as substrates.

From the above results it could be concluded that the purified LiP of *Aspergillus* sp. SIP 11 had a certain dye decolorization capacity and could be potentially useful for the development of industrial effluent treatment systems.

Table 4. Purification Protocol of LiP

Sl.No.	Purification Protocol	Reference
1	Ammonium sulphate precipitation Sephacryl 3-200 HR DEAE Sepharose CL 6B Mono Q HR-55	Shin et al., 1997
2	Ultrafiltration DEAE – Bio Gel A	Umezawa et al., 1986
3	Ultrafiltration DEAE Biogel A IEF	Hammeli et al., 1986
4	Ultrafiltration IEF	Ollikka et al., 1993
5	Ultrafiltration DEAE Biogel A IEF	Waldner et al. 1988
6	Ammonium sulphate precipitation Sephadex G-25	Vyas & Molitoris, 1995
7	Ultrafiltration Polybuffer Exchanger PBE-9	Paszczynski et al., 1986
8	Ultrafiltration DEAE-Biogel A Mono Q HR 5/5	Tien & Kirk,1984,1988 Kirk et al., 1986
9	Ultrafiltration DEAE Sepharose CL 6B Sephadex G10	deJong et al., 1992
10	Ammonium sulphate precipitation DEAE – Toyopearl 650M Sephadex G-100 Mono Q HR 16/10 column	Maltseva et al., 1991
11	Ammonium sulphate precipitation DEAE – A50 Sephadex Concanavalin – A Sepharose	Evans et al., 1984

Table 5. Characterization of LiPs from different Fungi

Sl. No.	Microorganism	Optimum pH	Optimum temp. (°C)	Mol.wt. (kDa)	K_m value	v_{max}	Purification fold/ recovery (%)	Izo enzymes	pI	Inhibitors	Ref.
1	*Phanerochaete chrysosporium* (BKM-F-1767)	2.5	39	38 – 46	60 μM = VA 80 μM = H_2O	-	40.5	H1, H2, H6, H7, H8*, H10	-	-	Tien & Kirk (1988)
2	*Pleurotus ostreatus*	3.0 – 3.5	25	71	32.9 μM = H_2O_2	8.37 μmol Min^{-1} mg = H_2O_2	9.9	-	3.0	1 μM of $Na_2S_2O_5$, KCN, NaN_3 cysteine	Shin et al. (1997)
3	*Phanerochaete chryosporium* (BKM 1767)	3.0	-	30 μM = H_2O_2	-	8.4 μM = H_2O_2	48	-	3.5	-	Tien & Kirk (1984)
4	Phlebia radiata 79	-	-	52 – 45	-	-	-	LIP 1,2,3	3.2, 3.9, 4.1	-	Niku Paavola (1990)
5	*Phlebia radiata* (L12-41)	-	-	39 – 40	-	-	-	L1, L2	3.1, 4.2	-	Hatakka et al. (1991)
6	*Phebia tremell osa* (2845)	-	-	35 – 46, 38 – 39	-	-	-	L1, L2, L3	3.1, 3.5, 4.0	-	Hatakka et al. (1991)
7	*Coriolus versicolor* (28-A)	3.0	-	50*, 25 – 73.5	-	-	3.5 fold	-	-	-	Dodson et al. (1987)
8	*Phanerochate chrysosporium*	2.3, 3.5, 3.2		41 – 42	85 – 140 μM=H_2O_2, 83 – 200 μM = VA	-	-	1, 2, 3, 4, 5	-	-	Glumoff et al. (1990)
9	*Phanerochaete chrysosporium* (BKM 1767)	-	-	38 – 43	86 – 480 μM = VA, 13 – 77 μM = H_2O_2	-	-	H1, H2, H6, H7, H8, H10	3.5 – 4.7	-	Farrell et al. (1989)
10	*Phanerochaete chrysosporium* (F-1767)	-	-	-	0.33 mM, 0.12 mM = VA 0.23 mM, 0.2 mM, 0.15 mM = H_2O_2	-	-	H2, H7, H8	3.8, 5, 4.65, 4.15	-	Ollikka et al. (1992)

*predominant isoenzyme, VA - Veratryl alcohol, *dominant band

Table 6. Purification of LiP

Fraction	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	262810	798.68	329.06	100	1
$(NH_4)_2SO_4$ precipitation	97694	246.7	396.00	37.2	1.2
DEAE Sepharose CL 6B	75042.7	12.06	6222.4	28.5	18.91
Biogel P-100	49122.8	6.2	7923	18.7	24.07

Table 7. Effect of metal ions on LiP

Metal ions	Inhibition (%)		Metal ions	Inhibition (%)	
	1mM	10mM		1mM	10mM
AgCl	17.5	27	$CoCl_2$	41	50
HgCl	4.5	17	NH_4MoO_3	46	95
$NiCl_2$	0	0	$MgCl_2$	47	50
K_2Cr_2O7	100	100	KCl	31	47
$CuCl_2$	67	68	$MnCl_2$	44	52
$CdCl_2$	40	49	$CaCl_2$	50	61
NaCl	10	28	$FeCl_3$	0	0

Table 8. Decolorization of different dyes by purified LiP

Dye	% Decolorization
Crystal violet	41± 5
Methylene blue	5 ± 1
Bromophenol blue	-
Methyl orange	36 ± 4
Poly R-478	adsorbed
RBBR	8 ± 2

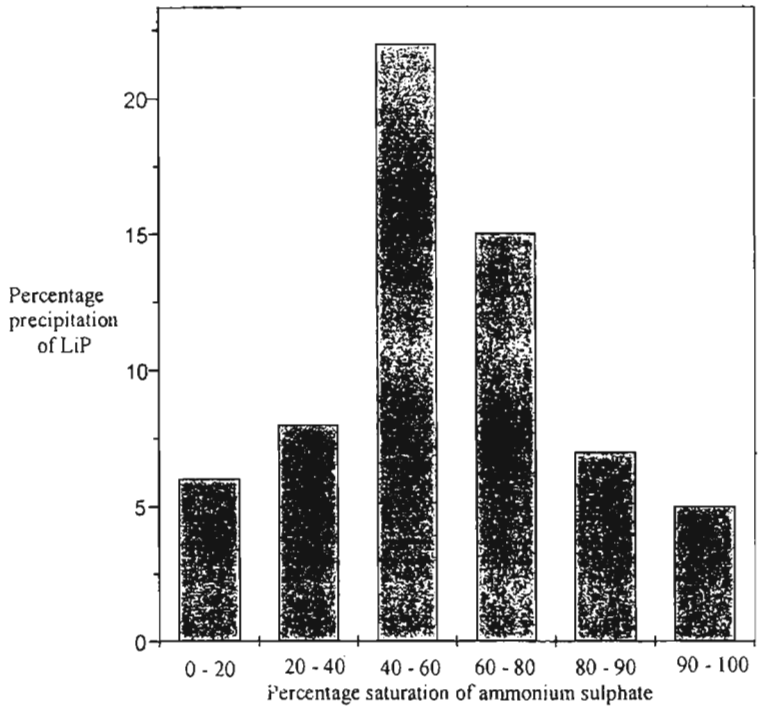


Fig. 41 Ammonium sulphate precipitation of LiP

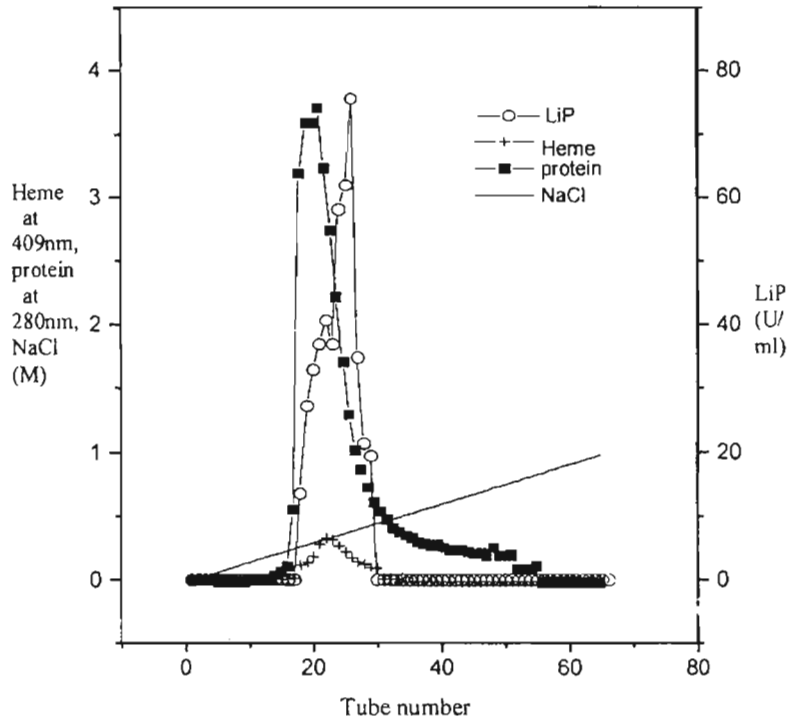


Fig. 42 Ion exchange of LiP using DEAE Sepharose CL 6B

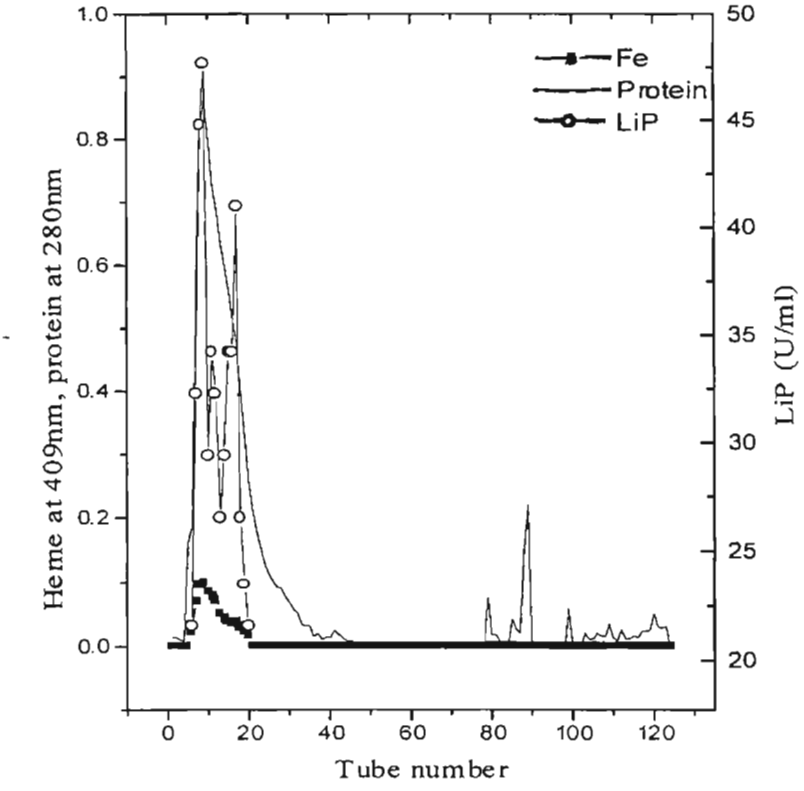


Fig. 43 Gel permeation chromatography of LiP using Biogel P 100

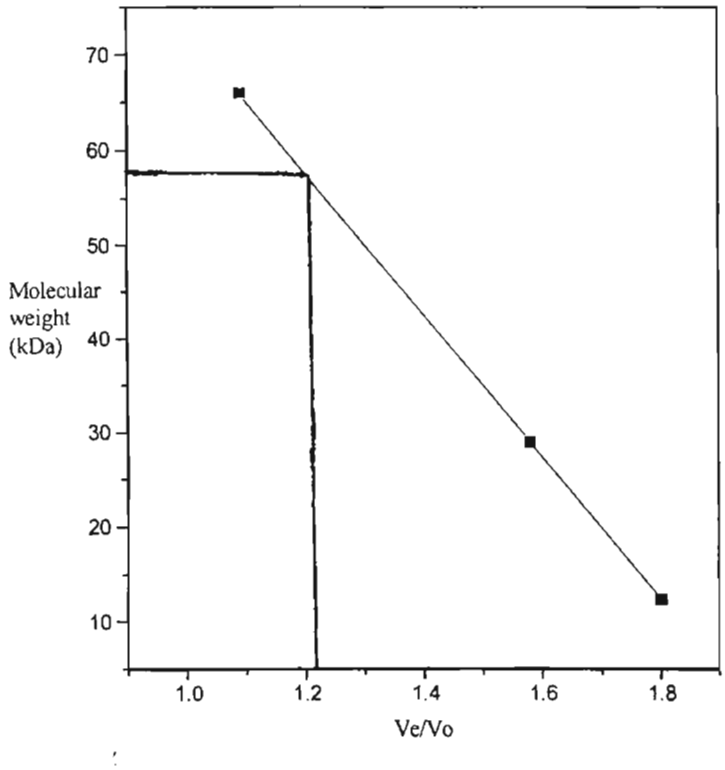


Fig. 44 Determination of molecular weight of LiP using GPC

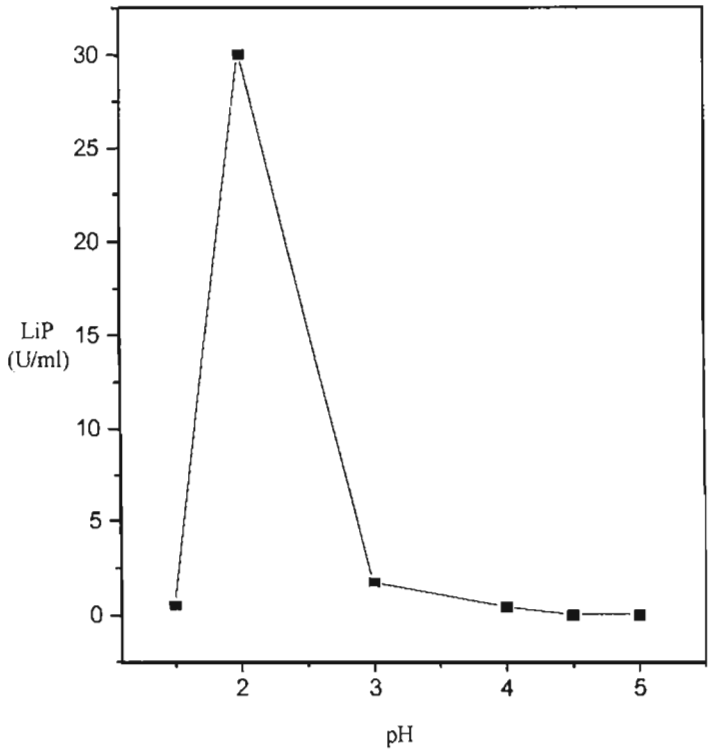


Fig. 45 Effect of pH on purified LiP

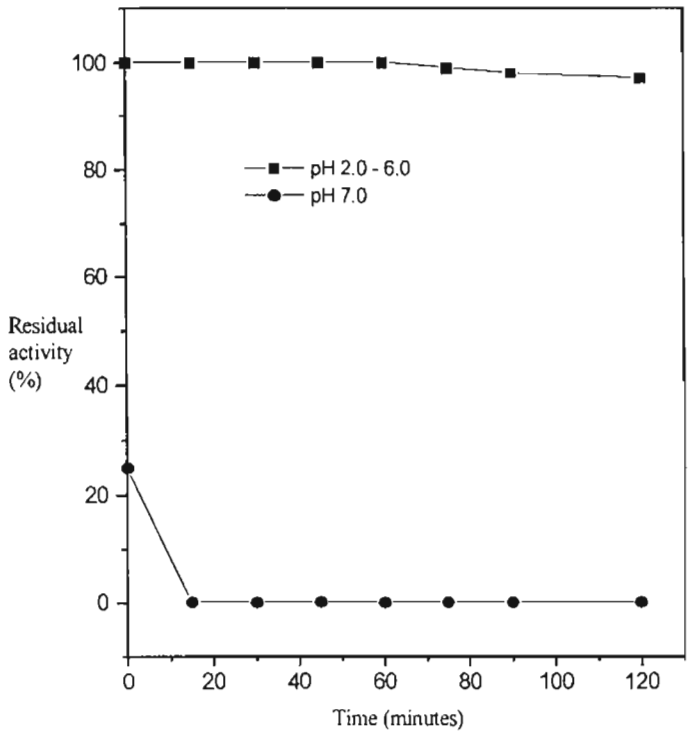


Fig. 46 Stability of LiP at different pHs

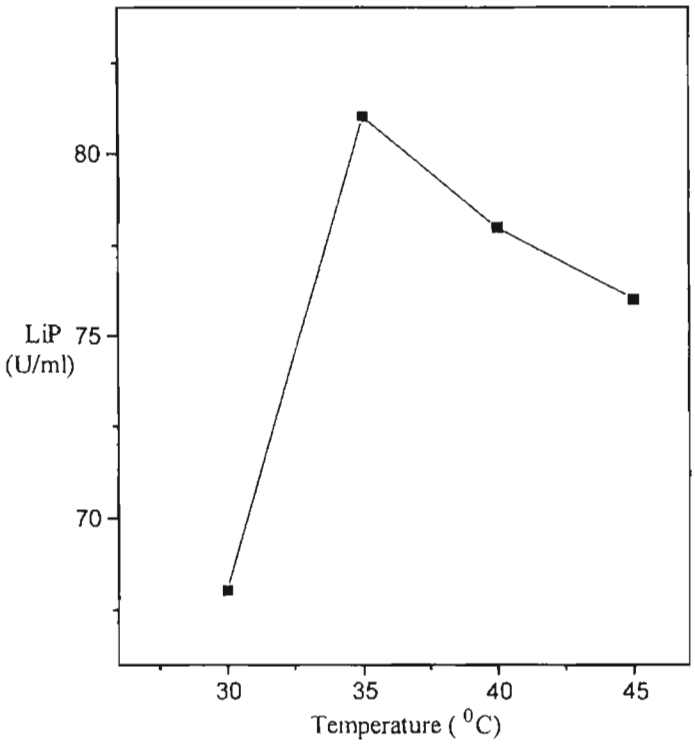


Fig. 47 Effect of temperature on purified LiP

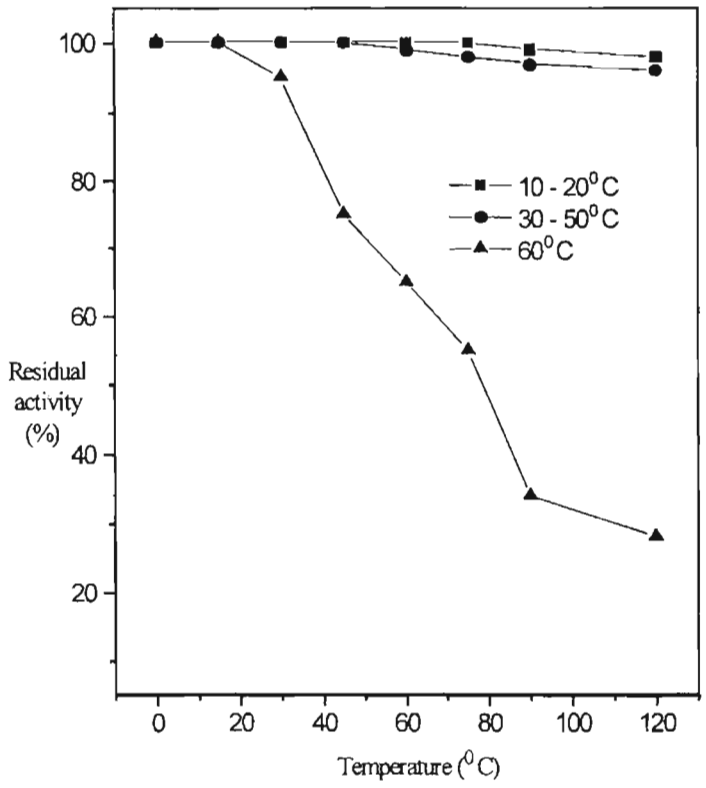


Fig. 48 Stability of LiP at different temperatures

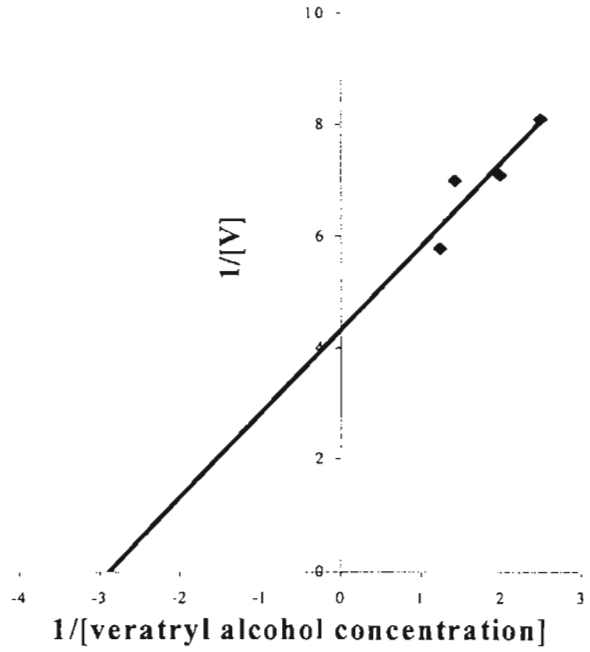


Fig. 49 Lineweaver Burk plot using veratryl alcohol as substrate

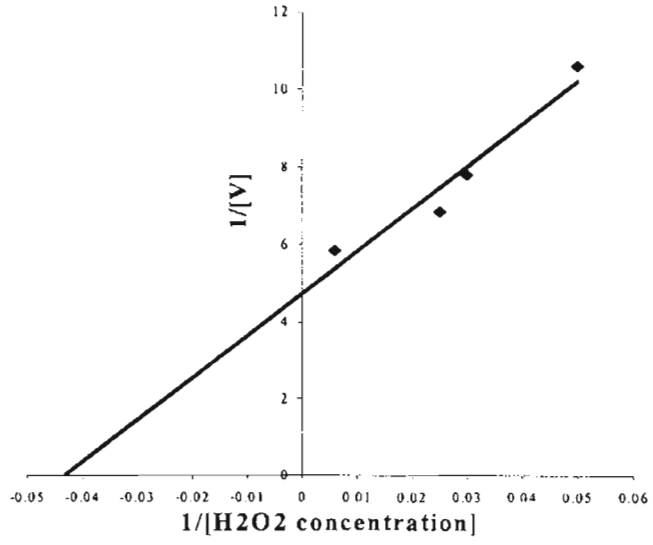


Fig. 50 Lineweaver Burk plot using H_2O_2 as substrate

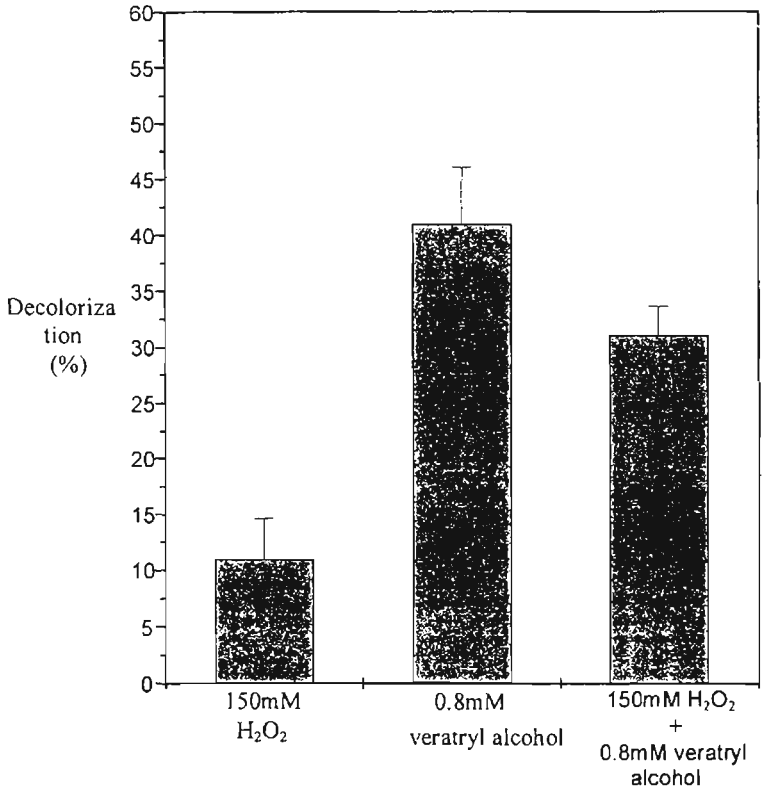


Fig. 51 Decolorization of crystal violet under different conditions

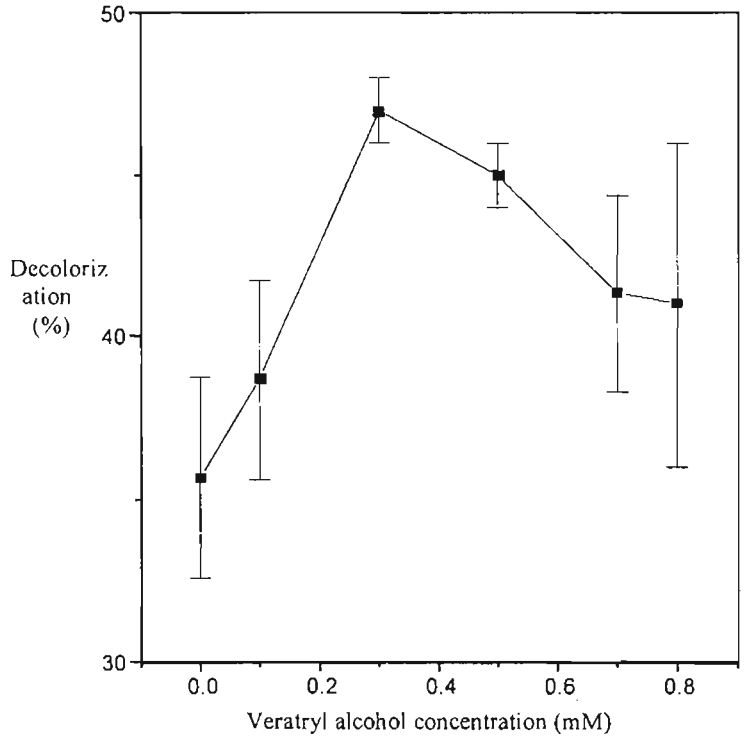


Fig. 52 Decolorization of crystal violet under different veratryl alcohol concentrations

Chapter 6

Summary and Conclusion

The continually growing worldwide hazardous waste problem is receiving much attention lately. The development of cost effective, yet efficient methods of decontamination are vital to our success in solving this problem. One such method that has become increasingly popular is bioremediation. The use of indigenous or introduced micro-organisms to decontaminate waste sites provides a very attractive economic solution.

Bioremediation using white rot fungi, a group of basidiomycetes characterized by their ability to degrade lignin by producing extracellular LiP, MnP and laccase have come to be recognized globally which is described in detail in **Chapter 1**. Because of the non-specificity of substrates for these enzymes recalcitrant pollutants like synthetic chlorinated pesticides, polyaromatic hydrocarbons, synthetic dyes, polychlorinated biphenyls and nitroaromatic explosives are all effectively degraded to CO_2. These organisms do not require preconditioning to a particular pollutant, can effectively grow in inexpensive agricultural wastes under nutrient deficient condition and could produce extracellular enzymes which are non-specific in their enzyme action. These features provide them with tremendous advantages over other micro-organisms. Besides, the lignin degrading ability make them useful in other biotechnological applications like the biobleaching of paper pulp and in the digestibility enhancement of fodder.

For the development of a highly efficient bioprocess using white rot fungi, it is necessary that they produce LiP, H_2O_2 and veratryl alcohol continuously and coordinately under acidic conditions. The slow growth of the fungi, the late production of LiP, only on the sixth and seventh day of incubation, the low stability of the enzyme and the need of an expensive compound, veratryl alcohol, in the medium make them rather unsuitable for large scale commercialisation. The industrial effluents are also highly saline

making it almost impossible for even the most potent of the white rot fungi to grow without subjecting the effluents to costly desalination processes.

In order to address the above problems, the isolation of new strains of marine microbes was initiated. **Chapter 2** deals with the isolation and screening of lignin degrading enzyme producing micro-organisms from mangrove area. Marine microbes of mangrove area has great capacity to tolerate wide fluctuations of salinities. Mangrove leaves consist of lignocellulosic structural polymers and soluble organics which form substrates for the growth of lignin degrading micro-organisms. Marine fungi, especially the Ascomycetes and the Deuteromycetes, are least studied in respect of their lignin degrading ability so that the probability of isolating a better strain was high. Primary and secondary screening for lignin degrading enzyme producing halophilic microbes from mangrove area resulted in the selection of two fungal strains from among 75 bacteria and 26 fungi. The two fungi, SIP 10 and SIP 11, were identified as *Penicillium* sp. and *Aspergillus* sp. respectively belonging to the Class Ascomycetes. Of the lignin degrading enzymes, only LiP was produced by this fungi with maximum activity of 67 U/ml on the tenth day of incubation by *Aspergillus* sp. SIP 11 and 21 U/ml on the eighth day by *Penicillium* sp. SIP 10 in aerated and agitated conditions. Crude LiP showed the following characteristics:

- Optimum veratryl alcohol concentration of 0.8 mM for *Aspergillus* sp. and 1 mM for *Penicillium* sp. SIP 10.
- Optimum H_2O_2 concentration of 150 mM, pH 3.0 and temperature 30°C.
- Stable for two hours at a pH range of 2.0 to 5.0 and a temperature range of 20°C to 60°C.

Of the two fungal isolates, as *Aspergillus* sp. SIP 11 produced highest LiP activity, this species was selected for further studies.

For maximizing the production of LiP by *Aspergillus* sp. SIP 11, optimization of cultural and nutritional parameters was done. This is dealt with in **Chapter 3**. The optimized conditions were found to be as follows:

- pH - 4.0, temperature - 30°C, agitation - 80 rev. min^{-1}, aeration per day - 4 hours.
- Glucose - 1%, KH_2PO_4 - 0.1%, $(NH_4)_2SO_4$ - 12.5mM, $MgSO_4.7H_2O$ - 0.1%, coir pith - 3%.
- Salinitiy of 35 g/kg.

Under these conditions, in a carbon and nitrogen sufficient medium, a maximum LiP activity of 345 U/ml (862.5 U/mg protein) was obtained on the second day of incubation in the absence of veratryl alcohol. Thus, a five fold increase in activity with an early production of the enzyme was obtained when compared to the non-optimized medium.

Because of the several advantages offered by SSF for the production of ligninolytic enzymes and also to determine its effectiveness over SmF in LiP production by *Aspergillus* sp. SIP 11, the fungus was grown in wheat bran under SSF conditions. **Chapter 4** is on the Optimization of the growth parameters during SSF of *Aspergillus* sp. SIP 11 by which an LiP activity of 180 U/g dry wt. was obtained on the third day of incubation with the following conditions:

- Wheat bran of particle size 420 μ - 500 μ at 50% moisture level.
- Temperature of 30°C and pH 4.0.

When lignin was provided as inducer, an LiP activity of 230 U/g dry wt. was produced on the fifth day of incubation. It was, therefore clear that highest LiP titre was produced by *Aspergillus* sp. SIP 11 in SmF than SSF.

The next task was the purification of LiP from the extracellular culture medium of *Aspergillus* sp. SIP 11 in SmF, its characterization and ability to decolorize synthetic dyes as given in **Chapter 5**. As LiP was known to be an anion with a pI of 3.3 to 4.7 and molecular weight range of 38 kDa to 43 kDa, the procedure employed for purification of LiP was in that sequence, ammonium sulphate precipitation, DEAE Sepharose CL 6B ion exchange chromatography and Biogel P 100 gel permeation chromatography. Specific activity of the purified LiP was 7923 U/mg protein. The purification fold was 24.07 while the yield was 18.7%. SDS PAGE of LiP showed that it was a low molecular weight protein of 29 kDa. Zymogram analysis using crystal violet dye as substrate confirmed the peroxidase nature of the purified LiP. Besides, it showed the following characteristics.

- Optimum pH of 2.0 and temperature 30°C.
- Stable for two hours over a pH range of 2.0 to 6.0 and a temperature range of 10°C to 50°C .
- K_m value of LiP using veratryl alcohol as substrate was 0.34 mM and V_{max} of 0.227 U/mg protein.
- K_m value of the enzyme using H_2O_2 as substrate was 23 mM and V_{max} of 0.21 U/mg protein.

Effect of different metal ions and inhibitors on purified LiP was determined. It was found that 1 mM of Cr^{2+} and 0.1 mM of sodium azide and EDTA completely inhibited the enzyme while Ni^{2+} and Fe^{2+} had no effect on LiP activity

The studies on the ability of purified LiP to decolorize different synthetic dyes was done. Among the dyes studied, crystal violet, a triphenyl methane dye was decolorized to the greatest extent. Two units of purified

LiP decolorized 47% of 50 mM crystal violet dye in 30 minutes in the presence of 0.3 mM veratryl alcohol.

The present isolate, *Aspergillus* sp. SIP 11, was therefore shown to be capable of producing high LiP titres on the second day of incubation under SmF, when grown in a medium containing the lignocellulosic substrate, coir pith. Coir pith, the waste product of coir fibre is generated during the extraction of fibre from coconut husk. For every tonne of fibre about two tonnes of coir dust is produced which not only puts strain on space but also cause environmental problems in rural areas of coconut growing regions like Kerala. To utilize this cheap waste for LiP production thus provides the twin advantage of environmental clean up and xenobiotic degradation. Besides, the fungus does not require veratryl alcohol in the medium. The halophilic nature of the fungus, which grow and produce LiP only under high saline conditions, make it better able to cope with the extreme salinities met in industrial effluents. The enzyme was also found to be stable over a wide range of pHs and temperatures. In SSF condition also, this fungus was able to produce LiP on the fifth day and in higher amounts than earlier reported. The potential uses to which this fungus can be put are, therefore vast. Immobilization of *Aspergillus* sp. SIP 11 on a suitable substrate and proper bioreactor designing can open up new vistas in bioremediation.

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APPENDIX

LIST OF PUBLICATIONS/PRESENTATIONS

1. Shamla Ahammed and P. Prema (2001) Influence of media nutrients on the synthesis of lignin peroxidase from *Aspergillus* sp. Appl.Biochem.Biotechnol. (in press).

2. Shamla Ahammed and P. Prema (2000) Effect of lignocellulosic substrates on lignin peroxidase production by *Aspergillus* sp. International Conference on microbial technology - trade and public policy, July 15 - 17, Hyderabad (Abstract).

3. Shamla Ahammed and P. Prema (2000) Optimization of growth parameters on lignin peroxidase production by *Aspergillus* sp. Emerging trends in biotechnology for the new millennium, February 26 - 27, Hyderabad (Abstract).

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