Characterization of laccase from *Streptomyces psammoticus*: an enzyme for eco-friendly treatment of environmental pollutants

Thesis submitted under the Faculty of Sciences
Cochin University of Science and Technology
for the award of the degree of

Doctor of Philosophy
in
Biotechnology

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राष्ट्रीय अंतर्विषयी विज्ञान तथा प्रौद्योगिकी संस्थान

(वैज्ञानिक एंव प्रौद्योगिकी अनुसंधान परिषद्)

(पहले क्षेत्रीय अनुसंधान प्रयोगशाला)

NATIONAL INSTITUTE FOR INTERDISCIPLINARY SCIENCE AND TECHNOLOGY

(Council of Scientific & Industrial Research)

(formerly Regional Research laboratory)

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CERTIFICATE

This is to certify that the work presented in the thesis entitled **"Characterization of laccase from *Streptomyces psammoticus*: an enzyme for eco-friendly treatment of environmental pollutants"** is based on the original research done by **Mrs. Niladevi K.N** under my guidance and supervision at Biotechnology Division, National Institute for Interdisciplinary Science and Technology (CSIR), Trivandrum 695 019, India, and no part of this work has been included in any other thesis for the award of any degree.

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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 F, in the Biotechnology Division of National Institute for Interdisciplinary Science and Technology (CSIR), Trivandrum, and no part of this work has been included in any other thesis submitted previously for the award of any degree.

NILADEVI. K.N

Acknowledgements

I express my sincere gratitude to my guide, Dr. P. Prema, Scientist F, Biotechnology Division, National Institute for interdisciplinary Science and Technology, Trivandrum, for her guidance, constant encouragement and support through out my research career.

It is my privilege to place on record my gratitude to Dr. T.K. Chandrasekhar, Director, National Institute for interdisciplinary Science and Technology, Trivandrum, for providing necessary facilities for the research.

I am greatly indebted to Dr. Ashok Pandey, Head, Biotechnology Division, NIIST, Trivandrum, for his timely help, encouragement and valuable suggestions during the research period.

I am thankful to Dr. Madhavan Nampoothiri, Dr. Rajeev Sukumaran and Dr. Sudheer Kumar, Scientists, Biotechnology Division, for their valuable suggestions. I am also thankful to Dr. Emilia Abraham, Mr. Ajit Haridas, Dr. V.B. Manilal and Dr. Luxmi Varma for the help and support provided.

My profound thanks are due to Mr. M.K. Chandran and Mrs. Soumini for helping me to do the scanning electron microscopy and NMR spectroscopy. I also thank all the technical and non-technical staffs of NIIST.

I express my love and gratitude to my friends, Mrs. Nicemol, Mrs. Asha Poorna, Mrs. Anisha, Mrs. Sheejadevi, Mrs. Priya, Mrs. Smitha, Mrs. Nimi, Ms. Deepthi, Ms. Sajisha, Ms. Sasikala, Ms. Deepa and Ms. Raji for their

help and good wishes. A special word of thanks to all my friends and colleagues at Biotechnology Division, NIIST.

The fellowship received from the Council of Scientific and Industrial Research (CSIR) is duly acknowledged.

My heart-felt gratitude to my parents, sisters, brother and in-laws for their sincere love, prayers and constant encouragement during the course of my studies.

I am deeply indebted to my husband Jyothi Kumar for his love, patience and whole hearted support during my research career. Words are not enough to express my love to my son Arun, who has been the constant source of inspiration for me to finish my research work in time.

Finally to my Lord, without whom nothing would be possible but with whom - everything is!

NILADEVI K.N.

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LIST OF ABBREVIATIONS

%	percentage
°C	Degree Celcius
µL	micro liter
µM	micro molar
µm	micron meter
µmol	micro mol
^{14}C	Radioactive Carbon
ABTS	2,2'–azino–di–[3–ethyl benzothiazoline–6–sulphonic acid
APPL	Acid Precipitable Polymeric Lignin
cm	centimeter
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
Da	Dalton
DAN	1,8 Diamino naphthalene
DCP	Dichlorophenol
DEAE	Diethyl aminoethyl
DHP	Dehydrogenation polymers
DMP	Dimethoxy phenol
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
E^0	redox potential
EDTA	Ethylene diamine tetra acetic acid
g	gram
g/L	gram per liter
gds	gram dry substrate
h	hour
H_2O	Water
H_2O_2	Hydrogen Peroxide

HBT/HOBT	1-Hydroxy benzo triazole
HVA	Humic acid vitamin agar
kDa	Kilo Dalton
K_m	Michaelis constant
L	Liter
Lac	Laccase
LiP	Lignin Peroxidase
LMS	Laccase mediator system
m	meta
M	Molar
mg	milligram
mg/L	milligram per liter
mg/mL	milligram per milliliter
min	minute
mL	milliliter
mM	milli molar
mm	millimeter
MnP	Manganese Peroxidase
mv	milli volt
MW	Molecular weight
MWCO	Molecular weight Cutoff Membrane
NaCl	Sodium chloride
nkat	nano katal units
nm	nanometer
NMR	Nuclear Magnetic Resonance
o	ortho
O_2	Oxygen
P	para
PAGE	Poly acrylamide gel electrphoresis
pH	Hydrogen ion concentration
*p*HBA	para hydroxybenzoic acid

pI	Isoelectric pH
ppm	parts per million
ppt	parts per thousand
PUF	Polyurethane foam
RBBR	Remazol Brilliant Blue R
rpm	rotations/revolutions per minute
SCA	Starch Casein Agar
SDS	Sodium dodecyl sulphate
Sec	second
SEM	Scanning Electron Microscopy
SmF	Submerged Fermentation
SSF	Solid-state Fermentation
TCA	Trichloro acetic acid
Temp	Temperature
TLC	Thin Layer Chromatography
U	Unit
U/g	Units per gram
U/mg	Units per milligram
U/mL	Units per milliliter
UV	Ultra violet
v/v	volume by volume
VA	Veratryl Alcohol
V_{max}	maximum reaction velocity
vvm	vessel volume per minute
w/v	weight by volume
WRF	White Rot Fungi

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K.N Niladevi, N Jacob and P Prema. Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: purification and characterization. *(Communicated to Process Biochem)*

POSTERS PRESENTED

K.N Niladevi and P.Prema. Isolation and Screening of actinomycetes for the production of lignin degrading enzymes. National Symposium on "Developments in Biotechnology – Emerging Trends and Challenges" and first BRSI convention, November 25-27, 2004, North Maharashtra University, Jalgaon.

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K.N Niladevi and P.Prema. Immobilization of laccase from *Streptomyces psammoticus* and its application in phenol removal using packed bed reactor.International conference on New Horizons in Biotechnology and 4th BRSI convention, November, 26-29,NIIST, Trivandrum.

TRAINING ATTENDED

"Emerging Trends in Bio-analytical Techniques" organized by Thapar Institute of Engineering and Technology, Patiala, Punjab, Nov – Dec 2003.

PREFACE

The theme of the present study is pollution abatement. The study is focused on the treatment of some of the important aquatic pollutants such as dyes and phenolic compounds using laccase from actinomycetes. Chapter 1 includes a brief introduction and review of literature on the subject of study. The second chapter of the thesis deals with the isolation and screening of actinomycetes for the production of lignin degrading enzymes. Optimization of various process parameters for laccase production in submerged and solid-state fermentation has been discussed in chapter 3 and 4 respectively. Chapter 5 includes the purification and characterization of laccase. Chapter 6 describes the application studies conducted using the strain *Streptomyces psammoticus* and the laccase produced by the organism. Chapter 7 includes the overall summary and conclusions of the study.

Chapter 1.

INTRODUCTION AND REVIEW OF LITERATURE

1. INTRODUCTION

The industrial revolution has played a massive role in changing the socio-economic scenario of the modern world. Despite the large numbers of merits of industrial revolution, that have made the human life more easy and comfortable, it remains a fact that industrial revolution is one of the major causes for the environmental pollution as we see it today. Throughout the world there are various types of pollution that interfere with the quality of life for all living creatures and with the natural functioning of the Earth's ecological systems. Although some environmental pollution is a result of natural causes, such as methane emissions from cattle and toxic materials expelled from volcanoes, most pollution is caused by human activities.

Comprising over 70% of the Earth's surface, water is undoubtedly the most precious natural resource that exists on our planet. As society has become more technologically advanced, pollution has evolved from being primarily biohazards in our water to containing an ever-expanding mixture of dissolved manufactured chemicals. In addition to the increasing complexity of pollution, the sources of pollution are now evident throughout every region of the world. In response to the increases and complexity of both human and industrial pollution, methods for treating pollution have also evolved. Primarily because of cost, however, advanced treatment technologies have been implemented only selectively and non-point sources of pollution remain virtually uncontrolled. As a result, a wide range of pollutants from a variety of sources is being discharged into our water resources. Because of the biological and chemical diversity and complexity of today's population, the environment simply cannot assimilate all of these potentially harmful discharges. Furthermore, pollution regulations around the world allow chemical pollution of water resources as long as ambient water quality criteria are not exceeded thus; pollution of our water resources has become unavoidable. Since water pollution by industrial effluents has become one of the inevitable outcomes of expanding industrialization, the scientific community is focusing their attention towards developing efficient methods for the treatment of effluents. In the last few

decades much effort has been expended in developing suitable methods for protecting the 'Mother Earth' from the harmful effect of diverse pollutants. Hence the current phase of researches is two dimensional; one is aiming at developing new technologies for the welfare of humankind while the other is focusing on finding out solutions for minimizing any after effect that may be caused by the new innovations made. Thus, these two phases of researches have to be carried out in parallel to sustain appropriate development.

1.1. SOURCES OF WATER POLLUTION

There are many causes for water pollution; but two general categories exist: direct and indirect contaminant sources. Direct sources include effluent outfalls from factories, refineries, and waste treatment plants that emit fluids of varying quality directly into the water bodies. Indirect sources include contaminants that enter the water resources from soil/groundwater systems and from the atmosphere via rainwater. Whatever be the source of pollution, the magnitude of pollution is usually measured based on the nature of the compounds contaminating the water resources. Aromatic compounds with diverse structure and nature are one of the threatening sources that contribute to much of the chemical pollution in water. This group includes different phenolic, non-phenolic and substituted poly aromatic compounds of varying toxicity. Dyes and phenolic compounds are the two categories of chemical pollutants that the researchers are more concerned about, probably due to their high toxicity and abundance in the effluents.

The major sources of chemical pollution are the effluents from different industries. It is well known that textile, leather and pulp mills discharge highly colored industrial wastewater, which contains appreciable concentrations of dyes belonging to different categories. Synthetic dyes are extensively used for dyeing and printing in industries. Over 10,000 dyes with an annual production over 7×10^5 metric tones worldwide are commercially available and 5-10 % of the dyestuffs are lost in the industrial effluents (Vaidya and Datye, 1982). Color is usually the first contaminant to be recognized in wastewater. A very small amount of dye in water

(10-50 mg/L) is highly visible and affects the aesthetic merit, water transparency and gas solubility of waterbodies. Many azo dyes, constituting the largest dye group, may be decomposed into potential carcinogenic amines under anaerobic conditions in the environment (Chung and Stevens, 1993). Color removal from wastewater is often more important than the removal of soluble colorless organic substances which usually contribute the major fraction of chemical or biochemical oxygen demand (Banat et al., 1996).

Phenols are the major organic constituents found in effluents of coal conversion processes, coke ovens, petroleum refineries, phenolic resin manufacturing, herbicide manufacturing, fiberglass manufacturing and petrochemicals. The terms ''Phenols'' or ''Total Phenols'' or ''Phenolics'' are used interchangeably either to denote simple phenol or a mixture of phenolic compounds in wastewater (Beszedits and Silbert, 1990). The concentration of phenols in effluents varies from 10 to 17 x 103 mg/L. In general, COD contributed by phenolic compounds in the effluents ranges from 40 % to 80 % of the total COD. Phenols are toxic, carcinogenic, mutagenic and teratogenic (Autenrieth et al., 1991). A phenol concentration of 1 mg/L or greater affects aquatic life. Therefore, in most cases stringent effluent discharge limit of less than 0.5 mg/L is imposed (Chang et al., 1995; Tay et al., 2001).

1.2. TREATMENT OF INDUSTRIAL EFFLUENTS

In general, there are several methods for the treatment of industrial effluents that includes physical, chemical and biological methods. Physical methods aim at removing solid or liquid pollutants based on their density difference from water. They are essentially wastewater clarification methods and remove suspended or floating solids or liquids. Physical methods of effluent treatment are reverse osmosis, electrodialysis, filtration, foam separation, porous-bed filtration, adsorption etc. They help remove fine particles, and organic and inorganic dissolved materials, resulting in better water quality for re-use or disposal. The chemical method includes treatments like oxidative processes,

ozonation, photochemical and electrochemical destruction. Although different types of physico-chemical methods of effluent treatment are prevalent, they suffer from several drawbacks such as high cost, high sludge production, formation of toxic by-products etc. The disadvantages of physico-chemical methods necessitate the need for developing alternate methods of effluent treatment. It is at this particular scenario that the significance of biological methods of effluent treatment takes the course.

The biological methods of effluent treatment have gained much attention in the recent years. The biological method of purifying effluents is now becoming increasingly important because of the possibility of complete oxidation and decontamination of many impurities including toxic ones. The method also requires relatively low operating cost and simple equipments. This method makes use of the activity of microorganisms or their metabolites. In cases where the microorganisms are being used, they make use of the impurities as a nutritive substrate and form harmless oxidation products such as H_2O and CO_2. Different groups of bacteria, actinobacteria, fungi and protozoa are the commonly used biological agents. Another approach of biological treatment involves the use of microbial metabolites, which includes mainly the oxidative enzymes produced by microorganisms. The oxidative enzymes are those groups of enzymes that catalyze the oxidation-reduction reactions in a system and come under the major group called oxidoreductase. These enzymes are highly active against a wide range of compounds and are effective in the treatment of effluents from different sources especially those containing dyes, phenols and other related compounds.

1.3. RELEVANCE OF LIGNIN DEGRADING ORGANISMS AND ENZYMES IN POLLUTION ABATEMENT

The oxidative enzymes widely used for effluent treatment are lignin peroxidase, manganese peroxidase and laccases, coming under the general category called lignin degrading enzymes or ligninolytic enzymes. These enzymes are mainly produced by fungi and bacteria to achieve the task of degrading the highly

complex aromatic polymer, lignin. The treatment of industrial effluents has been carried out either with the help of lignin degrading organisms or their enzyme systems. Extensive studies have been carried out on the subject over last few decades. White-rot fungi (WRF) are among the few groups of microorganisms capable of completely degrading polymers of phenolic origin, including lignin. The random nature of the structure of lignin requires its degradation to function non-specifically; consequently other compounds of aromatic structure, including many xenobiotic compounds, are also susceptible to degradation by ligninolytic enzymes, and it is this property that confers their bioremediation potential on these organisms (Davis and Burns, 1990). The efficiency of white rot fungi in the decolourization of industrial effluents have been well accounted in the literature. Jaouani et al have reported the decolourization of olive oil mill waste waters by the white rot fungi *Coriolopsis polyzona* (Jaouani et al., 2006). Immobilized laccase from *Lentinula edodes* has been reported to be involved in the partial decolorization and decreased toxicity of an olive mill effluent (Annibale et al., 1999). Phenol is not readily biodegradable and has been reported to be toxic or growth inhibitory to most types of microorganisms, even to those species that have the metabolic capacity of using it as a growth substrate (Annachhatre and Gheewala, 1996). Phenolic removal from olive oil mill wastewater using loofah-immobilized *Phanerochaete chrysosporium* has also been reported. The system removed 90 % 0f total phenols and 50 % of COD (Ahmadi et al., 2006). Ryan et al (2007) have investigated the role of different parameters such as fungal growth, culture age and activity and enzyme (laccase) production on bioremediation of phenolic wastewaters by *T. versicolor*. They have proposed that the time of addition of the phenolic effluents to fungal cultures play an important role in the degradation of phenols by the strain.

Decolourization of dye containing effluents by different fungi has also been established. Decolourization of both synthetic and real textile waste water by *Pleurotus flabellatus* has been reported by Nilsson et al (2006). Similar studies have been reported from other white rots such as *Thelephora* sp and *Trametes hirsuta* (Selvam et al., 2003; Rodriguez Couto et al., 2006). Next to fungi, actinomycetes are the widely used organisms for effluent treatments. Dye

degradation by actinomycetes has been well documented. Works on degradation of recalcitrant azo dyes by *Streptomyces* sp were undertaken by Paszczynski et al (1991) and the authors have reported that the linkage of a guaiacol molecule onto the azo dyes facilitated the degradation of recalcitrant dyes by five of the *Streptomyces* strains tested. The influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* sp has also been studied (Pasti-Grigsby et al., 1992). Decolorization of the industrial effluents containing reactive dyes, by actinomycetes strains were also proposed (Zhou and Zimmermann, 1993). The degradation of environmental pollutants by microorganisms is invariably effected with the aid of lignin degrading enzymes, either a single enzyme being involved in the process or a group of enzymes acting synergistically. The significance of lignin degrading enzymes in the treatment of industrial effluents is immense; which necessitates a thorough understanding of lignin, lignin degrading organisms and their enzyme systems.

1.3.1. Structure of Lignin

Lignin serves as the second major reservoir of fixed carbon sources in nature, next to cellulose, comprising 15 % of the earth's biomass (Hammel, 1992). True lignin is distributed widely but not universally throughout the Plant Kingdom. It is found in all vascular plants, where it is deposited in cell walls of supportive and water conductive tissues. The three primary polymers that make up plant cell walls consist of about 35 to 50 % cellulose, 20 to 35 % hemicellulose and 10 to 25 % lignin. Lignin fills the spaces in the cell wall between different plant polysaccharides by covalent linking and thereby conferring mechanical strength to the cell wall and by extension the plant as a whole (Chabannes et al., 2001).

The chemical nature of lignin is known largely from studies of its biosynthesis, work pioneered by Freudenberg and his co-workers between about 1930 and 1965. Unlike other biopolymers, lignin contains no readily hydrolysable bond recurring at periodic intervals along a linear backbone. Instead, lignin is a three dimensional amorphous polymer containing many different stable C–C and

C–O–C linkages between phenyl propanoid monomeric units. It consists of an apparently random complex of phenolic and non-phenolic compounds (Crawford, 1981). The most common bond in lignin, the β–O–4, is a bond starting at the middle carbon atom (b) of the propyl sidechain on one repeat unit, linking through the oxygen of the next repeat unit to the number 4 carbon atom of the aromatic ring of that repeat unit (Meister, 2002).

The structural complexity of lignin makes it one of the most recalcitrant molecule and its breakdown involves multiple biochemical reactions, that has to take place more or less simultaneously; cleavage of inter monomeric linkages, demethylations, hydroxylations, side chain modifications and aromatic ring fission followed by dissimilation of the aliphatic metabolites produced. Activity against lignin has been demonstrated in a relatively limited range of microorganisms and an additional challenge for potential ligninolytic microorganisms is the need to gain access to the substrate by penetration of plant tissues. Fungi as well as lignocellulose degrading actinomycetes, accomplish this task by hyphal invasion of the various cell wall layers (Vicuna, 1988). The molecular mass (MW) of lignin is high, about 100 kDa or more, which prevents its uptake inside the microbial cell (Eriksson et al., 1990). Thus, the biological degradation of macromolecular lignin must occur through the activity of extracellular enzymes.

1.3.2. Lignin degrading organisms

1.3.2.1. White Rot Fungi (WRF)

Of all the ligninolytic groups of microorganisms, the white rot fungi (WRF) are the most efficient lignin degraders (Kirk and Farrell, 1987; Eriksson et al., 1990). In contrast to other fungi and bacteria, white rot fungi degrade lignin more extensively and rapidly than any other known group of organisms. The term white rot fungi were derived from the appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance of the substrate. In the case of WRF, lignin is degraded during secondary metabolism during their secondary metabolism since lignin oxidation provides no net energy to the fungus; synthesis and secretion

of these enzymes are often induced by limited nutrient levels; mostly carbon and nitrogen (Wesenberg et al., 2003). White rot fungi can cause selective or non-selective delignification of wood. In selective delignification, lignin is removed without any marked loss of cellulose and in non-selective delignification all the major cell wall components are degraded (Eriksson et al., 1990; Blanchette, 1995).

Among the white rot fungi, the most studied lignin degrading system is that of *P. chrysosporium*. Lignin degradation by *P. chrysosporium is* a classical secondary metabolic activity induced particularly by nitrogen starvation. This organism secretes LiP, MnP and laccase for lignin degradation (Tien and Kirk, 1983; Glenn and Gold, 1985; Srinivasan et al., 1995). Also there are reports about the production of dioxygenase, catalase, aromatic acid reductase (Leisola and Fietcher, 1985), aryl alcohol dehydrogenase, and vanillate hydroxylase and quinone oxidoreductase (Buswell and Eriksson, 1988) by *P. chrysosporium* under different conditions. An extracellular multicopper oxidase with ferroxidase activity was also discovered recently in *P. chrysosporium* but the role if any, this newly identified enzyme have in lignocellulose degradation was not studied by the authors in detail (Larrondo et al., 2003). *P. chrysosporium* has been considered as the model organism for studying lignin degradation and the enzymology and molecular biology of this organism has been studied in detail and reported by many authors (Reddy and D'Souza, 1994; Cameron et al., 2000; Macarena et al., 2005). The gene encoding lignin peroxidase of *P. chrysosporium* was first cloned by Tien and Tu in the year 1987 (Tien and Tu, 1987) and thereafter many workers have concentrated on exploring the molecular biology of *P. chrysosporium* and the different genes encoding LiP and MnP of this organism were characterized (Gold and Alic, 1993; Alic et al., 1997; Gaskell et al., 1994). Similar works were carried out with other white rot fungi like *Trametes versicolor* (Johnsson et al., 1994; Johansson and Nyman, 1996), *Phlebia radiate* (Saloheimo et al., 1989), *Bjerkandera adusta* (Kimura et al., 1991), *Pluerotus eryngii* (Ruiz-Duenas et al., 1999), *Dichomitus squalens* (Li et al., 1999) and *Ceriporiopsis subvermispora* (Lobos et al., 1998).

White rot fungi produce lignin-degrading enzymes in different combinations and based on this the fungi can be placed into different groups such as fungi

producing Lip and MnP, MnP and laccase, LiP and laccase (Hatakka, 1994). The lignin degradation capability of the fungi may differ greatly according to the group to which they belong. A recent study by Boer et al (2004) has suggested that manganese peroxidase was the main ligninolytic enzyme produced by *Lentinula edodes* although other ligninolytic enzymes have also been already reported in the species. Different species of white rot fungus *Trametes* (*T. Versicolor, T. hirsuta,* and *T. ochracea*) were reported to be producing LiP and MnP along with laccase (Tomsovsky and Homolka, 2003). The lignin degrading ability of the white rot fungus *Elfvingia applanata* has also been revealed recently and the organism was found to be producing Manganese peroxidase and laccases under ligninolytic conditions, but failed to produce LiP (Ohkuma et al., 2001). Production of lignin modifying enzymes by co-cultivated white-rot fungi *Cerrena maxima* and *Coriolus hirsutus* has been reported by Koroleva et al (2002). Arora et al (2002) has studied the ligninolytic system of two *Phlebia* sp and found that they were capable of degrading lignin selectively and hence hold better prospects in various biotechnological applications than *P. chrysosporium*. Lignin degrading ability has also been attributed to the basidiomycete *Stropharia coronilla* that secretes MnP for benzopyrene degradation (Kapich et al., 2005). Reports on lignin degrading white rot fungi from the aquatic environment are sparse as compared to the land isolates. However qualitative screening of lignocellulose-degrading enzymes in selected marine fungi has been reported (Rohrmann and Molitoris, 1992). Lignin-Modifying Enzymes of the white rot fungus, *Flavodon flavus,* isolated from a coastal marine environment has been studied in detail by Raghukumar et al (1999). The same group has done considerable work on lignin degrading enzymes from marine and facultative marine fungi. Lignin modifying enzymes of *Coriolopsis polyzona* has also been established recently (Jaouani et al., 2006). Several isozymic forms of lignin degrading enzymes have been detected in *P. chrysosporium* cultures and a number of other white-rot fungi like *Trametes versicolor, Bjerkandera adusta, Phlebia radiata.*

1.3.2.2. Brown Rot Fungi

Brown rot fungi are usually defined as those wood-rotting fungi that decompose and remove wood carbohydrate, leaving a residue of modified lignin that is typically dark brown in colour and almost equal in weight to the lignin in the original wood (Kirk and Adler, 1970). Brown rot fungi are able to mineralize the methoxyl groups of lignin, but the mineralization of other parts is much lower (Buswell and Odier, 1987). They also introduce other chemical modifications into the lignin polymer such as the removal of R–O–CH3 side chains leaving phenols behind. The studies by Kirk (1975) have shown that there was probably a significant depletion of lignin during decay by the brown rot fungus *Gleophylum trabeum*. Other species like *Wolfiporia cocos* and *Laetiporus sulphoreus* were also reported to be capable of degrading *Eucalyptus grandis* wood to some extent (Machuca and Ferraz, 2001).

1.3.2.3. Soft Rot Fungi

Soft-rot fungi degrade wood in environments that are usually found to be unfavorable for white- or brown-rot fungi, generally in wet environments. Soft rot fungi attack moist wood, producing a characteristic softening of surfaces of the woody tissues. Among the soft rot fungi, *Aspergillus* species are the important lignin degraders. It has been reported that *A. fumigatus* was capable of liberating 38 % of the initial radioactivity labeled kraft lignins as $^{14}CO_2$ (Kadam and Drew, 1986). The lignocellulose degrading ability of the ascomycete, *Phaeosphaeria spartinicola* growing on decaying salt marsh grass *Spartina alterniflora* has also been reported (Newell et al., 1994). The soil fungi *Penicillium chrysogenum*, *Fusarium solani* and *Fusarium oxysporum* were found to be mineralizing 20–27 % of ^{14}C-milled wood lignin from wheat straw in 28 days (Rodriguez et al., 1996). Degradation of phenolic compounds was established in other species of *Aspergillus* such as *A. niger* and *A. terreus* (Garcia et al., 1999). The production of lignin

peroxidase by *Aspergillus* sp isolated from mangrove region has been reported by Shamla and Prema (Shamla and Prema, 2002). Kanayama et al (2002) have isolated a strain of *Aspergillus terreus*, which produced LiP, MnP, and phenol oxidase that were capable of lignin degradation.

1.3.2.4. Non-filamentous bacteria

Bacteria are known to display an ample metabolic versatility towards aromatic substrates. This characteristic applies for naturally occurring compounds such as lignin as well as for those of Xenobiotic origin. Bacteria of several genera, including *Pseudomonas, Alcaligenes* and *Arthrobacter,* were reported to be readily degrading the single-ring aromatic compounds that build up the lignin macromolecule (Crawford, 1981). However the extent to which bacteria are able to bring about the decay of the lignin polymer itself has not been properly assessed.

The ability of a Bacillus strain to convert ^{14}C–[side chain]–lignin of spruce to $^{14}CO_2$ was elucidated by Robinson and Crawford (Robinson and Crawford, 1978). The authors hypothesized that the initial release of $^{14}CO_2$ from the side chain labeled spruce lignins was due to the degradation of ^{14}C that have been incorporated into peripheral units of the lignin which were more susceptible to attack than highly condensed lignins. Studies by Odier and Monties (1978) have established that a *Xanthomonas* strain could decompose dioxane-lignin as a sole carbon and energy source and they also observed 77 % degradation of lignin in minimal medium after 15 days of growth. However, there was a view that it could have been low molecular weight fractions of the extractive dioxane lignins that were metabolized by *Xanthomonas* (Crawford, 1981). There are no available data on degradation studies using ^{14}C labeled lignins by this bacterial strain that prevents direct comparison with that of fungi, which are reported to cause 75 % mineralization of ^{14}C – DHP. Synthetic lignin transformation has also been observed in the marine bacterium *Sagitulla stellata.* The bacterium was found to be releasing 3.5 % and 1 % $^{14}CO_2$ from [β-^{14}C] DHP and [ring-^{14}C] DHP respectively, after 30 days of incubation (Gonzalez et al., 1997). It needs mentioning that

bacteria are able to degrade some of the structural components of lignin including side chains and methoxyl groups although the ability to decompose lignin is much less widely distributed among bacteria than fungi.

1.3.2.5. Filamentous bacteria (Actinomycetes)

Among bacteria, lignin degradation has been most extensively studied in actinomycetes, particularly *Streptomyces* species. Actinomycetes are a heterogeneous group of gram-positive bacteria, of which terrigenous saprophytes are the most common forms. The growth of most actinomycetes as branching hyphae is a trait shared by the filamentous fungi and their hyphal growth form is well suited to the colonization of plant biomass and they secrete a range of enzymes active against lignocellulose (McCarthy, 1987). Lignin degradation always accompanies growth in actinomycetes and is therefore presumed to be a primary metabolic activity. The primary degradative activity of actinomycetes is solubilization of lignin with low levels of mineralization to carbon dioxide (McCarthy, 1987). Haider et al (1978) have shown that *Nocardia* species were able to decompose lignin and to assimilate lignin degradation products as a carbon source. These strains were reported to be capable of releasing $^{14}CO_2$ significantly from the methoxyl group and transforming other carbons from the phenylpropane skeleton of lignin also into $^{14}CO_2$. Although lignin-degrading ability is observed among different genera of actinomycetes, the most promising lignin degraders are those coming under the genus *Streptomyces*. The list of actinomycetes involved in the production of lignin degrading enzymes is given in Table 1.1. The studies on lignin degradation by actinomycetes were initiated early in 1970s. Sutherland et al (1979) used scanning electron microscopy very successfully to show colonization of Doughlas fir phloem by the actinomycete *Streptomyces flavovirens*. Microscopic observations indicated that the non-lignified walls of parenchyma cells were attacked first, followed by attack on the thick walled, heavily lignified scleroids. Lignin degradation studies by Phelan et al (1979) have revealed that six

Streptomyces strains were capable of degrading specifically ^{14}C lignin labeled Douglas fir lignocelluloses.

Actinomycetes	Lignin source/substrate	Reference
Nocardia	Corn stalks	Trojanowski et al., 1977
S. viridosporus	Milled wood lignin	Crawford., 1978
S. badius	Lignocellulose	Phelan et al., 1979
S. flavovirens	Douglas fir wood	Sutherland et al., 1979
S. setonii	Corn stover	Pometto and Crawford, 1986
Actinomadura spp	Ball milled straw	Mason et al., 1988
S. diastaticus	Corn stover	Pasti et al., 1990
S. rochei	Corn stover	Pasti et al., 1990
Thermomonospora mesophila	Ball milled straw	Godden et al., 1992
S. chromofuscus	Lignocellulose	Goszczynski et al., 1994
S. thermoviolaceus	Lignocellulose	Iqbal et al., 1994
S. cyaneus	Wheat straw	Berrocal et al., 1997
S. griseus	Lignocellulose	Endo et al, 2003
S. *lavendulae*	Lignocellulose	Suzuki et al, 2003
S. coelicolor	NA*	Machczynski et al, 2004

NA* Information not available in the source material

Table 1.1. Lignin degrading actinomycetes

The results also showed that it was the aromatic ring components that were cleaved by the strains and a substantial percentage of the labeled ring carbons were released as $^{14}CO_2$ while the side chain components were attacked to only a limited degree. These observations suggest that the studied strains have the ability of ring opening within an intact lignin polymer. Studies by the same authors have established that a strain of *S. badius* could release about 13 % of a ^{14}C lignin labeled lignocellulose as $^{14}CO_2$. The degradation studies on lignin model compounds using six actinomycetes strains has been reported and the results have shown that all of the six strains used for the study could demethylate the substrates and oxidize Cα on the phenyl propane side chain while two strains; *Thermomonospora mesophila* and *S. badius* utilized the compounds and produced substantial amounts of monomeric products (Godden et al., 1992).

Although the above studies were capable of giving an insight into the lignin degradative ability of actinomycetes, valuable contribution in this area was given by Crawford (1978) who has done considerable works on the lignin degrading system of *Streptomyces viridosporus* T7A including the mechanisms involved. *S. viridosporus* T7A degraded lignin oxidatively and in the process released water soluble intermediates into the culture medium, which included single ring aromatic compounds and an oxidatively modified polymer – Acid Precipitable Polymeric Lignin (APPL) (Crawford et al., 1983; Pometto and Crawford, 1986). Studies by Crawford and Borgmeyer (1985) have also elucidated the differences between APPLs produced by *S. viridosporus* and another species, *S. badius* 252, which indicated that significantly differing mechanisms of lignin metabolism existed between these two ligninolytic species. *S. viridosporus* T7A produced APPLs by oxidative depolymerization while *S. badius* produced APPLs from the repolymerization of lower molecular weight intermediates of lignin degradation with the help of an extracellular phenol oxidase. The chemistry of the APPLs produced also differed. It was observed that the APPL of *S. baduis* was less lignin like and increased substantially in average molecular weight over time while that of *S. viridosporus* was observed to be more lignin like and was slowly modified further over time.

1.3.3. Ligninolytic enzymes

The extremely complex nature of lignin requires an array of oxidative enzymes to be involved in its complete degradation. The characteristics of these enzymes differ widely with the microbial sources. The efficiency of an organism to produce one or more of these enzymes also varies greatly among different microbial groups. Although a wide range of enzymes have been reported to be involved in the tedious process of lignin degradation only a few enzymes like Lignin peroxidase, manganese peroxidase and laccases play major role and each of these enzymes exhibits specific mode of action.

1.3.3.1. Lignin Peroxidase

This enzyme commonly known as ligninase is one of the most important enzyme involved in the degradation of lignin. It was discovered in 1983 from the WRF, *P.chrysosporium*. Since then, this enzyme has been demonstrated in wide variety of organisms including brown rot fungi, soft rot fungi and filamentous bacteria. LiPs are oligomannose type glycoprotein with a molecular weight range of 38 KDa to 43 KDa (Schmidt et al., 1990). The crystal structure of lignin peroxidase (LiP) from the white rot fungus *Phanerochaete chrysosporium* was refined by Choinowski, et al (1999). They have used the program MOLSCRIPT (Kraulis, 1991) to depict the structure of LiP isozyme with an isoelectric point of 4.15 (LiP 415), and according to their study the final model comprised 343 amino acid residues, 370 water molecules, the heme, four carbohydrates, and two calcium ions. The authors also reported the evidence for a radical formation at Trp171 using spin trapping, which supported the concept of Trp171 being a redox active amino acid and being involved in the oxidation of veratryl alcohol.

LiP is having relatively high redox potential, so the compounds with high redox potentials that are not oxidized by other enzymes are also oxidized by LiP. It is this particular character of LiP that makes it an important part of ligninolytic

system. LiP can oxidize both phenolic and non-phenolic compounds. This enzyme employs free radical chemistry to cleave the propyl side chain of lignin substructures (Schoemaker et al., 1985) and have been shown to depolymerize lignin invivo (Hammel et al., 1993). LiPs have the unusual ability to cleave the recalcitrant nonphenolic units that comprise approximately 90 % of lignin (Glenn et al., 1983; Tien and Kirk, 1983). The reactions catalysed by LiP include Cα-Cβ cleavage of the propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation and even aromatic ring cleavage of non-phenolic lignin model compounds (Tien and Kirk, 1984; Renganathan et al., 1985; Umezawa and Higuchi, 1987; Chun and Aust, 1995). This heme peroxidase has a classical peroxidase catalytic mechanism for which H_2O_2 is required. The native enzyme is oxidized by H_2O_2 and generates two-electron deficient compound I. Compound I can oxidize a compound and can be reduced to compound II, which is one electron deficient. A subsequent oxidation of another molecule by compound II returns the peroxidase to its native resting stage. When there is excess H_2O_2, it will combine with compound II of LiP, generating compound III, which is an inactive form of the enzyme. In many cases, the substrates are not directly accessible to heme of LiP and thus direct oxidation of substrate does not occur. In such cases involvement of redox mediator plays an important role. Veratryl alcohol is an excellent substrate for LiP and it acts as the redox mediator for indirect oxidation of other substrates. Veratryl alcohol stimulates oxidation by preventing enzyme inactivation (Valli et al., 1990) and it is oxidized by LiP to VA cation radical, which is a strong oxidant, and it acts as an electron transfer mediator in the catalytic reaction of LiP.

Lignin peroxidase is usually produced in combination with other lignin degrading enzymes like MnP and laccases. The most studied ligninolytic organism, *Phanerochaete chrysosporium* produced LiP along with MnP (Hatakka, 1994). *Chrysosporium purinosum*, considered to be a separate strain of *P.chrysosporium*, similarly produced LiP and MnP activities (Waldner et al., 1998). *Phlebia ochraceofulva* produced higher LiP activities along with laccases

but it failed to produce MnP even at elevated Mn (II) concentrations (Sarkanen et al., 1991). LiP is also produced by ascomycetes like *Aspergillus* sp (Shamla and Prema, 2002). There are also reports that several fungi which are efficient lignin degraders in nature and especially suitable for selective lignin degradation apparently did not produce LiP (Sarkanen et al., 1991). These include *Dichomitus squalens, Lentinus edodes, Rigidoporus lignosus* and *Sterreum hirsutum* (Paice et al., 1993). 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.

Lignin peroxidase has been well established among actinomycetes. The characterization of the extracellular peroxidase of *Streptomyces viridosporus* T7A was carried out by Ramachandra et al (1987) and they proposed that the lignin-oxidizing enzyme of *S. viridosporus* T7A, designated as actinomycete lignin peroxidase P3 (ALiP – P3) was a heme protein and the enzyme has been confirmed to be capable of catalyzing Cα – Cβ. cleavage of lignin substructure model compounds. Pasti et al (1990) have isolated actinomycete strains from the gut of higher termites and found that the peroxidases of *S. Chromofuscus* A2 were superior to that of *S. viridosporus* and the rates of lignin solubilization in both organisms were correlated and subjected to glucose repression. However, it was not confirmed by these researchers whether the superior lignocellulolytic activity of *S. chromofuscus* A2 was due to the activities of other enzymes acting in concert with the peroxidases. The inducible nature of actinomycetes peroxidase has been studied and the authors have concluded that the peroxidase production by actinomycetes could be induced by growth on straw, indulin AJ, Syringic acid, 3, 4-dimethoxycinnamic acids and vanillic acid. One of the interesting works on peroxidases of actinomycetes was carried out by Mason et al (2001) who have investigated the appearance of the secreted pseudoperoxidase of the thermophilic actinomycete, *T. fusca* BD 25. The enzyme from *T. fusca* was found to be associated with the appearance of a heme like spectrum and the species responsible for this spectrum was found to be a metallo-porphyrin. Based on the above-mentioned spectral studies, they proposed that lignin degrading heme peroxidases

were not secreted by actinomycetes and the spectra recorded from culture media were due to zinc or copper containing porphyrins and not the heme. The same porphyrin was observed by this group of workers in the growth medium of the lignin solubilizing actinomycete *S. viridosporus* T7A. These results were contradictory to the reports on heme peroxidases of actinomycetes. The authors have reported that the earlier reports on heme peroxidases of actinomycetes were due to the incorrect assignment of optical spectra to heme groups rather than to non-iron containing porphyrins. Thus the studies implies that the exact nature of peroxidases from actinomycetes needs to be studied more to get a thorough knowledge on the same and in the present form the information on actinomycetes peroxidases remains incomplete with several missing links.

1.3.3.2. Manganese Peroxidase

Manganese peroxidase is another important enzyme produced by the lignin degraders. It is also a heme peroxidase and requires H_2O_2 for its activity. The redox potential of the MnP – Mn system is lower than that of LiP and normally it does not oxidize non-phenolic lignin models however Maltseva et al has reported that the MnP from *Panus tigrinus* is able to degrade nonphenolic lignin model compounds (Maltseva et al., 1991). MnP shows a strong preference for Mn (II) as its reducing substrate (Glenn and old, 1985). MnP oxidizes Mn^{2+} to Mn^{3+}, which is stabilized by organic acid chelators viz; oxalate, malonate, glyoxylate etc and acts in turn as a low molecular mass, diffusible, redox mediator that attacks organic molecule and oxidizes various compounds nonspecifically via hydrogen and one electron abstraction. The organic acids also facilitate the release of Mn (III) from the active site of the enzyme. The crystal structure of manganese peroxidase (MnP) from the lignin- degrading basidiomycetous fungus *P. chrysosporium* showed that the enzyme has two structural calcium ions. MnP also has two N-acetylglucosamine residues N-linked to Asp131 that are readily visible in the electron density map. The active site, consisting of a proximal His ligand H-bonded to an Asp residue and a distal side peroxide-binding pocket consisting of a catalytic Histidine and Arginine. The 1.45 crystal structure of MnP complexed with

Mn (II) provided a more accurate view of the Mn-binding site including possible partial protonation of Glu 39 in the Mn-binding site and glycosylation at Ser 336 (Sundaramoorthy et al., 2005). The one electron oxidation of Mn (II) to Mn (III) in a multi step reaction cycle is as follows:

MnP + H_2O_2 ---- MnP compound I + H2O (Reaction 1)

MnP compound I + Mn (II) ---- MnP compound II + Mn (III) (Reaction 2)

MnP compound II + Mn (II) ---- MnP + Mn (III) + H_2O (Reaction 3).

This enzyme is mostly reported in WRF, where it is produced in combination with LiP or laccase. There are a number of efficient delignifying fungi that secrete MnP as the sole extracellular peroxidase, including *Lentinula edodes* (Leatham, 1986), *Bjerkandera adusta* (Wang et al., 2002), *Ceriporiopsis subvermispora* (Lobos et al., 1994), *P. sordida* (Ruttimann et al., 1994), *Dichomitus squalens* (Perie et al., 1996), *Pleurotus ostreatus* (Giardina et al., 2000) and *Rigidoporus lignosus* (Galliano et al., 1991). Some other ligninolytic fungi produce MnP in combination with lignin peroxidase. *P. chrysosporium*, *P. radiata*, *T. versicolor*, and *Nematoloma frowardii* are examples for LiP – MnP group (Hofrichter et al., 2001). A manganese peroxidase (MnP) from a wood-degrading fungus *Trichophyton rubrum* LSK was characterized earlier and the enzyme has the highest p*I* of 8.2 among MnPs reported so far and when compared with other MnPs, this MnP has been reported to be more stable in the presence of high concentrations of H_2O_2 (Bermek et al., 2004). Manganese mediated lignin degradation by *Pleurotus pulmonarius* (Camarero et al., 1996) have also been studied and the results clearly indicated the stimulation of lignin mineralization by Mn2+. Two phylogenetically and structurally divergent manganese peroxidases (Pr- MnP 2 and Pr- MnP 3) from *Phlebia radiata* were recently described by Hilden et al (2005). The Pr-MnP2 with a long C-terminal extension has the highest structural similarity with the crystal structure of *P. chrysosporium* MnP1, whereas the shorter Pr-MnP3 protein was structurally more related to lignin peroxidase. Some differences between both Pr-MnPs were observed including the presence of helix G* (only in Pr-MnP2) and helix B* (only in Pr-MnP3). Both *P. radiata* MnPs have 12 helices, the position of one of the additional helices, depicted B** in the *P.*

radiata proteins was similar to those found in most fungal class II peroxidases. However, the second additional helix, helix G* in Pr-MnP2 and helix B* in Pr-MnP3, occupies very different positions. Both models also showed four-disulphide bridges (Hilden et al., 2005). Production of Manganese peroxidase by actinomycetes is not well established although there are citations on few species. The studies on biodegradation of pesticides by actinomycetes suggested the probable production of MnP by a *Streptomyces* strain CCT 4916 that was able to degrade the herbicide diuron by oxidative reactions (Esposito et al., 1998). The studies by Zou and Schrempf (2000) revealed for the first time that a bacterial catalase-peroxidase has a heme-independent manganese-peroxidase activity. The catalase-peroxidase CpeB reported by them was found to be catalyzing the peroxidation of Mn (II) to Mn (III), independent of the presence or absence of the heme inhibitor KCN.

1.3.3.3. Laccase

Laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera* in 1883 (Reinhammar, 1984). It is a polyphenol oxidase, which belongs to the family of blue multicopper oxidases. These enzymes catalyze the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water. Laccases oxidize a broad range of substrates, preferably phenolic compounds. Laccases differ from LiP and MnP in that it does not require H_2O_2 to oxidize its substrates.

1.3.3.3.1. Classification of laccases

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

Catechol oxidase or *o*-dipenol: oxygen oxidoreductase (EC 1.10.3.1)

Laccase or *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2)

L-ascorbate oxidase (EC 1.10.3.3)

These different enzymes can therefore be differentiated on the basis of substrate specificity (Walker and McCallion, 1980). There is, however, difficulty in defining laccase according to its substrate specificity, because laccase has an overlapping range of substrates with tyrosinase. Catechol oxidases and tyrosinases have *o*-diphenol as well as cresolase activity (oxidation of L-tyrosine). Laccases have ortho and para diphenol activity, usually with more affinity towards the *p*-diphenols. Only laccases have the ability to oxidize syringaldazine (Thurston, 1994; Eggert et al., 1996).

1.3.3.3.2. Sources of laccases

Plant laccases

The first laccase to be reported was from a plant source, the Japanese lacquer tree *Rhus vernicifera,* belonging to the family Anacardiaceae. All the members of this family appear to contain laccase in the resin ducts and in the secreted resin (Huttermann et al., 2001). Laccases are encoded by multigene families in plants. However, the occurrence of laccases in higher plants appears to be far more limited than in fungi. The isolation and characterisation of laccases has been obtained mainly from dicot species, such as *Acer pseudoplatanus* (Sterjiades et al 1992), tobacco (Richardson and G.J. McDougall, 1997), *Arabidopsis thaliana* (McCaig et al, 2005) yellow-poplar (LaFayette et al, 1999) and cotton (Wang et al, 2004). *Pinus taeda* tissue has been shown to contain eight laccases, all expressed predominantly in xylem tissue (Sato et al., 2001). Other reports are those of Wosilait et al. (1954) on the presence of a laccase in leaves of *Aesculus parviflora* and in green shoots of tea (Gregory and Bendall, 1966). Five distinct laccases have been shown to be present in the xylem tissue of *Populus euramericana* (Ranocha et al., 1999). Laccases have also been characterized from a few monocots such as ryegrass and maize (Gavnholt et al 2002; Caparros-Ruiz et al 2006).

Fungal laccases

Laccase activity is widely distributed among different groups of fungi. Among fungi, different genera of white rot fungus are the leading laccase producers. Many workers have done extensive studies on the production of laccase from the fungi under differing conditions. Laccase production has been carried out by submerged as well as solid-state fermentation techniques (Tong et al., 2007; Janusz et al., 2007; Rodriguez Couto and Sanroman, 2005). Solid-state fermentation has been considered more suitable for laccase production by due to their filamentous nature. However, there are few designs available in the literature for bioreactors operating in solid-state conditions for laccase production. This is principally due to several problems encountered in the control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture. SSF lacks the sophisticated control mechanisms that are usually associated with SmF. Control of the environment within the bioreactors is also difficult to achieve, particularly temperature and moisture.

The production of laccases under solid-state fermentation conditions by different fungal strains is summarized in Table 1.2. The enhanced formation of laccase by the white rot fungi *Trametes pubenscens* in the presence of copper was studied by Galhaup et al (2001). The influence of copper on laccase production has been reported earlier in *Neurospora crassa* (Huber and Lerch, 1987). A blue laccase of litter decaying basidiomycete *Stropharia rugosoannulata* capable of oxidizing Mn^{2+} in the presence of Mn^{3+} chelators has also been suggested (Schlosser and Hofer, 2002). The important laccase producing white rot fungi are *Phlebia radiata* (Niku-Paavola et al., 1990), *Ganoderma lucidum* (Perumal, 1997), *Trametes versicolor* (Swamy and Ramsay, 1999), *Cyathus stercoreus* (Sethuraman et al., 1999), *Pycnoporus cinnabarinus* (Otterbein et al., 2000), *Pleurotus ostreatus* (Palmieri et al., 2000), *Pycnoporus sanguineus* (Pointing et al., 2000), *Coriolus hirsutus and C. sonatus* (Koroleva et al., 2001), *Trametes modesta*

Support	Fungal strain	Reference
Bagasse	*T. versicolor, Pycnoporus cinnabarinus*	Pal et al., 1995; Meza et al., 2005
Banana waste	*P. ostreatus, P. sajor-caju*	Reddy et al., 2003
Canola roots	*Cyathus olla*	Shinners-Carnelley et al., 2002
Corn	*Lentinus edodes, P. pulmonarius*	D'Annibale et al., 1996; Li et al., 2000
cotton	*P. ostreatus, P. chrysosporium*	Jaszek et al., 1998; Sik and Unyayar, 1998
Sawdust	*Coriolus hirsutus*	Elisashvili et al., 2001
Wheat bran	*P. pulmonarius, Ganoderma lucidum*	Marques de Souza et al., 2002; Murugesan et al., 2007
Wheat straw	*Phlebia radiata, P. ostreatus, P. pulmonarius*	Vares et al., 1995; Baldrian et al., 2002; Marques de Souza et al., 2002
Wood	*Ceriporiopsis subvermi-spora, P. ostreatus*	Ferraz et al., 2003; Pradeep and Datta, 2002.
Orange peelings	*Trametes hirsuta*	Rosales et al., 2007
Banana Skin	*T. pubescens*	Osma et al., 2007
Groundnut shell	*P. ostreatus*	Mishra and Kumar, 2007
Grape seeds	*T. hirsuta*	Rodriguez-Couto et al., 2006
Coconut flesh	*T. hirsuta*	Rodriguez-Couto and Sanroman, 2005
Chestnut shell	*Coriolopsis rigida*	Gomez et al., 2005
Barley bran	*C. rigida*	Gomez et al., 2005
Kiwi fruit waste	*Trametes hirsuta*	Rosales et al., 2005

Table 1.2. Production of laccase by different fungal strains under solid-state fermentation

(Nyanhongo et al., 2002), *Bjerkandera adusta, Pleurotus sajor caju* (Reddy et al., 2003) and *Panus tigrinus* (Zavarzina et al., 2004). The ascomycete fungi like *Podospora anserine* (Frese and Stahl, 1992), *Trichophyton rubrum* (Jung et al., 2002), *Botryophaeria* sp (Alves-Da-Cunha et al., 2003), *Physisporinus rivulosus* (Hakala et al., 2005) also exhibits laccase activity. Laccase activity has also been reported from *Agaricus bisporus* (Smith et al., 1998), the hyphomycete, *Chalara paradoxa* (Robles et al., 2000) and ascomycetes such as *Mauginiella* sp (Palonen et al., 2003), *Trichoderma atroviride, Trichoderma harzianum* (Holker et al., 2002) and *Melanocarpus albomyces* (Kiiskinen and Saloheimo, 2003). Gonzales et al (2002) have reported that the fungus *Petriellidium fusoideum* produces laccase as the only enzyme involved in lignin metabolism and the mineralization of synthetic lignins by the laccase of this organism has also been discussed.

Bacterial laccases

There is increasing evidence for the existence of typical multi-copper laccases in prokaryotes (Alexandre and Zhulin, 2000; Claus, 2003). Recent rapid progress in the whole genome analysis suggests that laccases are widespread in bacteria (Sharma et al., 2007). Corresponding genes have been found in gram-negative and gram-positive bacteria, including species living in extreme habitats, e.g. in *Oceanobacillus iheyensis* or *Aquifex aeolicus* (Deckert et al., 1998), *Thermus thermophilus* (Miyazaki, 2005) and in the archaebacterium *Pyrobaculum aerophilum* (Fitz-Gibbon et al., 2002). Laccases have been identified also in *Pseudomonas stutzeri* (Kumar et al. 2005). The first convincing data for a prokaryotic laccase activity was presented for *Azospirillum lipoferum* (Givaudan et al., 1993). It is a multimeric enzyme, composed of a catalytic subunit and one or two larger chains. *Marinomonas mediterranea* is a melanogenic marine bacterium expressing both an SDS-activated tyrosinase and a laccase (Sanchez-Amat and Solano, 1997). The laccase, which was heterologously expressed in *Escherichia coli*, revealed the typical copper-binding domains of laccases and two additional potential copper-binding sites near the N-terminus (Sanchez-Amat et al., 2001). A

laccase-like enzyme activity has been detected in spores of a *Bacillus sphaericus* strain (Claus and Filip, 1997). Recently the spore protein CotA of *Bacillus subtilis* has been recognized to be a laccase (Hullo et al., 2001, Jones and Henriques, 2002; (Martins et al., 2002). Laccase-like activity has also been found in other bacteria, e.g., CopA protein from *Pseudomonas syringae* (Mellano and Cooksey, 1988) and PcoA protein from *Escherichia coli* (Brown et al., 1995). Recently, a protein encoded by ORF bh2082 of *Bacillus halodurans* C-125 has been identified as a potential bacterial laccase by genome mining. The enzyme showed an alkaline pH optimum with syringaldazine as the substrate (Ruijssenaars and Hartmans 2004). Dalfard et al (2006) have demonstrated laccase activity in a newly isolated *Bacillus* strain HR03 which exhibited laccase activity along with cresolase and cathecolase activities.

Actinomycete laccases

Although some specific phenol oxidases (Yoshimoto et al., 1985) (tyrosinases) has been described in actinomycetes, the presence of phenol oxidases, possibly involved in the lignin degradation have been reported only in a few *Streptomyces* species. Extracellular phenol oxidase produced by *S. badius* has been implicated in lignin metabolism, which involved the production of low molecular weight intermediates of lignin degradation and the repolymerization of the intermediates to produce APPL (Borgmeyer and Crawford, 1985). The phenol oxidase secreted by *S. cyaneus* CECT has been reported as capable of solubilizing and mineralizing the lignin fraction of lignocellulose at the rate of 44.96 % and 3.41 % respectively after 21 days of incubation and it has been cited as the first report on laccase activity produced by a *Streptomyces* strain during growth in SSF (Berrocal et al., 1997). The presence of a laccase like phenol oxidase (Epo A) has been reported in *S. griseus* (Endo et al., 2003) and the enzyme shared many of the characters of typical laccase but failed to oxidize compounds like guaiacol and syringaldazine that were known model laccase substrates. A recent work by Arias et al (2003) elucidated the potential of extracellular laccases from *Streptomyces* for use in combination with

mediators for biobleaching of Kraft pulps. Suzuki et al (2003) have identified a thermo stable laccase from *S. lavendulae*. The structural details of actinomycete laccases were also unveiled in the recent years. Machczynski et al (2004) have identified a new family of laccases – the two domain laccases in *S. coelicolor* that lacked the second domain at the substrate binding cleft and it has also been claimed to be the first known paramagnetic NMR spectrum for the trinuclear copper cluster of laccase. The results of the above study are of great significance as it revealed the occurrence of a new laccase family in *Streptomyces,* which could instigate the search for different types of laccases from actinomycetes.

Insect laccases

Laccases have been detected in the cuticles of several dipterans like *D. virilis* (Yamazaki, 1969) and *L. cuprina* (Barrett, 1987), as well as in the lepidopteran *B. mori* (Yamazaki, 1972). In insects, several forms of laccases have been identified. "Laccase-1" and "laccase-2" have been identified in *Manducca sexta* and *Tribolium castaneum (*Arakane et al. 2005, Dittmer et al. 2004). Suderman et al., (2006) have investigated the role of laccase in insect cuticle sclerotization. Different isoforms of laccase have also been documented in *Drosophila melanogaster* and *Anopheles gambiae* (Arakane et al. 2005). In *Anopheles gambiae,* 5 different forms of laccases have been identified (Dittmer et al. 2004). A feature that is unique to insect laccases (relative to fungal and plant laccases) is a longer aminoterminal sequence characterized by a region with conserved cysteine, aromatic and charged residues (Dittmer et al. 2004). Hattori et al. (2005) have detected laccase in the salivary glands of the green rice leafhopper, *Nephotettix cincticeps.*

1.3.3.3.3. Functions of laccases

Laccases are distributed widely among fungi, bacteria and also in higher plants. Hence the properties of different laccases show a great deal of divergence

according to the source of its origin. It has been proposed that plant laccases could be implicated in the polymerization step of the lignification process. The possibility that laccases are involved in the lignification process in higher plants was first raised by Freudenberg (1958) and confirmed by Sterjiades et al (1992). This group did suggest, however, that laccase was involved only in the early stages of lignification, while peroxidases were involved later. Bao et al. (1993) showed that laccase activity was correlated with lignification of xylem in *P. taeda.* In the cases when laccase activity has been correlated with lignification, it has been located in or near the cell walls of lignifying cells. In other tissues, such as leaf or stem tissue, neither the cellular nor the sub-cellular location has been determined except in the case of the resin ducts of the Anarcardiaceae. Hence there is a lack of information about some of the basic factors relating to the exact function of laccases in plants (Mayer and Staples, 2002).

Information on the role of fungal laccases is more abundant in the literature. Fungal laccases play important role in physiological processes related to pathogenesis, morphogenesis, i.e. fruitbody development, pigmentation and to cell detoxification. One of the laccases of the edible mushroom *Lentinula edodes* has been assigned a role in fungal morphogenesis (Zhao and Kwan, 1999), and laccase has been reported to be specifically expressed in the green-spored conidia of *A. nidulans* (Aramayo and Timberlake, 1990; Clutterbuck, 1972). *Aspergillus fumigatus*, a filamentous fungus producing bluish-green conidia, is an important opportunistic pathogen that primarily affects immuno-compromised patients. Conidial pigmentation of *A. fumigatus* significantly influences its virulence. The conidial pigmentation of this fungus has been reported as being regulated by a cluster of genes such as abr1 and abr2. The abr1 gene (aspergillus brown 1) possessed two signatures of multicopper oxidases while the abr2 gene product showed homology to the laccase of *Aspergillus nidulans* (O'Hara and Timberlake,

1989). Similarly, laccase has been identified as responsible for the virulence of the encapsulated fungus *Cryptococcus neoformans*, which is a human pathogen (Williamson, 1997). In *C. neoformans*, laccase is present as a tightly associated cell wall enzyme that is readily accessible for interactions with host immune cells (Zhu et al., 2001). The grapevine mould, *Botrytis cineria* produces a laccase that is necessary for pathogenesis, and the role of the laccase is presumably related to detoxification of toxic defence metabolites produced by the plant (Bar-Nun et al., 1988). Laccases have also been shown to be important for pathogenesis in the chestnut blight fungus *Cryphonecteria parasitica* (Choi et al., 1992; Mayer and Staples 2002). Laccases have also been proposed to participate in fungal morphogenesis in *Armillaria* sp, *Lentines edodes* and *Volvariella volvacea* (Worral et al., 1986; Leatham and Stahmann, 1981; Chen et al., 2004).

The bacterial laccases have been assigned specific roles in relation to their location in the cell. The cot A laccase of *Bacillus subtilis*, which is a component of the endospore coat was found to have a role in the appearance of a brown pigment characteristic of colonies in the late stages of sporulation (Donovan et al., 1987; Rogolsky, 1968) which appears to protect spores against UV light (Hullo et al., 2001). The CopA protein from *Pseudomonas syringae* and PcoA protein from *Escherichia coli* have been shown to be important for bacterial copper resistance (Mellano and Cooksey 1988; Brown et al. 1995). EpoA from *Streptomyces griseus* appears to have a role in morphogenesis in *Streptomyces* sp (Endo et al., 2002).

Laccase is hypothesized to play an important role in insect cuticle sclerotization by oxidizing catechols in the cuticle to their corresponding quinones, which then catalyze protein cross-linking reactions (Dittmer et al, 2004). Salivary laccase from the green rice leafhopper has been reported to be involved in the rapid oxidization of toxic monolignols, resulting in the formation of nontoxic polymers that allows the insect to feed successfully (Hattori et al. 2005).

1.3.3.3.4. Structure of laccases

The overall structure of laccases comprises three cupredoxin-like domains; A, B and C, that are about equal in size. (Ducros et al., 1998; Piontek et al., 2002). All three domains are important for the catalytic activity of laccases: the substrate-binding site is located in a cleft between domains B and C, a mononuclear copper centre is located in domain C, and a trinuclear copper centre is located at the interface between domains A and C. The crystal structure of laccases from different fungi has been elucidated. Ducros et al (1998; 2001) reported the crystal structure of a laccase from the fungus *Coprinus cinereus*. This was found to be a copper type-2-depleted form in which the putative T2 copper was completely absent and therefore was in a catalytically incompetent state. The difficulties in successfully crystallizing the active form of laccase have been unanimously attributed to the occurrence of extensive microheterogeneity, presumably caused by variable glycosylation of the enzyme. Unfortunately, deglycosylation to obtain high quality diffracting crystals of the *C. cinereus* laccase (CcL) resulted in the loss of copper. Antorini et al (2002) have carried out the purification and crystallization of laccase isozymes from two white rot fungi, *Trametes versicolor* and *Pycnoporus cinnabarinus*. *T. versicolor* laccase was crystallized in two crystal forms, both with the orthorhombic space group P212121, which diffracted to 1.9 and 2.95 Å resolution, respectively. The crystals of *P. cinnabarinus* laccase belonged to the monoclinic space group C2 and diffracted to at least 2.2 Å resolution. All the laccase crystals were suitable for X-ray structure determination and contained full complement of copper ions. (Antorini et al., 2002). The laccase isozymes from *T. versicolor* had a sequence identity of about 70% to the laccase from *C. cinereus*, but the value dropped to about 55% for *P. cinnabarinus* laccase isozymes with the *C. cinereus* laccase (Antorini et al., 2002).

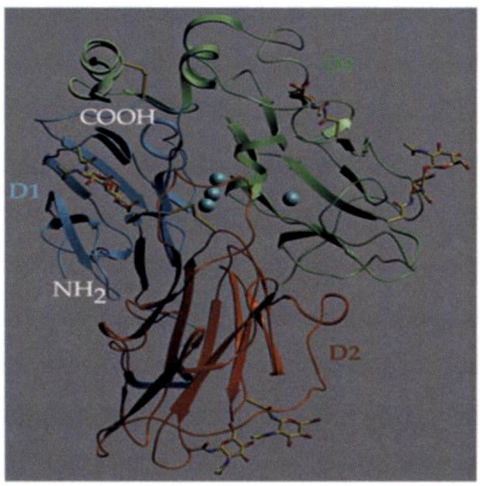


Fig. 1.1. Ribbon diagram of laccase from *Trametes versicolor* (TvL)

The arrangement of the domain structure is depicted in different color coding (D1–D3). Copper ions are drawn as blue spheres. Carbohydrates and disulfide bonds are included as stick models (Piontek et al., 2002)

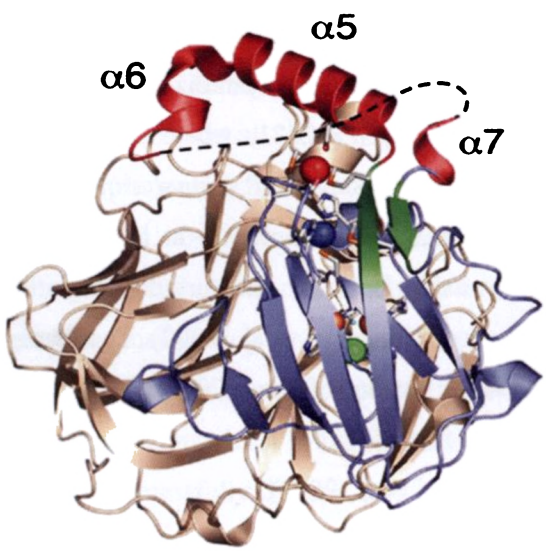


Fig. 1.2. Ribbon diagram of laccase from *Escherichia coli* (CueO)

The domain 3 of CueO is shown in blue and red (the antiparallel connections of the first and second β-strands of domain 3 are highlighted in green). Red, blue, green, and orange spheres indicate the labile (regulatory) Cu (rCu), type I Cu, type II Cu and type III Cu ions respectively

The crystal structure of a *T. versicolor* laccase (TvL) in its oxidized, copper-complete state has been described by Piontek et al (2002). This structure has given insight into the coordination of all the four copper centers in the fully active enzyme. The geometry of the trinuclear copper cluster in TvL was similar to that found in the ascorbate oxidase and that of mammalian ceruloplasmin structures, suggested a common reaction mechanism for the copper oxidation and the O2 reduction (Piontek et al., 2002). The TvL structure was a monomer organized in three sequentially arranged domains and had dimensions of about 65 x 55 x 45 Å3. Each of the three domains was of a similar β-barrel type architecture. The tri-nuclear copper cluster (T2/T3) was embedded between domains 1 and 3 with both domains providing residues for the coordination of the coppers. The third domain had the highest helical content with one 3_{10}-helix and two ∝-helices located in the connecting regions between the strands of the different β-sheets. Finally, at the C-terminal end of domain 3, three sequentially arranged ∝-helices complete the fold. A 13-aminoacid- long ∝-helix at the C-terminal portion was stabilized by a disulfide bridge to domain 1 (Cys-85–Cys-488), and a second disulfide bridge (Cys-117–Cys-205) connected domains 1 and 2. Both N-terminal and C-terminal amino acids benefited from hydrogen bonding networks to the rest of the protein, which provided sufficient rigidity so that excellent electron density was observed for these regions in the crystal structure.

The structure of *Rigidoporus lignosus* laccase (RlL) containing a full complement of copper ions has been elucidated by Garavaglia et al (2004). Structural comparison between RlL and TvL revealed only subtle differences. RlL folded into three sequentially arranged domains, each of them with a β-barrel type topology. As observed in all laccases, the type-1 copper was located in domain 1 whereas the trinuclear copper cluster (T2/T3) was embedded between domains 1 and 3. The structure was stabilized by two disulfide bridges: Cys85-Cys487 located between domain I and III; and Cys117-Cys210 connecting domains 1 and 2. The crystal structure of CueO (Fig. 2) from *Escherichia coli* has also been determined (Roberts et al., 2002; Roberts et al., 2003). Differing from other MCOs, the substrate-binding site of CueO is deeply buried under a methionine-rich helical

region including α-helices 5, 6, and 7 that interfere with the access of organic substrates (Kataoka et al., 2007). The asymmetric unit contained two Δα 5–7 CueO molecules (molecules A and B), which were related by a pseudo-2- fold symmetry. The overall structure of Δα5–7 CueO (molecule A) comprised three domains as other MCOs. Molecules A and B have essentially the same fold, although the C-terminal 6xHis-tag region of molecule B was disordered (Kataoka et al., 2007).

The crystal structure of another bacterial laccase which exists as a component of the spore coat of *Bacillus subtilis* has been disclosed by Enguita et al (2003). The overall CotA fold comprises three cupredoxin-like domains. The cupredoxin fold is mainly formed by an eight-stranded β-barrel, comprising two β-sheets composed by four strands, arranged in a sandwich conformation. The first domain comprises eight strands organized in a β-barrel form, starting with a coiled section which is absent in plant and fungal multicopper oxidases such the laccase from *C. cinereus* and ascorbate oxidase. However, a similar coiled section is present in the *E. coli* CueO protein (Roberts et al., 2002). Domain 2 of CotA acts as a bridge between domains 1 and 3. but a short α-helical fragment, encompassing residues 177–182, makes the connection between domains 1 and 2, whereas a large loop segment including residues 341–368 links domains 2 and 3. In both the structures of CotA and CueO, this region represents an external connection between domains 2 and 3, whereas in plant and fungal multicopper oxidases the corresponding link is made through an internal connection. Therefore, this feature may be a characteristic of the prokaryotic variants of these enzymes. Domain 3 of CotA not only contains the mononuclear copper center, but also contributes to the formation of the binding site of the trinuclear copper center, which is located in the interface between domains 1 and 3. Moreover, domain 3 includes the putative substrate binding site, located at the surface of the protein, close to the type I mononuclear copper center. A protruding section formed by a loop and a short α-helix, comprising amino acids from 434 to 454, forms a lid-like structure over the substrate binding site. No similar element has been found in the previously analyzed multicopper oxidases with known three-dimensional structure. Therefore, this structural elemental has been reported as a distinctive feature of CotA (Enguita et al., 2003).

1.3.3.3.5. Active site of laccases

The blue multicopper laccases typically employ four copper ions at their active site (Fig. 1.3). Type I copper confers the typical blue colour to multicopper proteins, which results from the intense electronic absorption caused by the covalent copper–cysteine bond. Due to its high redox potential of ca. þ790 mV, type 1 copper is the site where substrate oxidation takes place. Type 2 copper shows no absorption in the visible spectrum and reveals paramagnetic properties in EPR studies. It is strategically positioned close to the type 3 copper, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm (oxidized form) and by the absence of an EPR signal as the result of the anti-ferromagnetic coupling of the copper pair. The type 3 copper center is also the common feature of another protein superfamily including the tyrosinases and haemocyanins (Decker and Terwilliger, 2000). The typical coordination of type-1 coppers in blue multi copper oxidases and in the small copper enzymes consists of two histidines, one cysteine, and one axial methionine and is therefore 4-fold. Axial coordination has been considered to be one factor affecting the redox potential of copper enzymes. Mutational studies on azurin showed that the substitution of methionine by a leucine resulted in an increase of the E^0 by about 0.1 V (40). In CcL, which had a redox potential of 550 mV, the axial position was occupied by a leucine, whereas in TvL, with a redox potential of 800 mV, there was a phenylalanine in the corresponding position. Thus, it has been speculated that a phenylalanine in the axial position was responsible for the very high E^0 of TvL (Piontek et al., 2002).Type 2 and type 3 copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place. Type 2 copper is coordinated by two and type 3 copper atoms by six histidines. The strong anti-ferromagnetical coupling between the two type 3 copper atoms, is maintained by a hydroxyl bridge.

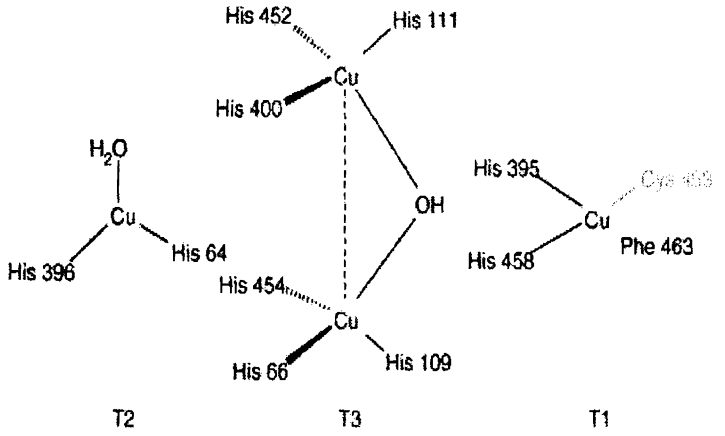


Fig. 1.3. Active site of laccase. Model of the catalytic cluster of the laccase from *Trametes versicolor* made of four copper atoms (Piontek et al., 2002)

Type I (T1) copper confers the typical blue colour to the protein and is the site where substrate oxidation takes place. Type 2 (T2) and Type 3 (T3) copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place

Multiple sequence alignments of more than 100 laccases resulted in identification of four ungapped sequence regions, L1–L4, as the overall signature of laccases, distinguishing them within the broader class of multi-copper oxidases (Kumar et al., 2003). The amino acid ligands of the trinuclear cluster are the eight histines, which occur in a highly conserved pattern of four HXH motifs. In one of these motifs, X is the cysteine bound to the T1 copper while each of the histidines is bound to one of the two type 3 coppers. Intra-protein homologies between signatures L1 and L3 and between L2 and L4 suggested the occurrence of duplication events (Claus, 2004). In the structure of RlL, the mononuclear Cu1 center was trigonally planar coordinated by two ND atoms from His457 and His396 and by a SG atom of Cys452, with coordination distances similar to those observed in other fungal laccases (Garavaglia et al., 2004). Type-1 copper center

usually has an additional axial ligand provided by Leu or Phe. In RlL, Leu462 occupies such a position with its CD2 atom at 3.76 A ° from Cu1, which confirmed that no additional axial ligand for type-1 copper was present in laccase. Cu1 lies therefore within the plane formed by the two nitrogen atoms and the sulfur ligands. The trinuclear copper center of RlL lies between domains 1 and 3 at about 13 A° from the protein surface. The distance between the two T3 copper ions observed in RlL, appears to be longer than usually observed in other blue multi-copper oxidases. In RlL the two T3 copper ions are 5.1 A ° apart, about 1 A ° more than in TvL (Piontek et al, 2002) and AO (Messerschmidt et al., 1992). RlL was a high E^0 enzyme, showing a redox potential of 730 mV (Bonomo et al., 1998). When comparing the coordination distances for the type-1 copper center among different laccases, it was noted that RlL showed the longest value for the Cu1–His distance (2.20A°) (Garavaglia et al., 2004). Hence the authors concluded that the distance between the type-1 copper and the coordinating nitrogen atoms is a key factor for the modulation of the redox potential in laccases.

Recently, Hakulinen et al (2006) have investigated the effect of X-ray radiation upon the crystal structure of a recombinant laccase from *Melanocarpus albomyces* through the use of crystallography and crystal absorption spectroscopy. It has been reported that the trinuclear site has an elongated electron density amidst coppers, suggesting dioxygen binding. Two crystal structures of rMaL were refined at 2.0A ° resolution from data sets collected rapidly (1.5 h) and slowly (8 h) with synchrotron radiation. At the trinuclear site, several differences between the low, and the high-dose structures were observed. The distance of type-3 coppers from each other was 4.7 and 4.8–4.9A° for the low, and the high-dose structures, respectively. In addition, the distance of the opposite chloride ligand of the T2 copper was 2.5 Å in the low-dose structure but clearly longer in the high-dose structure, approximately 2.9 Å The authors have concluded that X-ray radiation could alter the active site of laccase from *M. albomyces*. The spectral studies showed no band at 590 nm due to the reduction of T1 copper during the long X-ray measurements and the colour of the crystal changed from blue to colourless. Structural comparison between CueO from E. coli and three typical fungal laccases

revealed an obvious difference in the substrate binding pocket. In fungal laccases, the substrate binding pockets are fully open and type I copper is exposed to solvent. However in CueO, an additional α-helix from Leu351 to Gly378 is located over type I copper and makes the substrate binding pocket smaller. The in vitro laccase substrates such as ABTS might not be preferred by CueO due to this extra α-helix (Li et al., 2007). Several different types of recombinant CueO (rCueO) have also been expressed. The active site of engineered CueO of *E. coli* (Δα5–7 CueO) has been reported to include a bridging group between type III Cu atoms. In the case of Δα5–7 CueO, an oxygen atom has been satisfactorily refined as a bridging species with an acceptable temperature factor at the trinuclear Cu center (Kataoka et al., 2007). On the other hand, a Cl^- ion bridged between type III Cu atoms in recombinant CueO (rCueO), although the OH^--bridged form of rCueO has been reported very recently (Li et al., 2007).

1.3.3.3.6. Mode of action

Direct oxidation

Substrate oxidation by laccase is a one-electron reaction generating a free radical. As one electron oxidation of a substrate is coupled to a four-electron reduction of oxygen, the reaction mechanism cannot be straightforward (Thurston, 1994). The Cu atoms arranged at three different sites play an essential role in the catalytic mechanism of laccase. There are three major steps in laccase catalysis. The first step of the catalytic cycle involves the formation of a fully reduced laccase in which all four coppers are in a reduced state. Molecular oxygen then oxidizes the fully reduced laccase, presumably via a peroxy intermediate, and is reduced to water. (Shin et al., 1996; Solomon et al., 1996; Lee et al., 2002). In a typical oxidation of a substrate by laccase, there is a one-electron reaction that generates a free radical which is typically unstable. The free radical has several options for further reaction. A second enzyme-catalyzed oxidation may take place which converts a phenol to a quinone or it may undergo a non-enzymatic reaction such as hydration, disproportionation or polymerization to produce amorphous insoluble

melanin-like products (Thurston, 1994). Molecular oxygen acts as the electron acceptor to remove protons from the phenolic hydroxyl groups. This reaction gives rise to phenoxy radicals that can spontaneously rearrange, which can lead to fission of carbon-carbon or carbon-oxygen bonds of the alkyl side chains, or to cleavage of aromatic rings (Marzulla et al., 1995; Salas et al., 1995).

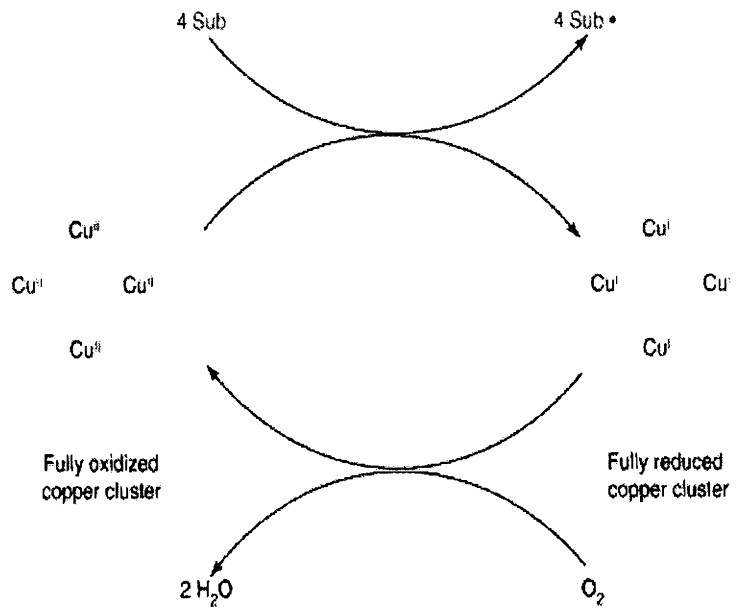


Fig. 1.4. Schematic representation of a laccase catalytic cycle

(Riva, 2006)

In the reaction two molecules of water from the reduction of one molecule of molecular oxygen and the concomitant oxidation (at the T1 copper site) of four substrate molecules to the corresponding radicals

The suitability of a chemical compound as a laccase substrate depends on two factors. Firstly, the substrate must dock at the T1 copper site, which is mainly determined by the nature and position of substituents on the phenolic ring of the substrate, especially those with bulky side chains (Xu 1996; Bertrand et al., 2002). Secondly, the redox potential (E^0) of the substrate must be low enough, because the rate of a laccase catalyzed reaction has been shown to depend on the difference between the redox potentials of the enzyme and the substrate (Xu et al 2000; Xu et al., 2001). The redox potential of the substrate is determined by its chemical structure and nature of the substituents. Different substituents have different impact on (E^0) depending on their ability to withdraw or donate electrons. Methoxy substituents are electron donating and increase the electron density at the phenoxy group, thus making it more readily oxidized (Xu 1996, Garzillo et al., 1998).

Laccase mediator system

There are instances in which the substrates of interest cannot be oxidized directly by laccases, either because they are too large to penetrate into the enzyme active site or because they have a particularly high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of so-called 'chemical mediators', which are suitable compounds that act as intermediate substrates for the laccase, (Fig. 1.5) whose oxidized radical forms are able to interact with the bulky or high redox-potential substrate targets (Riva, 2006). Approximately 100 different potential mediator compounds have been described for the LMS, but ABTS and HBT (1-Hydroxybenzotriazole) remain the most commonly used (Bourbonnais et al., 1995; Bourbonnais et al., 1997; Johannes and Majcherczyk, 2000). Synthetic mediating substrates are heterocyclic compounds belonging to the general classes of phenoxazinones, phenothiazines or phenoxybenzothiazoles (Eggert et al., 1996). Natural mediators include phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol. The use of natural mediators proved to be as efficient as the commonly used ABTS and HBT (Johannes and Majcherczyk, 2000).

Activity of laccase-mediating substrate systems towards compounds depends on a combination of two main factors: the redox potential of the enzyme and the stability and reactivity of the radical generated by oxidation of the nediating substrate (Bourbonnais et al., 1998). Several hypotheses have been proposed for the mechanism of mediating substrate systems. The first hypothesis is that the mediating substrate can act as a redox mediating substrate, ie; reversible. It is thought that laccase oxidizes the mediating substrate and this oxidized form of the mediating substrate can oxidize the substrate, and is consequently reduced back to its non-oxidized form: the species responsible for the oxidation of the substrate would be the oxidized mediating substrate (Li et al., 1998). Another hypothesis is that active intermediates are generated during the oxidation of the mediating substrate by laccase. These intermediates can abstract hydrogen. In such a reaction the mediating substrate would be continuously consumed (Li et al., 1998) and not be reversible. According to the results of the study performed by Li et al. (1998) the first hypothesis is more probable.

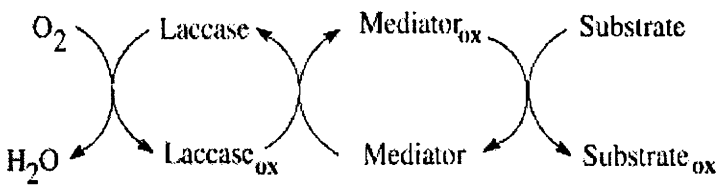


Fig. 1.5. Schematic representation of role of mediators in laccase catalyzed reactions (Fabbrini et al., 2002)

One of the first reports of a mediated system was by Bourbonnais and Paice (1990) using the laccase from *T. versicolor*. It was shown that laccase was able to cleave a nonphenolic dimer in the presence of ABTS. The presence of the mediating substrate, ABTS, prevented and reversed the polymerization of kraft lignin by *T. versicolor* laccase (Bourbonnais et al., 1995). The oxidation of ABTS

by laccase produces the stable dark green cation radical, ABTS+ and then the dication ($ABTS^{2+}$) is formed. Both oxidized species were relatively stable and the reactions are highly reversible. It was observed that the dication was the intermediate responsible for the oxidation of non-phenolic compounds such as veratryl alcohol. The cation radical was shown to react only with the phenolic structures. *T. versicolor* laccase has a redox potential of 585 mV. This is 300 mV below the redox potential of $ABTS^{+}/ABTS^{2+}$ couple. Laccase can slowly oxidize ABTS to $ABTS^{2+}$ provided that the reaction is driven forward by the subsequent reaction of the dication with veratryl alcohol or some other compound (Bourbonnais et al., 1998). It is clear that the enzyme must produce $ABTS^{2+}$ for the oxidation of nonphenolic compounds to occur but oxidation by $ABTS^{+}$ is limited to phenoiic compounds.

HBT is oxidised by laccase to form a nitroxide cation radical (Call and Mucke, 1994; Bourbonnais et al., 1997). The nitroxy radical intermediate was not stable and decayed rapidly but was shown to catalyze the oxidation of veratryl alcohol to veratraldehyde. The nitroxy radical formed when HBT is oxidized by laccase is a potent electrophile that easily abstracts hydrogen. So it is clear that mediating substrates work to increase the substrate range of laccase by increasing the difference in redox potential. The laccase/HBT system has given good results in trials done for the bleaching of pulp and has the ability to oxidise the nonphenolic β-O-4- linked subunits that are predominant in lignin as well as β-1 linked dimers (Bourbonnais et al., 1997, Srebotnik and Hammel, 2000; Xu et al., 1997; Ander and Messner, 1998). This mediator however has been reported as unable of acting as a recyclable mediator (Li et al., 1998). Delignification by the laccase/HBT system is not fully understood but as in the case of ABTS, HBT is small enough to access lignin.

1.3.3.3.7. Properties of laccases

pH and temperature

The catalytic performance of laccases is greatly influenced by their activity and stability at different pH and temperature conditions. The pH activity profiles of laccases are often bell shaped, with optima around 4-6, when measured with phenolic substrates (Palmieri et al., 1993; Eggert et al., 1996, Xu 1997; Chefetz et al., 1998; Garzillo et al., 2001). The variation in pH optima may be due to changes to the reaction caused by the substrate, oxygen or the enzyme itself (Xu, 1997). The decrease in laccase activity in neutral and alkaline pH values is affected by increasing hydroxide anion inhibition, because as a small anion, hydroxide ion is also a laccase inhibitor (Xu, 1997). The hydroxide anion (OH^-) binds to the T2/T3 coppers and results in the inhibition of the laccase activity due to the disruption of the internal electron transfer between the T1 and T2/T3 centres. On the other hand, the increasing pH decreases the redox potential of the phenolic substrate, which makes the substrate more susceptible to oxidation by laccase (Xu, 1997). The bell-shaped pH profile is thus the result of two opposite effects: increasing ΔEo [laccase-substrate] and inhibition by hydroxide anion. In contrast to their activity, the stability of laccases is generally highest at pH values around 8-9 (Nishizawa et al., 1995; Xu et al., 1996; Chefetz et al., 1998). Saito et al (2003) has reported that laccase of a fungus belonging to the family Chaetomiaceae was stable at pH 4.5-9.

The optimum temperature of laccases usually ranges between 30-60 °C. Laccase from *T. versicolor* has been reported to have a temperature optima at 60 °C (Call and Mucke, 1997) while the laccase from the actinomycte strain, *S. cyaneus* has a temperature optima of 70 °C. Temperature stabilities of laccases vary considerably, depending on the source organism. In general, laccases are stable at 30-50 °C and rapidly lose activity at temperatures above 60 °C (Xu et al., 1996, Heinzkill et al., 1998; Schneider et al., 1999, Palonen et al., 2003). The most thermostable laccases have been isolated from bacteria; the half-life of *Streptomyces lavendulae* laccase was 100 min at 70 °C (Suzuki et al., 2003) and

that of *Bacillus subtilis* CotA was 112 min at 80 °C (Martins et al., 2002). The laccases isolated from a strain of *Marasmius quercophilus* (Farnet et al., 2000) were found to be stable for 1 h at 60 °C.

Substrate specificity

The low substrate specificity is one of the most important characters that have made laccase useful in varying industrial applications. Laccase is an important oxidant for aromatic rings substituted with electron donating groups, such as phenolics and aromatic amines. These are the preferred electron rich substrates of laccase. There is some difficulty in defining laccase by its reducing substrate. Laccase has an overlapping substrate range with tyrosinase, another type of Cu-containing oxidase, but laccase does not oxidize tyrosine itself (Thurston, 1994). Thus laccases are non-specific regarding to their reducing substrate, and the range of substrates oxidized varies from one laccase to another (Thurston, 1994).

The reducing substrate spectrum for laccase is diverse as long as the redox potentials are not too high (>IV) (Gianfreda et al., 1999). While laccase has low specificity for its reducing substrates, it has a strong preference for its oxidizing substrate, O2 (Gianfreda et al., 1999). Thus laccase can oxidize *o*- and *p*-diphenols, aminophenols, methoxyphenols, polyphenols, polyamines, lignin, some organic ions, aryl diamines and a considerable range of other compounds (Thurston, 1994; Call and Mucke, 1997; Gianfreda et al., 1999). In general, laccases show more affinity towards *p*-diphenols than the *o*-diphenols and very less reactivity has been observed generally with the meta-substituted phenols (Jolivalt et al., 1999).

Carbohydrate content

All laccases characterized to date are glycoproteins and this characteristic imparts resilient properties to laccases. The growth conditions of the organism can have a marked effect on the amount of glycosylation, as seen in a study of chloroperoxidase (Pickard and Hashimoto, 1988). The total carbohydrate content of

Organism	pH	Temp (°C)	pI	MW (kDa)	Carbohydrate (%)	Substrate	Km (mM)	Reference
Magnaporthe grisea	6	30	NA	70	NA	Syringaldazine	0.118	Iyer & Chattoo, 2003
Xylaria polymorpha	2.5	55-60	3.1	67	NA	ABTS	0.02	Liers et al., 2007
Trametes trogii	2.0	NA	4.5	62	NA	ABTS	0.05	Zouari-Mechichi et al., 2006
Trametes sp	4.5	55	3.4	61	NA	Catechin	0.19	Motoda, 1999
Trametes sanguinea	5.0	60	3.5	62	9.1	DMP	NA	Nishizawa et al., 1995
Dichomitus squalens	3.0	NA	3.5	66	10.5	DMP	NA	Perie et al., 1998
Sclerotium rolfsii	2.4	62	5.2	59	NA	ABTS	0.22	Ryan et al., 2003
Coriolus hirsutus	2.5	45	7.4	73	11	ABTS	0.05	Shin and Lee, 2000
Trichophyton rubrum LKY-7	3.0	NA	4.0	65	NA	ABTS	0.04	Jung et al., 2002
Trametes villosa	2.7	NA	3.5	63	10	ABTS	NA	Yaver et al., 1996
Pleurotus ostreatus	6.5	50	3.0	55	7.4	Guaiacol	0.18	Okamoto et al., 2000
S. cyaneus	4.5	70	5.6	75	NA	ABTS	0.38	Arias et al., 2003
S. griseus	6.5	40	5.3	114	NA	DMP	0.42	Endo et al., 2003
S. lavendulae	4.5	50	NA	73	NA	Catechol	0.04	Suzuki et al., 2003
Bacillus subtilis	3.0	75	7.7	65	NA	ABTS	0.10	Martins et al., 2002

NA – Information not available

Table 1.3. Properties of laccases from different organisms

laccases usually varies from 1 % to 15 % depending on the source of the enzyme *P. eryngii* has two isoenzymes with carbohydrate content of 1 % and 7 % (Munoz et al., 1997). *C. subvermispora* laccases has an approximate sugar content of 15 % and 10% for LI and L2 isoenzymes respectively (Fukushima and Kirk, 1995). *C.* zonatus laccase has been reported to have 10 % carbohydrate per mole of protein (Koroljova et al., 1999).

Relative glycosylation levels can impart different properties to enzymes. It has been demonstrated that the carbohydrate moiety of the laccase of *T.* versicolor imparts resistance to proteolytic attack and elevated temperatures (Yoshitake et al., 1993). The resistance to proteolytic attack can be attributed to the specificity of proteases as it will specifically cleave the peptide bonds in the primary structure but the carbohydrate can obscure the peptide bonds, thereby conferring resistance. The carbohydrate can also confer thermophilic stability by additional hydrogen bonding between the carbohydrate portion and the protein. Some of the important properties of laccases from different organisms are summarized in Table 1.3.

1.3.3.3.8. Applications of laccases

Laccases have received much attention from researchers in last decades due to their ability to oxidise both phenolic and nonphenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes (Couto and Herrera, 2006). The important applications of laccases include detoxification of dye containing industrial effluents from textile and leather industries (Wong and Yu, 1999; Couto 2007), pulp biobleaching (Arias et al., 2003), use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics (Couto and Herrera, 2006). Laccases also find application in food industry that includes beverage (wine, fruit juice and beer)

processing, sugar beet pectin gelation, baking etc (Minussi et al., 2002). The major sectors where laccases are being used are as follows:

Dye decolourization

The application of laccases in dye decolourization has increased in recent years and many studies have been demonstrated for dye decolourization using both crude and purified forms of laccase. Some of the chemicals serving as redox mediators facilitate the dye degrading activity of laccase and enhance its specificity to wide range of dyes. Laccases have been used for the decolourization of dyes belonging to different categories such as azo, anthraquinone, heterocyclic, triphenylmethane dyes etc. Laccases have been extensively studied for their degradation of azo dyes (Zille et al., 2005). Chivukula and Renganathan (1995) have reported the oxidation of phenolic azo dyes by the laccase from *Pyricularia oryzae.* Laccase from *Thelephora* sp. has been reported to decolourize azo dyes at different rates. The enzyme decolourized 19% of orange G, 12.0% of congo red and 15.0 % of amido black 10B (Selvam et al., 2003). Purified laccase from *Trametes hirsute* has been reported as capable of degrading triarylmethane, indigoid, azo, and anthraquinonic dyes. Anthraquinonic dyes and indigo carmine (Acid Blue 74) were degraded more than twofold faster than the azo dyes by the purified laccase (Abadulla et al., 2000). Nyanhongo et al (Nyanhongo et al., 2002) have screened the ability of laccase from different Trametes sp to decolorize eight synthetic dyes (anthraquinone, azo, indigo and triarylmethane). All the tested dyes were decolorized by the laccase from *T. modesta* most efficiently under acid conditions (pH 3–6) but the optimum pH for decolorization of the individual dye varied. The four crude fungal laccases differed remarkably in their decolorization efficiency of eight different synthetic textile dyes. Specificity of *T. hirsuta* laccase towards different dye structures has been investigated by Couto et al., 2006. The enzyme decolourized dyes at different rates and 83 % decolourization of the azo dye methyl orange was attained with the enzyme. In another study, Cameselle et al., 2003 have observed that the azo dye methyl orange was easily degraded than the triphenyl methane dye bromophenol

blue. This behaviour was explained in terms of the structure and size of each dye, the first dyes to degrade are the ones with a low number of aromatic rings. Laccases immobilized on different supports have also been implemented in dye decolourization. Zamora et al (2003) have reported the decolourization of different reactive dyes by immobilized laccase. Laccase from *Sclerotium rolfsii* immobilized on alumina pellets has been employed for the decolourization of wool azo dye Diamond Black PV 200 (Ryan et al., 2003). The decolourization of the commonly used anthroquinone dye RBBR by laccases has also been cited (Hou et al., 2004).

Pulp biobleaching

The major problem encountered in the pulping process is the characteristic brown colour of the pulp due to the presence of residual lignin. The chemical processes are usually employed in the industries for the removal of lignin and hemicellulose from the pulp thereby the brightness of the pulp is achieved. Although the chemical treatments are effective method to achieve this task, the high cost and related pollution harms make them unattractive. Hence, there arises the necessity for alternate methods of pulp bleaching. Biobleaching can be defined as an ecofriendly treatment of pulp that involves either microorganisms or their enzymes. This process helps in the selective removal of lignin and hemicellulose components without degrading cellulose. Xylanases are the most widely used enzyme for biobleaching however, the role of laccases in the process has also been elucidated in the recent years.

The use of laccase mediator system along with chemical treatments has also been suggested for biobleaching. Sealey and Ragauskas (1998) have demonstrated that the laccase/n-hydroxybenzotriazole bleaching system can very effectively remove lignin from kraft pulps via a series of oxidative degradation reactions. Balakshin et al (2001) have studied the biobleaching of pulp with dioxygen in laccase-mediator system. The authors have concluded that rate of pulp delignification in the laccase mediated system is determined by the oxidation of a mediator with laccase or/and by the reaction of the oxidized mediator with the

residual lignin in pulp. Laccases from three different white rot fungi; *P. cinnabarinus*, *T. versicolor* and *Pleurotus eryngii* have been reported in the efficient bleaching of high-quality flax pulp in a totally-chlorine-free (TCF) sequence that involved laccase-mediator system. The treatment resulted in high brightness and low lignin content (Camarero et al., 2004).

The laccase from *S. cyaneus* has been employed for the biobleaching of eucalyptus kraft pulp in the presence of ABTS and it has been reported by the authors that the process has increased the brightness of the pulp by 2.2% (Arias et al., 2003). The potential of thermostable laccase from the fungus *P. cinnabarinus* along with the chemical mediator (1-hydroxybenzotriazole) to improve totally chlorine-free (TCF) bleaching of *Eucalyptus globulus* kraft pulps has been investigated by Ibarra et al (2006). The authors have claimed that the new TCF sequence including the laccase stage permitted to improve eucalypt pulp delignification to values around kappa 5 compared to kappa 7 using only TCF chemical reagents. In a similar way, the final brightness obtained, over 91 % ISO, was 3–4 points higher than that obtained in the chemical sequences. The studies by different groups prove that laccase-mediator system is an efficient alternative for chemical methods involved in pulp bleaching. A recent study by Camarero et al (2007) has established the potent role of natural laccase mediators in pulp biobleaching. The authors have claimed that the natural mediators such as acetosyringone and syringaldehyde enabled over 15% increase of final brightness and a decrease of final kappa number similar to that obtained by synthetic laccase mediators.

Food industry

In the food industry laccase finds application in different processes like fruit juice processing, wine stabilization, sugar beet pectin gelation, baking and in improving food sensory parameters (Minussi et al., 2002). Wine stabilization is one of the main applications of laccase in the food industry (Minussi et al., 1999). The color and taste of the wines depend particularly on the phenolic compounds present in

different types of wines (Brenna & Bianchi, 1994). The polyphenols present in musts and wines should be selectively removed to prevent any alterations in taste and colour imparted mainly by the oxidation of polyphenols. Different methods have been used in order to prevent the decolorization and flavor alteration in wines, such as the removal of phenolic groups with polyvinylpolypyrrolidone (PVPP), and the use of sulfur dioxide to block oxidizers, among others (Minussi et al., 2002). An alternative for the physicalchemical adsorbents could be the use of enzymes that selectively target specific polyphenols during the madeirization process. These polyphenolic substances would be oxidized by the enzyme, polymerized and then removed by clarification (Zamorani, 1989). One enzyme studied for this purpose is laccase (Cantarelli, 1986). There are several studies in the literature which states that laccase treatment promotes wine stabilization (Cantarelli & Giovanelli, 1990; Plank and Zent, 1993; Servili et al., 2000). According to Cantarelli (1986), mutant laccase from *Polyporus versicolor* (optimum pH 2.7) eliminated up to 70% catechin and 90% of anthocyanidins in a model solution in 3 h of treatment. The studies by Maier et al (1990) have shown that wines made by laccase treatment were the best, suggesting that a stable and high quality wine can be made with little or no added SO_2. The feasibility of using laccase for phenol removal from white must have also been suggested (Minussi et al., 2007).

It is well known that browning, both enzymatic and chemical, is one of the major faults in beverages (Giovanelli and Ravasini, 1993). Various pre- and post treatments are available to avoid post-turbidity and discoloration of fruit juices. Various enzymatic treatments have been proposed for fruit juice stabilization, including the use of laccase (Piacquadio et al., 1998). Stutz (1993) proved that is possible to produce clear and stable juices/ concentrates with a light colour by means of ultrafiltration and laccase, without any large additional investment. Ritter and Dietrich (1996) and Piacquadio et al (1998) have reported that the use of laccase improves stability in apple juice. There is a considerable interest in the food industry for finding new functional ingredients. Sugar beet pectin is a food ingredient with specific functional properties. It may form gels by an oxidative cross-linking of ferulic acid (Norsker et al., 2000). Micard and Thibault (1999)

showed that is possible to crosslink the beet pectin through the oxidative coupling of the feruloyl groups using laccase. In the baking industry, the use of laccase results in an increased volume, an improved crumb structure and softness of the baked product, as well as increased strength, stability and reduced stickiness and thereby improved machinability of the dough. The effect on the dough has been found to be particularly good when poor quality flour has been used (Minussi et al., 2002)

Phenol removal

Removal of phenols from industrial aqueous effluents is an important practical problem, because virtually all phenols are toxic and their presence in a number of industrial waste waters is a health hazard. The use of free laccase, tyrosinase and peroxidase, which catalyse the oxidative coupling of phenol compounds resulting in the formation of water insoluble oligomeric and polymeric products which are then removed by sedimentation or filtration, has been proposed (Shuttleworth and Bollag, 1986). The use of enzymes has been suggested as an alternative method for other physico-chemical methods that suffer from serious drawbacks such as high cost and formation of hazardous by-products (Atlow et al., 1984).

The efficiency of laccases in the degradation of individual phenolic compounds as well as phenolic mixtures has been proved (Lante et al., 2000; Krastanov, 2000). The decolourization of phenolic effluents from different industrial sources has been reported (Davis and Burns, 1990). The effect of reactive co-substrates such as guaiacol and 2, 6-dimethoxyphenol on the removal of chlorinated phenols by horseradish peroxidase (HRP) and a laccase from the fungus *Trametes versicolor* has been investigated by Roper et al. (1995). They found that the addition of co-substrates will enhance the precipitation of phenols by laccase. Annibale et al (2000) have reported the efficiency of an immobilized laccase from *Lentinula edodes* in removing the phenolics from olive mill waste water. The authors have reported that the laccase immobilized on Eupergit C was able to remove the phenols efficiently.

Aromatic compound degradation

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds that consist of fused aromatic rings and do not contain heteroatoms or carry substituents. These compounds can be point source pollutants (e.g. oil spill) or non-point source (e.g. atmospheric deposition) and are one of the most widespread organic pollutants. Some of them are known or suspected carcinogens, and are linked to other health problems. The degradation of PAHs are of great significance in maintaining a proper ecosystem for human life. The laccase–mediator systems have been applied for the degradation of environmental xenobiotics such as polycyclic aromatic hydrocarbons (PAHs). Oxidation of anthracene and benzo[a]pyrene by laccase from *Trametes versicolor* has been reported by Collins et al (1996). The oxidation of five polycyclic aromatic hydrocarbons; anthracene, benzo(α)pyrene, fluoranthene, phenanthrene and pyrene by laccase from *Coriolus hirsutus* in the presence of the redox mediators has been suggested by Cho et al (2002). The authors have reported 40 % degradation of the least oxidizable PAH, pyrene by laccase within 1 h. The ability of laccase from *Pleurotus ostreatus* to degrade different PAhs has been investigated by Pozdnyakova et al (2006). The PAH degradation by the enzyme differed with the type of the aromatic compound as well as with the mediator used in the study (Pozdnyakova et al., 2006).

Denim washing

Denim washing includes one of the new areas of application for laccases. Cellulase enzymes are usually used in denim garment processing to get stone wash look on to the denim garments. Laccases are new generation enzymes for the finishing of denim jeans. Laccase can be used for bleaching delicate denim fabrics as well as for making fashionable effects without compromising the strength of the fabric. Campos et al. (2001) reported the degradation of indigo both in effluents and on fabrics using purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii* in combination with redox-mediators and reported that bleaching of fabrics by the

laccases correlated with the release of indigo degradation products. Pazarlogliu et al (2005) showed that a phenol-induced laccase from *Trametes versicolor* was an effective agent for stonewashing effects of denim fabric without using a mediator. Moreover, they found that *T. versicolor* laccase without a mediator was more effective than commercial laccase.

Cosmetics industry

Laccases find application even in production of cosmetics. Laccase-based hair dyes are less irritant and easier to handle than current hair dyes, since laccases replace H_2O_2 as an oxidizing agent in the dye formulation (Roure et al., 1992; Aaslyng et al., 1996; Lang and Cotteret, 1999). More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed (Golz-Berner et al., 2004).

Biosensors

A biosensor is a device for the detection of an analyte that combines a biological component with a physicochemical detector component. The function of a biosensor depends on the biochemical specificity of the biologically active material. Enzymes, antibodies, DNA, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. A number of biosensors containing laccase have been developed for different purposes such as immunoassays (Bauer et al., 1999), glucose determination (Wollenberger et al., 1986), aromatic amines (Simkus and Laurinavicius, 1995) and phenolic compound determinations (Freire et al., 2002; Vianello et al., 2004; Jegan Roy et al., 2005).

1.4. CONCLUSION

The growing concern about the increasing environmental pollution has led the scientific community to reorient their research attitudes. The term 'eco-friendly' has received much attention in the recent years and strict regulations have been made to assure that the new technologies should be environmental friendly. It is high time to consider effective and harmless methods for pollution abatement too. The use of biological methods is a promising alternate to the harsh physico-chemical methods usually employed for many of the industrial purposes, including pollution abatement. The use of microorganisms and their enzymes; especially the oxidative enzymes like laccases are nowadays widely used for these purposes and the success of these processes keeps the researches on characterization of enzymes from newly identified organisms at a great pace.

1.5. OBJECTIVES AND SCOPE OF THE PRESENT STUDY

Scope of the present study was confined to the following objectives

- Isolation and screening of actinomycete cultures for the production of lignin degrading enzymes
- Identification of the selected strain
- Optimization of cultural and nutitional parameters for laccase production in submerged fermentation (conventional and statistical methods)
- Optimization of process parameters for laccase production under solid-state fermentation (SSF)
- Enhanced laccase production in SSF using inducers and packed bed bioreactors
- Purification and characterization of laccase
- Application studies on dye degradation and phenol degradation using the selected strain and laccase
- Use of cell immobilization and enzyme immobilization technologies for application studies.

Chapter 2.

ISOLATION AND SCREENING OF ACTINOMYCETE CULTURES

2.1. INTRODUCTION

The diversity of form in the actinomycetales is well recognized, due to the sustained generation of environmental isolates for pharmaceutical screening and much interest was given to this group of organisms over the years for obtaining antibiotic compounds. Besides antibiotic production, another important trait of actinomycetes that has been studied is their ability to degrade lignin – the most complex biopolymer. Biodegradation of lignin has been considered as a significant phenomenon since it plays a critical role in the carbon cycling of earth. Most of the knowledge concerning the pathway of lignin breakdown comes from the study of lignin degradation by the white rot basidiomycete, *Phanerochaete chrysosporium* (Farell et al., 1989), where it is a complex secondary metabolic process mediated by the action of several extracellular enzymes. Lignin peroxidase (LiP, EC.1.11.1.14.), commonly known as ligninase is the most important lignin degrading enzyme followed by laccase (EC.1.10.3.2.) and Manganese peroxiase (MnP, EC.1.11.1.13.). Lignin degrading enzymes have many potential industrial applications including delignification of pulp, textile dye decolourization, effluent detoxification etc. Since the discovery of extracellular lignin – degrading enzymes of white rot fungi, considerable effort has been expended in searching for analogous enzymes from different groups of organisms including actinomycetes. Crawford et al (1983) were the first group to identify and characterize LiP from actinomycetes. *Streptomyces viridosporus* T7A was the most studied lignin degrading actinomycete and most of the enzyme characteristics were well studied in this organism. Actinomycetes are adapted to grow in soil, sediment and on solid substrates including wood. Their primary carbon sources in the soil are insoluble and polymeric, necessitating the secretion of a range of extracellular enzymes as their hyphae penetrate and colonize the substrate (McCarthy and Williams, 1992). The studies carried out in this group of organisms by Pometto and Crawford (1986) has proved that they secrete an extracellular LiP. Since then the screening of actinomycetes for lignin degrading enzymes has been carried out by different groups. Mercer et al (1996) had used chemiluminescence analysis system for screening ligninolytic actinomycetes.

Actinomycetes are generally considered as terrigenous bacteria because of their wide distribution and abundance in soil. The distribution of actinomycetes in the aquatic environments remained largely undescribed for many years and most of the workers suspected the indigenous nature of aquatic actinomycetes because these bacteria produce resistant spores that are known to be transported from land into sea and other aquatic bodies where they can remain dormant for many years. But the recent studies have shown that actinomycetes can be isolated from mangrove swamps and other coastal environments (Moran et al., 1995) and there are evidences that actinomycetes can be recovered even from ocean sediments (Mincer et al., 2002). The study on halophilic organisms for lignin degradation is a field comparatively not much explored, even in the case of best studied fungal groups. Only a few groups have attempted on revealing the potentials of marine and mangrove fungi (Raghukumar et al., 1999; Shamla and Prema, 2002) while the reports on halophilic actinomycetes in this aspect is still rare.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection and isolation of cultures

Sediment and soil samples were collected in sterile containers from different marine and mangrove areas. Samples were also collected from decaying logs found in the intertidal and swamp regions. A total of 59 samples were collected from different stations along the West coast of India. Samples (1g wet weight) were serially diluted in saline solution preheated to a temperature of 55 °C for six minutes. The dilutions were plated on starch casein (Kuster and Williams, 1964) and humic acid vitamin (Hayakawa and Nonumura, 1987) agar plates. Cycloheximide at a concentration of 100 mg/L was incorporated into the medium to minimize fungal contamination. The plates were incubated at 30 °C for two weeks. The isolates that showed tough leathery colonies on the isolation media were selected, purified by streak plate method and then subjected to Gram's staining and observed under an oil immersion microscope. The gram positive, filamentous isolates were selected and maintained on starch casein agar medium by storage at 4 °C.

2.2.2. Screening of the strains for ligninolytic enzyme production

2.2.2.1. Qualitative screening (Plate assay method)

Primary screening of the strains was done by plate assay method. Dyes such as Polymeric dye R-478 (Poly R, Sigma) and Remazol brilliant blue R (RBBR, Sigma) (100, 300 & 500 mg/L) were incorporated in the agar medium that contained (g/L) peptone- 5, glucose- 20 and agar-20. Agar plates were also prepared with lignin model compounds like cinnamic acid, vanillic acid, ferulic acid, (5 g/L each), and guaiacol (0.5mM, 1mM and 5mM). Plates having the lignin model compounds as the sole source of carbon (excluding glucose) were also used for screening. The isolates were spot inoculated on the plates and incubated at 30 °C for two weeks and observed for clear zones around the colonies.

2.2.2.2. Quantitative screening (submerged fermentation)

The strains were grown in 250 mL shake flasks containing 100 mL minimal salts – yeast extract nutrient medium with rice straw (10 g/L) as the substrate. The composition of liquid medium was same as that recommended by Ramachandra et al (1987). Adequate aeration was provided by agitation at 150 rev/min at 30 °C and incubated for 7 days. Strains grown under the same conditions for 48 hrs were used as the inoculum. The above medium was used for the screening of strains for LiP and laccases, while the medium for MnP production was modified by adding 0.5 mM $MnSO_4$.

2.2.3. Enzyme profile of the selected strain

Qualitative screening for different enzyme production by the selected strain was done by plate assay method. Agar plates were prepared by incorporating the corresponding substrate in minimal salt medium. The clear zones were visualized by the methods specific for each of the enzyme. The substrates used for plate assay were carboxy methyl cellulose (cellulase), pectin (pectinase), oat spelt xylan (xylanase), casein (protease), starch (amylase). Production of amylase was visualized by flooding the plates with iodine solution while pectinase production was visualized using

cetrimide. Congo red was used in the case of xylanase. Protease production was detected by observing the clear zones formed around the colony.

2.2.4. Enzyme assays

The culture filtrate was removed at 24 h intervals from each of the culture flasks and centrifuged at 10,000 x g for 20 min at 4 °C. The supernatant obtained after centrifugation was used for the enzyme assays and protein estimation.

2.2.4.1. Lignin peroxidase

Extracellular LiP activity was assayed with 2,4 – dichlorophenol (2,4 – DCP, Sigma) as the substrate (Tuncer et al., 1999). A total volume of 1.0 mL of reaction mixture contained 200µL each of 0.1 M potassium phosphate buffer (pH 7.0), 25 mM 2,4 – DCP, 16 mM 4 – amino anti pyrine and culture filtrate. The reaction was initiated by the addition of 200 µL of 50 mM H_2O_2 and the reaction was monitored for 1 minute at a wavelength of 510 nm. Calculation of enzyme activity used an absorption coefficient of 21,647 M^{-1} cm^{-1}.

2.2.4.2. Manganese peroxidase

Determination of MnP activity was done 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 0.2 M sodium phosphate buffer (pH 7.5) with 1mL of culture filtrate. The reaction was started by 50 mM H_2O_2 addition. One unit of enzyme activity was defined as the increase in absorbance at 470 nm per minute. ($\varepsilon = 26 \times 10^3$ M^{-1} cm^{-1}).

2.2.4.3. Laccase

The laccase activity was measured by monitoring the oxidation of 500 µM 2,2'–azino–di–[3–ethyl benzothiazoline–6–sulphonic acid] (ABTS) buffered with 0.2 M sodium phosphate buffer (pH 7.5) at 420 nm for 1 minute (Boubonnais and Paice,

1990) The reaction mixture (3 mL) contained 1 mL of culture filtrate. One unit of enzyme activity was defined as 1 micro mole of ABTS oxidized per minute ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$).

The enzyme activities were calculated using the following formula:

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

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

V = Volume of assay solution in the cuvette

ε = Extinction coefficient

v = Volume of the sample

t = Time in minutes

2.2.5. Analytical methods

2.2.5.1. pH

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

2.2.5.2. Biomass

The growth of the culture was observed by measuring the turbidity of the culture broth as an increase in absorbance at 600 nm.

2.2.5.3. Protein estimation

Total soluble protein was determined by Lowry's method (Lowry et al., 1951) using bovine serum albumin as the standard.

2.3. RESULTS AND DISCUSSION

2.3.1. Isolation of actinomycete strains

There are many recommended media for the isolation of actinomycetes from soil and other samples. Starch casein agar and humic acid vitamin agar were the two media used in the present study. Despite the media used, the actinomycetes were found to be slow growing with an average incubation time of one week. The selective isolation of actinomycetes on humic acid vitamin agar has been reported to be much better than that obtained with other media like starch casein agar (Hayakawa and Nonumura, 1987) and similar result was observed in the present study also. Humic acid has certain extent of structural similarity to lignin model compounds which enabled the better isolation of ligninolytic actinomycete strains on HV agar. The addition of cycloheximide in the isolation media and the heat pretreatment of samples served to minimize the contamination by fungi and other bacteria respectively.

Isolate no.	Type of the sample	Morphology / Colour	Pigmentation
NJP 9 A	Clay	Smooth, gray	Dark brown
NJP17	Sand	Serrated, white	Dark yellow
NJP 21	Sediment	Serrated, white	Yellow pigment
NJP 27	Sediment	Smooth, green	No pigment
NJP 28	Sand	Smooth, pink	Brown pigment
NJP 41	Sediment	Serrated, gray	Dark gray
NJP 41A	Sediment	Serrated, blue	Bluish black
NJP 41A4	Sediment	Serrated, gray	Yellow pigment
NJP 43	Sediment	Smooth, gray	No pigment
NJP 46	Sediment	Smooth, light gray	Dark gray
NJP 47	Sediment	Smooth, gray	Cloudy gray
NJP 47A2	Sediment	Smooth, gray	No pigment
NJP 47B1	Sediment	Serrated, gray	Pink
NJP 47 B	Sediment	Smooth, gray	Bright yellow
NJP 47B4	Sediment	Smooth, white	Pink
NJP 48	Sediment	Smooth, gray	Gray pigment
NJP 49	Sediment	Serrated, pink	Brown pigment
NJP 52B	Sand	Smooth, bright green	No pigment
NJP 53A	Sand	Smooth, gray	Yellow pigment
NJP 58A	Sand	Smooth, white	No pigment

Table 2.1. Characteristics of the isolated actinomycetes cultures

On the solid agar media for isolation, the different isolates could be distinguished by their colour and morphology. The Gram's staining revealed that all the isolates were Gram positive, thin filaments and in most cases the filaments were broken into small fragments or coccoid structures. Many of the isolated cultures showed the production of diffusible pigments, a characteristic feature of actinomycetes. Twenty actinomycete cultures were isolated from a total of 59 soil samples collected from the marine and mangrove regions. Samples collected from mangrove regions yielded more actinomycete cultures as compared to the marine samples. The details of the characteristic features of the isolates are given in Table 2.1.

2.3.2. Qualitative screening

Dye-based plate assay is the common method used for the screening of lignin degrading fungi (Kiiskinen et al., 2004). The degradation of the dye or other lignin model compounds and the formation of clear zone around the colony on the agar plates could be correlated to the ability of the strains to produce lignin-degrading enzymes. The results of the screening of actinomycetes on agar plates using different lignin model compounds and dyes are given in Table 2.2. Although the degradation of RBBR was suggested as a screening method for actinomycetes by Pasti and Crawford (1991), no such significant correlations could be achieved in the present study with dye incorporated agar plates. However the dyes like Poly R and RBBR could sustain the growth of the cultures in the agar plates in the presence and absence of glucose.

Isolates	Poly R	RBBR	Guaiacol	Cinnamic acid	Vanillic acid	Ferulic acid
NJP 41	-	-	++	-	-	-
NJP 46	-	-	+	-	-	-
NJP 49	-	-	+++	-	-	-
NJP 43	-	-	++	-	-	-
NJP 47	-	-	+	-	-	-
NJP 48	-	-	++	-	-	-

Table 2.2. Result of plate assay method for ligninolytic enzymes production using dyes and lignin model compounds

The degradation of different model compounds like syringic acid, vanillic acid, ferulic acid etc in the liquid culture medium by lignin degrading actinomycetes was reported by Ball et al (1989). However, there are not many reports on the incorporation of these model compounds in the solid screening media of actinomycetes. In the present study, ferulic acid, vanillic acid and cinnamic acid could sustain the growth of cultures in the agar plates only in the presence of glucose and no clear zone was observed in those plates even after two weeks of incubation. Among the different lignin model compounds tried, only guaiacol at a concentration of 1mM showed positive results in the form of purple coloured zone around the colonies after an incubation period of 7 days (Fig. 2.1.) The colour change was observed in the presence as well as in the absence of glucose which indicated the ability of the cultures to use guaiacol as the sole source of carbon. Four of the actinomycete cultures (NJP 41, 49, 43 and 48) were able to give positive result in the guaiacol incorporated agar plates, where as, two cultures (NJP 46 and 47) showed more growth in the guaiacol plates even though the colour change was not prominent. Since guaiacol is a compound that could be degraded by LiP, MnP as well as laccase, the exact nature of the enzyme that effected the utilization of the compound was not obvious at the primary screening stage.

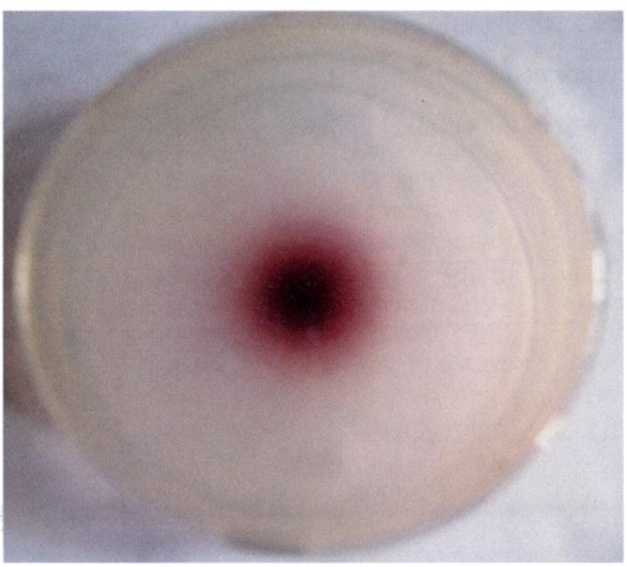


Fig 2.1. Primary screening for ligninolytic enzyme production using guaiacol

2.3.3. Quantitative screening

Rice straw, having a lignin content of approximately 17 % was used as the substrate for enzyme production by submerged fermentation. Lignin is a complex biopolymer which makes its transport across the membrane into the cell difficult and the degradation of it is mediated mainly by extracellular enzymes, though intracellular enzymes are also there whose role is only less significant. Hence the focus of the study was on extracellular ligninolytic enzymes. The ligninolytic enzyme profile of the selected isolates in submerged fermentation is given in Figure 2.2. The production of LiP by six out of the twenty isolates indicated that LiP was the predominant ligninolytic enzyme of the isolated actinomycetes confirming earlier reports (Crawford et al., 1983). Laccase was produced by four out of the twenty isolated cultures. One of the cultures, NJP 49 produced MnP at lower levels along with higher titres of LiP and laccase.

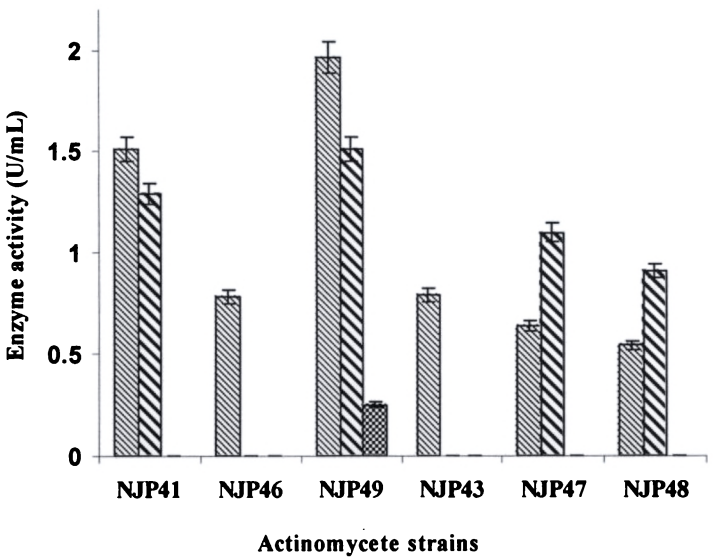


Fig. 2.2. Ligninolytic enzyme profile of the selected isolates in submerged fermentation

Rice straw was used as the substrate. pH 7.5; Temperature 30 °C

It was found contradictory that the cultures that were unable to give clear zones in the dye containing agar plates could give enzyme activity in the liquid production medium. Unlike fungi, there are no dependable plate assay methods for the screening of lignin degrading actinomycetes, which is one of the chief obstacles that restricts many workers from exploring this group of organisms for their enzyme potentials. The fact that the titer of peroxidases and oxidases produced by actinomycetes are low compared to that produced by fungi attributes to this incongruence. In this context, a more precise and sensitive technique like spectrophotometric analysis could solve the problem as indicated by the results of quantitative screening.

The production of LiP by several strains of actinomycetes has been cited in the literature. Characterization of the extracellular peroxidase of *Streptomyces viridosporus* T7A was carried out by Ramachandra et al (1987) and they proposed that the lignin-oxidizing enzyme of *S. viridosporus* T7A, designated as actinomycete lignin peroxidase P3 (ALiP – P3) was a heme protein and the enzyme have been confirmed to be capable of catalyzing Cα-Cβ cleavage of lignin substructure model compounds. The occurance of LiP has been established in many other actinomycetes such as *S. setonii, S. chromofuscus, S. thermoviolaceus, Thermomonospora fusca* etc.

Laccases are the second major ligninolytic enzyme of actinomycetes. Laccases have been reported from *Streptomyces* sp viz; *S. badius, S. cyaneus, S. griseus* etc and the enzyme titers obtained in the present study were comparable with that of the reported strains (Yoshimoto et al., 1985; Borgmeyer and Crawford, 1985). MnP is comparatively a least reported enzyme among actinomycetes and the studies on MnP of actinomycetes is still in its infancy. Esposito et al (1998) have suggested the probable production of MnP by a *Streptomyces* strain CCT 4916 that was able to degrade the herbicide diuron by oxidative reactions. However, the study doesn't reveal the quantitative data on MnP produced by the strain. In this respect the present study was successful in quantifying the amount of all the three major lignin degrading enzymes, including MnP, produced by NJP 49.

2.3.4. Enzyme profile of the selected strain

In addition to the production of LiP, MnP and laccase, the strain NJP 49 was found to be producing other commercially important enzymes such as amylase, pectinase and protease (Figs. 2.3a, b, c). The clear zone formed around the spot inoculated culture was taken as the measure for detecting the enzyme production. From the Figure 2.3, it is clear that the strain is a potent producer of pectinase, amylase and protease. However the plate assays performed for cellulase and xylanase yielded negative results.

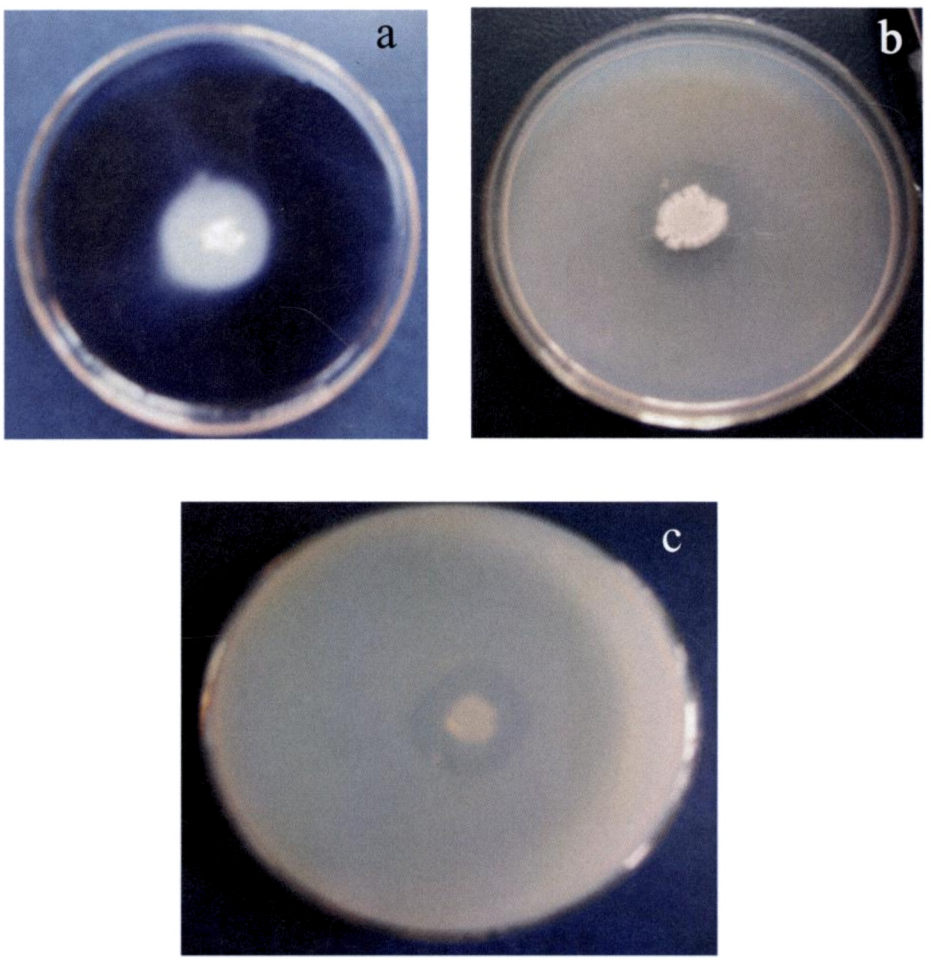


Fig. 2.3. Enzyme profile of *S. psammoticus* using plate assay method

(a) Amylase; (b) Protease; (c) Pectinase

2.3.5. Identification of the selected strain

The strain NJP 49 that was capable of producing all the three major ligninolytic enzymes was selected as the best strain for future works. The growth pattern of NJP 49 in starch casein agar tube and plate is shown in Figures 2.4a and 2.4b. The strain NJP 49 was identified as *Streptomyces psammoticus* by Institute of Microbial Technology (IMTECH), Chandigarh. Figure 2.5a and 2.5b shows the structure of *S. psammoticus* as revealed by Gram's staining and scanning electron microscopy respectively. Growth properties and biochemical characteristics of *S. psammoticus* are given in Table 2.3.

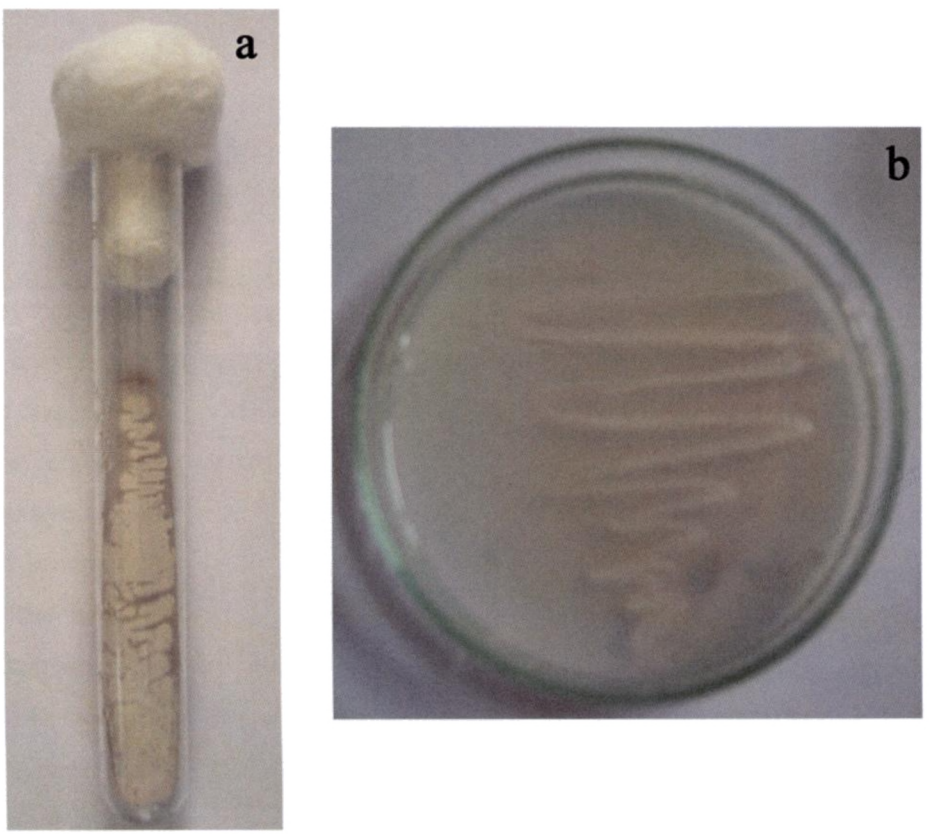


Fig. 2.4. Growth of NJP 49 (*S. psammoticus*) in starch casein agar (SCA) medium

(a) SCA slant culture of NJP 49; (b) SCA plate culture of NJP 49

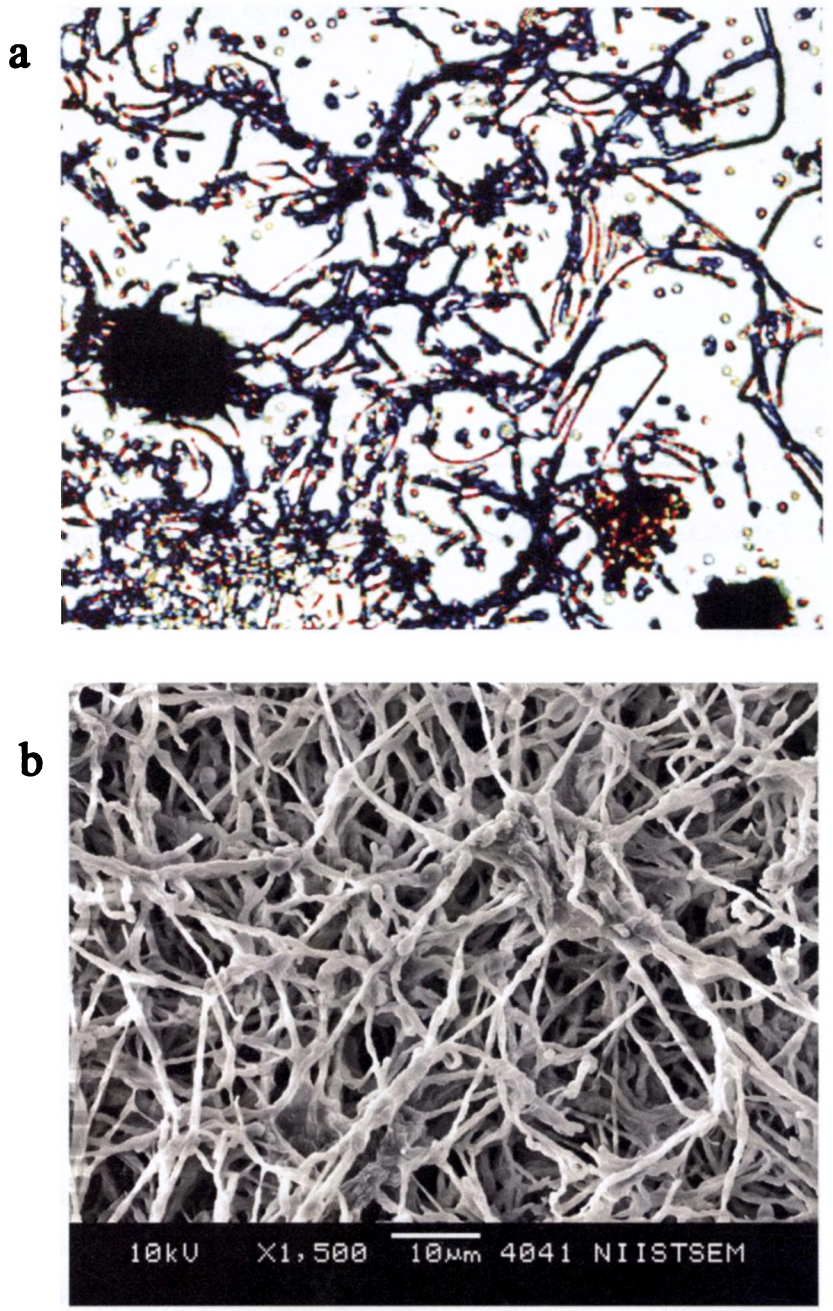


Fig. 2.5. Morphology of *S. psammoticus*

(a) Light micrograph; (b) Scanning electron micrograph

Parameter	Description
Colony morphology	Gram positive filaments with pink aerial mycelium and hard substrate mycelium, tough leathery texture, serrated margin.
Growth temperature	25-37 °C
Growth pH	5 - 9
Growth on NaCl	2-5%
Anaerobic growth	Nil
Biochemical characteristics	Catalase and oxidase positive. Degrades starch, casein and citrate
Carbohydrate utilization	Utilizes glucose, arabinose, fructose, mannitol and salicin

Table 2.3. Growth and biochemical characteristics of *S. psammoticus*

2.4. CONCLUSION

Twenty actinomycete cultures were isolated from the samples collected from marine and mangrove regions. The mangrove regions proved to be an ideal source for isolating lignin degrading actinomycetes. *S. psammoticus,* the potent isolate was found to be capable of producing all the three major lignin degrading enzymes viz; LiP, MnP and laccase, which is an unusual character among actinomycetes. Even though the enzyme titers of LiP produced by *S. psammoticus* was found to be higher than that of laccase, the latter was selected for the future works owing to its importance in industrial sectors. LiP and MnP are evaded from most of the commercial applications due to the requirement of external supply of H_2O_2 for their action. Laccases act on a wide variety of substrates and the low substrate specificity of laccases makes this enzyme suitable for a wide range of industrial applications.

Chapter 3.

OPTIMIZATION OF LACCASE PRODUCTION BY STREPTOMYCES PSAMMOTICUS IN SUBMERGED FERMENTATION

3.1. INTRODUCTION

Owing to its vivid biotechnological applications, studies on laccase producing organisms have been intensified in the recent years and the optimization of laccase production from different microorganisms is being carried out by several groups. The ever-increasing demand for this enzyme requires the production process to be economical. Identifying inexpensive raw materials for enzyme production could be viewed as a solution to make the entire process cost effective and further enhancement using inducers may add to the benefit.

The production of laccases from different microorganisms by submerged fermentation has been reported (Prasad et al., 2005; Tong et al., 2007; Janusz et al., 2007). Submerged fermentation is the cultivation of organisms in liquid culture media. This mode of fermentation has many advantages with respect to the purity of the product and easiness in controlling different process parameters and scale-up processes. The optimization of physico–chemical conditions is inevitable in any fermentation process and it is usually performed by varying the levels of one independent variable while fixing other variables at a certain level. The optimization of various process parameters by conventional method for laccase production has been reported (Songulashvili et al., 2007). However, the conventional method of optimization is laborious and time consuming and often interaction effects are overlooked, which demands the need for a more powerful technique by which multiple variables can be optimized in relatively few experiments.

Statistical Experimental designs are powerful tools for searching the key factors rapidly from a multivariable system. Plackett - Burman design (Plackett and Burman, 1946) is one such method that has been frequently used for screening multiple factors at a time. This experimental design is particularly useful for initial screening as it is used for the estimation of only the main effects. The significant factors obtained from the screening experiments could be further optimized by employing response surface methodology that enables the study of interaction effects among different variables. Optimization of media components for the production of laccase by response surface methodology has been reported in the case of different

fungal strains (Trupkin et. al., 2003), (Vasconcelos et. al., 2000) and (Nyanhongo et. al., 2002).

3.2. MATERIALS AND METHODS

3.2.1. Medium and cultural conditions for submerged fermentation (SmF)

The basal media used for laccase production had the following composition (g/L): Yeast extract – 6.0, $(NH_4)_2SO_4$ - 0.1, $MgSO_4$ - 0.1, $CaCO_3$ - 0.02, $CuSO_4$ – 0.001, pH -7.5 and 1 mL of trace elements solution. The trace elements solution contained 0.1 % $FeSO_4$, 0.09 % $ZnSO_4$ and 0.02 % $MnSO_4$, pH 7.5. Adequate aeration was provided by agitation at 150 rev/min at 30 °C for 4 days. The culture grown under the same conditions for 48 h was used as the inoculum for enzyme production.

3.2.2. Optimization of cultural and nutritional parameters in SmF (Single parameter optimization)

Various agro industrial residues like rice straw, rice bran, wheat bran, sugarcane bagasse, coir pith and coffee pulp (each 10 g/L) were screened for the production of laccases in submerged fermentation. The influence of salinity on laccase production by the mangrove isolate *S. psammoticus* was determined by altering the level of distilled water in the medium with different proportions of seawater (SW) with a salinity of approximately 35 g/L. Medium containing distilled water and 25 g/L NaCl was also included in the study as the control for the experiments on salinity. The initial pH of the medium was changed from 4 to 10 using 1M HCl and 1M NaOH to study the effect of initial media pH. Temperature optimization was done by incubating the culture flasks at temperatures ranging from 28–40 °C. Agitation rate (100–200 rpm) and inoculum size (5–25 %) were also varied to study their influence on laccase production. The inoculum contained 5 x 10^6 CFU/mL. The effect of organic and inorganic nitrogen sources was studied and the sources used were yeast extract, peptone, tryptone, corn steep solid, ammonium sulphate, sodium nitrate, ammonium chloride and potassium nitrate. The organic sources were added at a

concentration of 6 g/L while the inorganic nitrogen sources were included in such a way to contain 0.002 % Nitrogen.

3.2.3. Effect of inducers

In order to study the effect of inducers on enzyme production, various aromatic compounds such as gallic acid (3, 4, 5 - trihydroxy benzoic acid), ferulic acid (4-hydroxy, 3 - methoxy cinnamic acid), guaiacol (2 – methoxy phenol), veratryl alcohol (3,4 dimethoxy benzyl alcohol), catechol (Benzene – 1, 2 diol), pyrogallol (Benzene – 1, 2, 3 triol), para anisidine (*p* - methoxy aniline), and vanillic acid (4 - hydroxy, 3- methoxy benzoic acid) were incorporated in the fermentation medium. Besides these aromatic compounds $CuSO_4$ was also included in the study. All the inducers were added at 1 mM concentration at the time of inoculum transfer. Catechol, vanillic acid and copper sulphate were dissolved in sterile water while all the other inducers were dissolved in 50 % ethanol and used for the study.

3.2.4. Statistical optimization of laccase production in SmF

The optimization of laccase production was carried out using statistical design of experiments in two steps. The first step involved the screening of variables and the second step involved the optimization of significant variables. Plackett-Burman design, a widely used fractional factorial method was adopted for the screening of cultural and nutritional parameters influencing laccase production by *S. psammoticus* in submerged fermentation. All the experiments were carried out in triplicates according to a design matrix, which was based on the number of variables to be investigated. Each row of the matrix represented a trial and each column represented an independent factor whose levels were varied. The total number of trials to be carried out was $n + 1$, where n was the number of variables under study. Each variable was evaluated at two levels, a high (+) and a low (-) level. For the present study the selected variables included physical factors such as pH, temperature, inoculum size, incubation period and agitation. The nutritional factors studied were carbon source, nitrogen source, $MgSO_4$, $(NH_4)_2SO_4$, $CuSO_4$ and trace elements. The

eleven variables were evaluated by twelve experiments and the levels of each variable were determined based on prior experience with the system. The significant variables were identified by the analysis of the Plackett-Burman experiments and their levels were further optimized for enhanced laccase production by employing a Box-Behnken design (Box and Behnken, 1960). Each selected variable was analyzed at three levels – low, medium and high coded as –1, 0 and +1 in a total of 54 runs. The behavior of the system was explained by a second order polynomial equation (Eqn. 1)

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response, β_0 is offset term, β_i is linear effect, β_{ii} is squared effect, β_{ij} is interaction effect, and Xi is dimensionless coded value of independent variables under study. This design was used to evaluate the main effects, interaction effects and quadratic effects and to optimize the levels of parameters for enhancing laccase production. The statistical software Design Expert (version 6.0.6, Stat–Ease, Minneapolis, USA) was used for experimental design and data analysis.

3.2.5. Biomass estimation

The biomass was estimated by measuring intracellular protein concentrations (Arias et. al., 2003). Cells were harvested by centrifugation at 5,000 x g for 10 min, washed with distilled water and resuspended in 0.1 M sodium phosphate buffer (pH 7.0). The cells were disrupted using a cell homogenizer and the intracellular protein content was estimated by Lowry's method (Lowry et. al., 1951).

3.3. RESULTS AND DISCUSSION

3.3.1. Single parameter optimization

3.3.1.1. Substrate screening

Among the different agro industrial residues screened for laccase production, coffee pulp was the most suitable substrate for enzyme production by *S. psammoticus* in submerged fermentation (Fig. 3.1). Rice straw and wheat bran were also identified as

promising substrates while the production in other substrates like coir pith and sugarcane bagasse were very low. Coffee pulp has been reported in the production of various industrially important enzymes such as pectinase, tannase, caffeinase, etc (Pandey et al., 2000). Use of coffee pulp for ligninolytic enzyme production by fungi has also been reported in the recent literature (Mata et al., 2005). The result of the present study implying coffee pulp, as the best substrate for laccase production was not an astonishing result. The presence of significant amount of polyphenols in coffee pulp (Pandey et al., 2000) could very well substantiate the enhanced production of laccase, which is a polyphenol oxidase. Rice straw also had given considerable yield owing to the fact that it contains up to 12 -17 % of lignin.

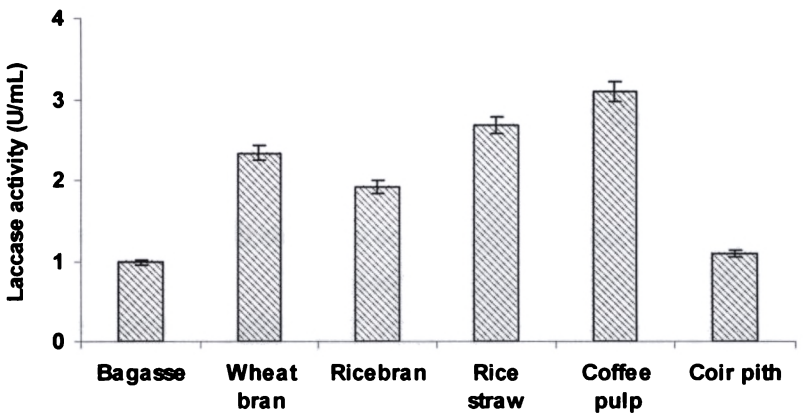


Fig. 3.1. Production of laccase (▨) by *S. psammoticus* on different substrates

The cultural conditions were pH - 7.5; Temperature – 30 °C; Agitation – 150 rpm; Inoculum size – 10 %

3.3.1.2. Effect of salinity of the medium

Salinity was a significant parameter influencing the growth and enzyme production by this isolate. Maximum laccase yield was obtained when 50 % of the distilled water in the production medium was replaced with equal volume of seawater with a salinity of approximately 35 g/L (Fig. 3.2). *S. psammoticus*, the culture used in the present

study was a mangrove isolate and hence maintaining a moderate level of salinity in the production medium was essential for enzyme production by this organism. The enzyme yield was low in the medium that contained distilled water and 2.5 % NaCl which implied that the enzyme production was influenced by some of the elements present in the seawater other than sodium chloride.

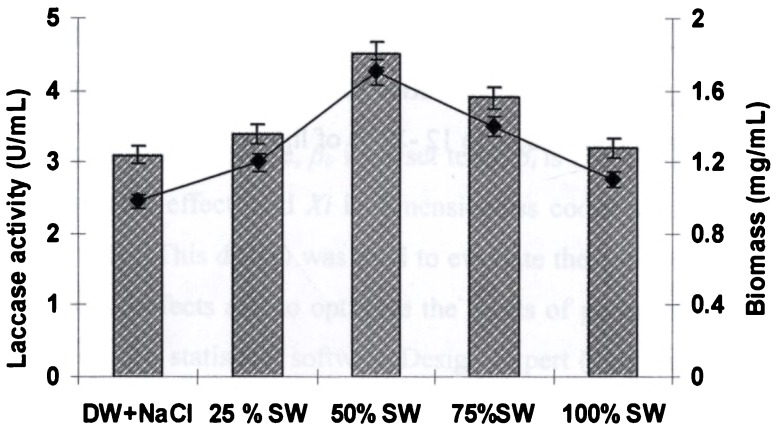


Fig. 3.2. Influence of salinity on production of laccase (▨) and biomass (◆) by *S. psammoticus* in SmF

Coffee pulp was used as the substrate. The cultural conditions were pH - 7.5; Temperature – 30 °C; Agitation – 150 rpm; Inoculum size – 10 %

3.3.1.3. Effect of medium pH and incubation temperature

Results of pH optimization studies indicated that pH 7.5 was the optimum for laccase production (Fig. 3.3). The enzyme production was found to be increasing gradually with the increase in initial pH, reached the maximum at 7.5 and then decreased at higher pH values. The figure depicted that pH in the neutral to alkaline range was the ideal condition for laccase production by this strain. It is probable that, being a mangrove isolate, this organism could exhibit some unusual characteristics such as the expression of laccase activity in the alkaline range of 7.5 as compared to the laccases from other bacterial sources such as *Bacillus subtilis* (Martins et al., 2002)

and *Streptomyces cyaneus* (Arias et al., 2003) that are reported to be active in the acidic pH. Fig. 3.3 also shows the change in media pH over the period of fermentation. The initial media pH shifted towards the higher pH as the enzyme production started. The observed result was of much significance because most of the reported laccases from the fungal sources show drop in production media pH during the course of the experiment (Kaal et al., 1995). The ability of the culture to grow and produce laccases in media of high pH values makes this enzyme more suitable for industrial applications. The optimum temperature for laccase production was observed at 32 °C (Fig. 3.4). Even though 32 °C was the optimum, considerable enzyme yield was obtained also at 30 °C while the activity was almost reduced to half at higher temperature of 40 °C.

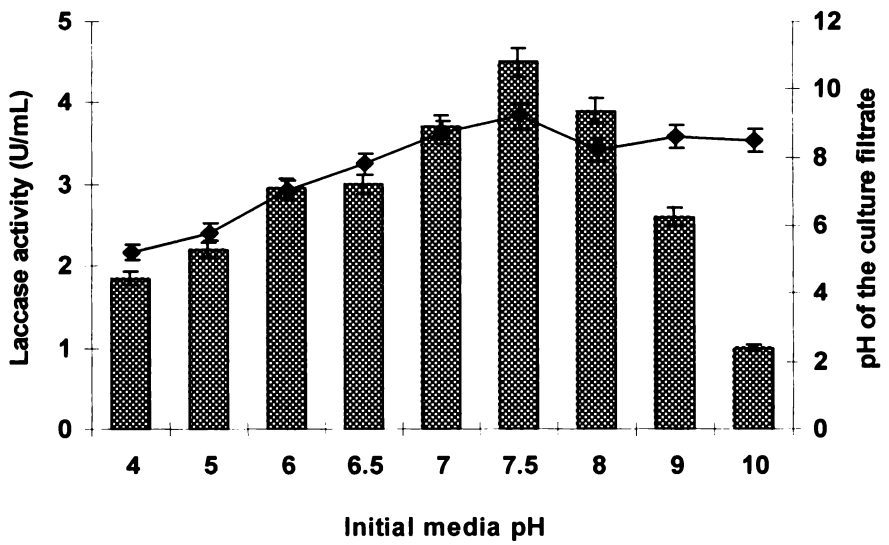


Fig. 3.3. Effect of initial pH of medium on production of laccase (▩) by *S. psammoticus* and pH of culture filtrate (◆) at the hour of maximum production

Coffee pulp was used as the substrate. The cultural conditions were Temperature – 30 °C; Agitation – 150 rpm; Inoculum size – 10 %

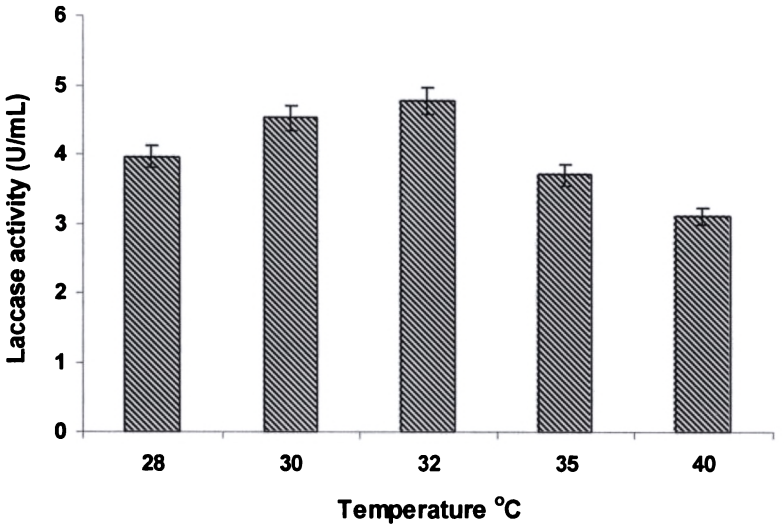


Fig. 3.4. Production of laccase (▨) by *S. psammoticus* in SmF at different incubation temperatures

The cultural conditions for the experiment were pH – 7.5; Agitation – 150 rpm; Inoculum size – 10 %. Coffee pulp was used as the substrate.

3.3.1.4. Effect of agitation rate

The agitation rate influenced the enzyme production in such a way that the yield increased with increase in agitation up to 175 rpm and decreased at 200 rpm although the effect was not drastic (Fig. 3.5). The enhancement in enzyme production at higher agitation rate was due to the better aeration in the well-agitated flasks that was essential for the growth and enzyme production by *S. psammoticus*, which is an aerobic organism. The drop in enzyme yield at 200 rpm could be attributed to the possible damage that may occur in the filamentous structure of the organism and thus hindering the enzyme production.

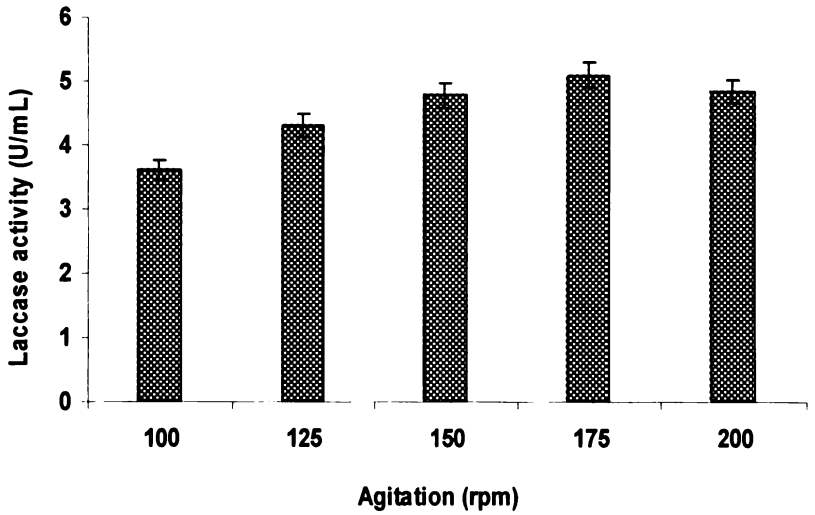


Fig. 3.5. Effect of agitation rate on production of laccase (▩) by *S. psammoticus*

The cultural conditions for the experiment were pH – 7.5; Temperature – 32 °C; Inoculum size – 10 %. Coffee pulp was used as the substrate.

3.3.1.5. Influence of inoculum size

The optimization of inoculum size revealed that an inoculum size of 15 % was the optimum for laccase production (Fig. 3.6). An inoculum size below this level was inadequate for enzyme production while the higher levels resulted in rapid depletion of nutrients in the media and reduced the enzyme yield. Also higher cell density would lead to the production of inhibitory metabolites that may be interfering with the enzyme production.

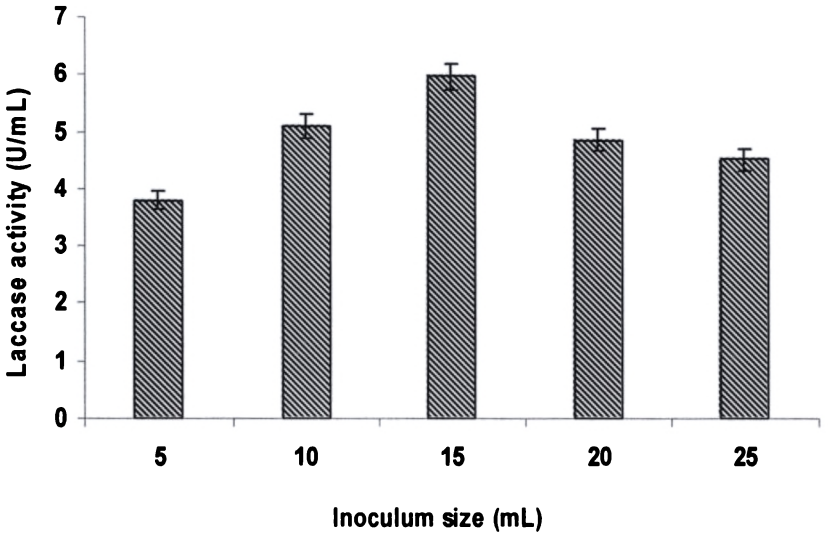


Fig. 3.6. Effect of inoculum size on laccase (▨) and biomass production (◆) by *S. psammoticus*

The cultural conditions for the experiment were pH – 7.5; Temperature – 32 °C; Agitation – 175 rpm. Coffee pulp was used as the substrate.

3.3.1.6. Effect of nitrogen sources

Results of the present study indicated that laccase production was higher in the organic nitrogen sources than the inorganic sources studied (Fig. 3.7). Among the organic sources studied yeast extract was the best for laccase production. Yeast extract was included as the nitrogen source in the basal media that was used for all the experiments. Replacement of yeast extract with other nitrogen sources failed to enhance laccase yield, which indicated that yeast extract is the suitable nitrogen source for laccase production by *S. psammoticus*.

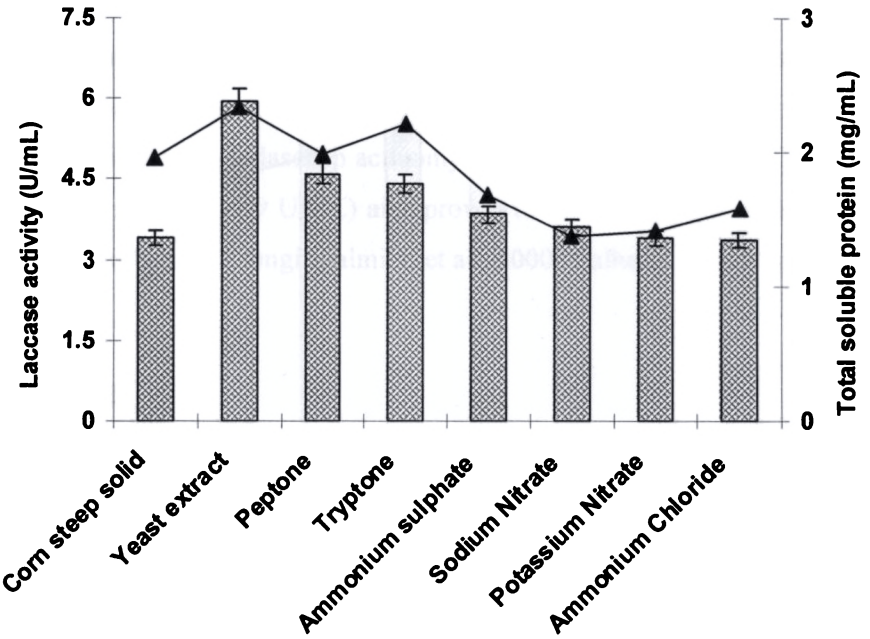


Fig. 3.7. Effect of nitrogen sources on laccase production (▩) and total soluble protein (▲) by *S. psammoticus* in SmF

The cultural conditions for the experiment were pH – 7.5; Temperature – 32 °C; Agitation – 175 rpm; Inoculum size – 15 %. Coffee pulp was used as the substrate.

3.3.1.7. Time course of laccase production in SmF

The time course of laccase (Fig. 3.8) clearly indicated that maximum enzyme yield and biomass could be attained at 48 h and considerable production of enzyme and biomass was retained at 60 h. The results also confirmed that there was a linear correlation between biomass and laccase production and these results were in compliance with the earlier reports that the ligninolytic enzyme production by actinomycetes is strictly a growth associated primary metabolic activity (McCarthy, 1987).

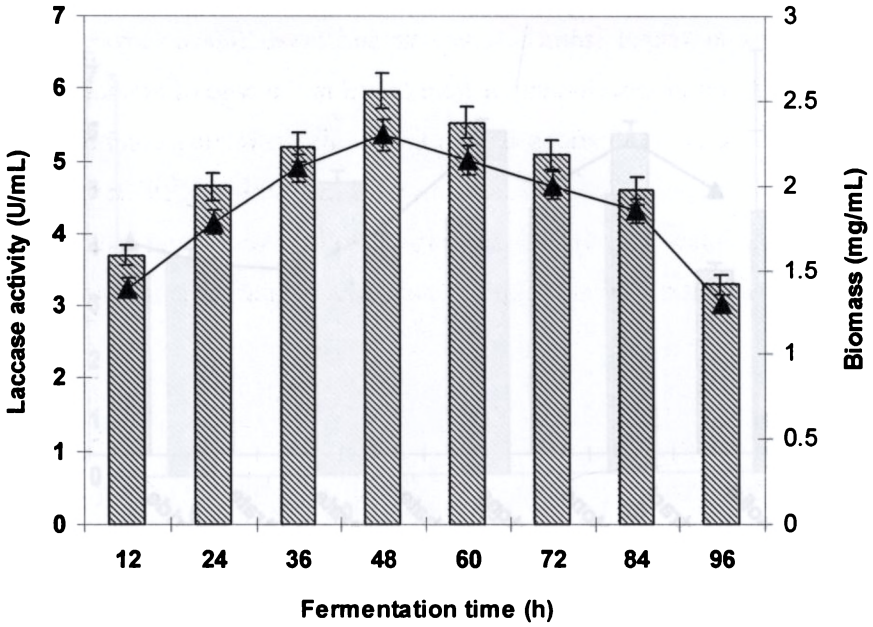


Fig. 3.8. Time course of laccase production (▨) and biomass (▲) by *S. psammoticus in SmF*

The cultural conditions for the experiment were pH – 7.5; Temperature – 32 °C; Agitation – 175 rpm; Inoculum size – 15 %. Coffee pulp and yeast extract were used as the substrate and nitrogen source respectively.

3.3.1.8. Effect of inducers

Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms (Leonowicz et al., 2001; De Souza et al., 2004) and the nature of the compound that induces laccase activity differs greatly with the species. The results of the studies on different laccase inducers indicated that all the inducers included in the study were capable of enhancing laccase production by *S. psammoticus* (Fig. 3.9). Among the different aromatic compounds pyrogallol (9.6 U/mL) and para anisidine (9.2 U/mL) substantially enhanced the production. Catechol, gallic acid, and veratryl alcohol also induced laccase production although to lesser extent than pyrogallol and para anisidine. The aromatic inducers used in the present study were either lignin related compounds having structural similarity to

lignin or polyphenols and most of these compounds have been reported as effective laccase inducers in fungal strains (Revankar and Lele, 2006). However, no other comparative data on laccase induction were found in the case of actinomycetes even though there were some citations on the use of lignin related compounds for the production of lignin peroxidases in actinomycetes (Spiker et al., 1992; Godden et al., 1992). Copper sulphate (7.9 U/mL) also proved to be a promising inducer as it was established in most of the fungi (Palmieri et al., 2000; Galhaup and Haltrich, 2001).

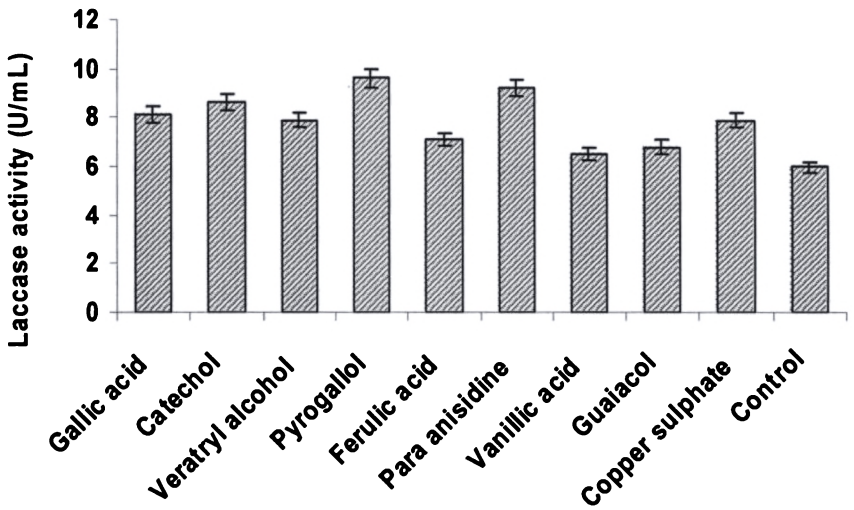


Fig. 3.9. Effect of different inducers on laccase production ( ) by *S. psammoticus* in SmF

The cultural conditions for the experiment were pH – 7.5; Temperature – 32 °C; Agitation – 175 rpm; Inoculum size – 15 %. Coffee pulp and yeast extract were the substrate and nitrogen source respectively.

3.3.2. Statistical optimization

The design matrix selected for the screening of variables for laccase production by statistical optimization and the corresponding responses in terms of enzyme yield are given in Table 3.1.

Variables											Lac U/mL
X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	
5.0	35	72	$1x10^8$	125	15	5	0.5	1.2	0.00	5	1.3
5.0	35	72	$5x10^7$	175	5	1	0.5	1.2	0.02	15	6.8
8.0	35	72	$5x10^7$	175	15	1	1.0	0.4	0.00	5	6.5
8.0	27	72	$1x10^8$	125	15	1	0.5	0.4	0.02	15	2.0
5.0	35	24	$5x10^7$	125	15	5	1.0	0.4	0.02	15	6.0
8.0	35	24	$1x10^8$	175	5	5	0.5	0.4	0.00	15	4.3
5.0	27	72	$1x10^8$	175	5	5	1.0	0.4	0.02	5	1.2
8.0	35	24	$1x10^8$	125	5	1	1.0	1.2	0.02	5	7.2
8.0	27	24	$5x10^7$	175	15	5	0.5	1.2	0.02	5	2.1
8.0	27	72	$5x10^7$	125	5	5	1.0	1.2	0.00	15	1.2
5.0	27	24	$1x10^8$	175	15	1	1.0	1.2	0.00	15	6.4
5.0	27	24	$5x10^7$	125	5	1	0.5	0.4	0.00	5	1.8

Table 3.1. Plackett – Burman design matrix for the screening of process variables

All the variables were studied at two levels (Low and High levels). The experiments were performed in triplicates and the mean values were presented. The variables $X_1, X_2, X_3, \ldots\ldots X_{11}$ represent pH (5 and 8), Temperature (27°C and 35°C), Incubation time (24 hours and 72 hours), Inoculum size ($5x10^7$ CFU and $1x10^8$ CFU), Agitation rate (125 rpm and 175 rpm), carbon source (5 g/L and 15 g/L), Yeast extract (1 g/L and 5 g/L), $MgSO_47H_2O$ (0.5 mM and 1.0 mM), Ammonium sulphate (0.4 mM and 1.2 mM), Copper sulphate (0.00 mM and 0.02 mM) and Trace elements (5mL/L and 15 mL/L) respectively.

The main effects of parameters on laccase production was estimated by subtracting the mean responses of parameters at their lower levels from their corresponding higher levels and dividing by the total number of experimental runs. The adequacy of the model was tested and the parameters with statistically significant effects were identified using the Fisher's test for analysis of variance (ANOVA). The analysis of variance for the selected factorial model showed that the model was significant with a Model F – value of 20.91 (Table 3.2).

Source	Sum of squares	Degrees of freedom	Mean squares	F value	*Prob*> F
Model	66.82	6	11.14	20.906	0.0021
Temperature (B)	25.23	1	25.23	47.365	0.0010
Incubation time (C)	6.45	1	6.43	12.115	0.0017
Agitation rate (E)	5.07	1	5.07	9.518	0.0273
Yeast extract (G)	17.76	1	17.76	33.348	0.0022
$MgSO_4$ (H)	8.67	1	8.67	16.277	0.0100
Trace elements (L)	3.63	1	3.63	6.815	0.0476
Residual	2.66	5	0.53		
Corrected total	69.48	11			

Table 3.2. Analysis of variance for Plackett – Burman factorial model

Factors such as Incubation temperature (B), Incubation time (C), Agitation rate (E), concentrations of Yeast extract (G), $MgSO_4$ (H) and Trace elements (L) had confidence level greater than 95 % (P>F $\leq$ 0.05) and were considered to have a significant effect on the response and selected for further studies.

Factors having a confidence level greater than 95 % (P>F $\leq$ 0.05) were considered to have a significant effect on the response and were selected for further studies. Incubation temperature with *Prob* > F value of 0.0010 was found to be the most influencing factor followed by concentrations of yeast extract (0.0022) and $MgSO_4.7H_2O$ (0.0100) in the medium, incubation time (0.0176), agitation (0.0273)

and trace elements (0.0476) (Table 3.2). Out of the six significant variables identified, yeast extract and incubation time were exerting negative influence while the other factors such as temperature, agitation rate, concentrations of $MgSO_4$ and trace elements exerted positive influence on enzyme production as indicated by the effect estimates (Fig. 3.10).

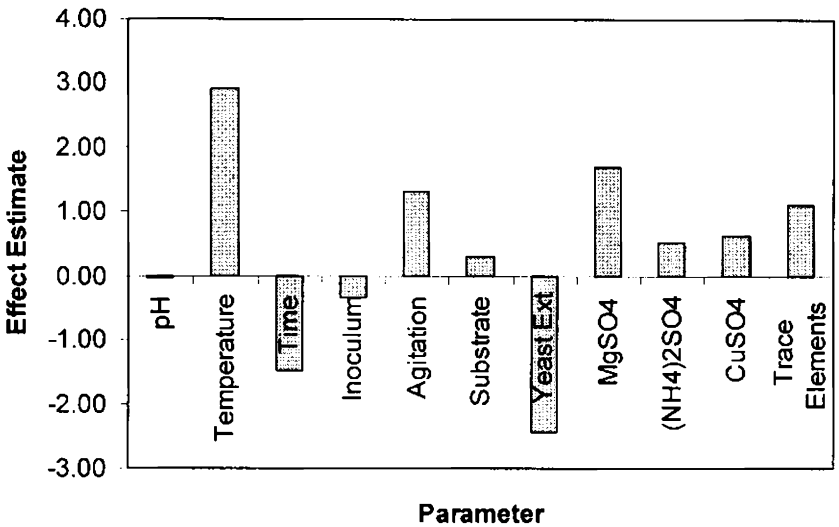


Fig. 3.10. Effect of different operational variables on laccase production.

Positive and negative influence of different variables on laccase production by *S. psammoticus* as screened with Plakett – Burman design.

The six parameters identified as having significant effects on laccase production by the screening experiments were studied using Box Behnken design for optimization of their levels for enhancing enzyme yield (Table 3.3). The levels of other parameters were fixed at their middle levels for the experiments. Table 3 shows the observed and predicted responses for laccase production.

Temp (°C)	YE (g/L)	Mg SO_4 (mM)	Incubation (h)	Agitation (rpm)	Trace Elements (mL/L)	Bio mass (mg/mL)	Laccase U/mL	
							Observed	Predicted
37	1.6	1.0	40	165	12	1.2	8.5	9.4
37	1.9	1.25	38	165	14	1.4	8.9	9.0
34	1.6	1.0	38	150	10	2.8	14.0	14.0
34	1.6	0.75	38	180	12	2.6	13.9	14.0
31	1.9	I.0	36	180	12	2.2	10.5	10.5
31	1.9	1.25	38	165	14	2.0	11.2	11.5
31	1.9	1.0	40	150	12	2.1	12.3	12.4
34	1.9	.0	38	165	12	2.7	14.6	14.6
31	1.9	0.75	38	165	14	1.9	12.2	9.8
31	1.9	1.0	36	150	12	2.0	12.2	12.1
31	1.6	1.0	40	165	12	2.1	12.5	11.5
37	1.9	1.0	40	150	12	1.3	8.6	8.1
37	1.9	1.0	36	180	12	1.5	9.2	9.4
34	1.9	1.25	40	165	14	2.5	14.3	14.6
37	1.9	0.75	38	165	14	1.6	10.0	9.8
34	1.9	0.75	40	165	10	1.9	13.9	14.2
34	1.9	1.25	36	165	14	2.3	14.2	13.7
37	2.2	1.0	40	165	12	1.1	9.1	8.4
34	2.2	1.0	38	150	14	1.6	13.9	14.1
34	1.6	1.25	38	180	12	2.2	14.1	13.6
34	1.9	1.0	38	165	12	2.0	14.6	14.6
34	1.9	0.75	40	165	14	2.6	15.0	15.1
37	1.9	1.0	40	180	12	1.2	10.5	10.1
34	2.2	0.75	38	150	12	1.9	13.9	14.3
34	1.9	1.25	36	150	10	2.4	14.3	14.2
34	1.6	1.0	38	180	14	2.6	14.7	14.6
34	1.6	1.25	38	165	12	2.0	13.8	13.6
34	2.2	1.25	38	165	12	2.8	14.4	14.3
34	2.2	1.25	38	165	12	2.4	15.0	14.8
37	1.9	1.25	38	180	10	1.3	8.5	8.7
34	1.9	1.0	38	180	12	2.5	14.8	14.6

31	1.9	1.25	38	165	10	1.7	12.0	12.1
34	2.2	0.75	38	150	12	2.8	14.8	14.8
31	1.9	1.0	40	165	12	1.8	11.6	12.1
34	1.9	1.0	38	165	12	2.4	14.9	14.6
34	2.2	1.0	38	165	10	2.2	15.0	14.9
31	2.2	1.0	36	165	12	1.9	13.0	12.3
31	2.2	10	40	165	12	1.4	12.8	12.6
34	1.9	0.75	36	165	14	2.3	14.6	14.5
37	2.2	1.0	36	150	12	1.1	8.4	9.0
31	1.9	0.75	38	165	10	1.6	11.8	11.7
34	1.9	0.75	36	180	10	2.4	14.5	14.1
37	1.9	1.0	36	165	12	1.3	9.0	8.7
37	1.6	1.0	36	165	12	1.4	8.8	8.6
34	2.2	1.0	38	165	10	2.5	14.7	14.8
31	1.6	1.0	36	165	12	1.5	8.9	9.8
34	1.9	1.0	38	165	12	2.2	14.2	14.6
37	1.9	0.75	38	150	10	1.3	9.1	8.8
34	1.9	1.0	38	165	12	2.5	14.6	14.6
34	1.9	1.25	40	165	10	2.6	14.5	14.6
34	1.6	1.0	38	150	14	2.2	14.2	14.0
34	1.6	0.75	38	150	12	2.0	13.9	14.0
34	1.6	1.0	38	180	10	2.5	13.8	13.4
34	2.2	1.0	38	180	14	2.8	15.2	15.2

Table 3.3. Box-Behnken design matrix showing the experimental and predicted values of laccase production along with the experimental values of biomass

Regression analysis of the data was performed for testing the adequacy of the proposed quadratic model and the following second order polynomial equation was derived (Eqn. 2)

$$Y = 14.62 - 1.35X_1 + 0.38X_2 - 0.100X_3 + 0.25X_4 + 0.12X_5 + 0.096X_6$$
$$- 4.04X_1^2 - 0.23X_2^2 - 0.16X_3^2 - 0.092X_4^2 + 0.00417X_5^2 + 0.050X_5^2$$
$$- 0.52X_1X_2 - 0.11X_1X_3 - 0.21X_1X_4 + 0.56X_1X_5 + 0.21X_1X_6$$
$$+ 0.075X_2X_3 - 0.35X_2X_4 + 0.12X_2X_5 - 0.21X_2X_6$$
$$+ 0.063X_3X_4 + 0.000X_3X_5 - 0.20X_3X_6$$
$$+ 0.34X_4X_5 + 0.11X_4X_6 + 0.29X_5X_6 \quad (2)$$

where, Y is the predicted response, X_1, X_2 ..... X_6 are the coded values for the variables. Fisher's test for the analysis of variance done on the experimental data indicated that the model was highly significant with and F value of 37.85 and P>F of 0.0001 (Table 3.4).

Source	Sum of squares	DF	Mean square	F value	*Prob*> F
Model	274.52	27	10.17	37.85	<0.0001
Temperature (A)	43.74	1	43.74	162.82	<0.0001
Yeast extract (B)	3.45	1	3.45	12.84	0.0014
Incubation time (D)	1.5	1	1.5	5.58	0.0259
Temperature · Temperature (A^2)	168.02	1	168.02	625.44	<0.0001
Temperature · yeast extract (AB)	2.21	1	2.21	8.21	0.0081
Temperature ·Agitation (AE)	2.53	1	2.53	9.42	0.0050
Residual	6.98	26	0.27		
Corrected total	281.51	53			

Table 3.4. Analysis of variance for the response surface quadratic model

The model terms X_1, X_2, X_4, X_1^2, X_1X_2 and X_1X_5 exhibited confidence level above 95% (*Prob* > F value less than 0.05). This indicated that the linear effects of incubation temperature, yeast extract concentration and incubation time, the quadratic effect of incubation temperature and the interaction effects of temperature and yeast extract and temperature and agitation were significant model terms. The model had an R^2 value of 0.975189 indicating a good correlation between observed and predicted responses. For a good statistical model R^2 value should be close to 1.0 where a value >0.75 indicates the aptness of the model. Also the model indicated that the predicted R^2 value of 0.87425 was in reasonable agreement with the adjusted R^2 value of 0.949423. A lower value of the coefficient of variation indicates a greater reliability of the trials and in this case a value of 4.10 % demonstrated that the model was reliable for determination of the optimal levels of the variables and their interaction effects.

Three dimensional response surface curves generated and analyzed by Design Expert software demonstrated that temperature, yeast extract concentration in the medium and incubation time had significant linear effects on the system. Interactions between temperature and yeast extract; and temperature and agitation also had significant effects. Temperature was found to have the most significant effect on laccase production by *S. psammoticus* indicated by a *P* > F value of 0.0001. Figure 3.11 shows the interactive effect between temperature and yeast extract. The yield was found to be slightly increasing with the increase in yeast extract concentration in the temperature range of 31-34 °C. Beyond this level of temperature the concentration of yeast extract failed to enhance the enzyme production.

The interaction between temperature and yeast extract concentration (Fig. 3.11) indicated that temperature is having a profound effect on laccase production almost masking the effect of yeast extract. The biomass production was also observed to be increasing in the temperature range of 31-34 °C (Table 3.3) that might have resulted in the depletion of yeast extract as well as other nutrients in the medium and hence increased concentrations of yeast extract was required by the organism to continue the enzyme production. The effect of nitrogen sources on laccase production by different organisms appears to be greatly controversial (Collins and Dobson, 1997). Although it was established that the ligninolytic enzyme production by

The temperature was found to be one of the crucial factors for enzyme and biomass production by *S. psammoticus* and better aeration and mixing provided by higher agitation rates would always be favourable for enzyme production. However, very high agitation rates may sometimes break the mycelia and thereby hinder with the proper growth and enzyme production by this filamentous organism. From the 3D surface curves it could be observed that the higher levels of agitation rate and yeast extract along with temperature in its optimal range could be the ideal condition for maximum laccase yield from this strain.

3.4. CONCLUSION

Laccases are the most extensively studied group of enzymes among oxidases. Even though the ligninolytic potential of actinomycetes had been established long before (Phelan et.al., 1979), the studies on lignin degrading enzymes from actinomycetes are still in its infancy compared to fungi. The single parameter optimization of various cultural and nutritional parameters for the production of laccase by *S. psammoticus* showed that the enzyme production by this isolate is governed by parameters such as salinity of the production medium and alkaline pH. These remarkable properties make this organism a best candidate for biotechnological applications especially in the areas where alkaline conditions are preferred. Moreover the organism produced laccases within a short incubation period of 48 h. The single parameter optimization yielded a three fold enhancement in laccase production than the un-optimized medium. Optimization of process parameters for enzyme production by statistical approaches is relatively a new trend and it has been widely accepted due to the promising results. In the present study, the initial screening by Plackett–Burman design and further optimization by Box–Behnken method resulted in enhanced production of laccase from *S. psammoticus* in submerged fermentation. Using the statistical approach, seven fold increase in enzyme production as compared to the un-optimized medium was obtained. Also, the

statistical approach proved to be a better method of optimization by yielding a 2.5 fold increase in laccase production as compared to that obtained with single parameter optimization. Hence it can be concluded that response surface methodology is a valuable and dependable tool for the optimization of laccase production from *S. psammoticus* and actinomycetes in general.

Chapter 4.

OPTIMIZATION OF LACCASE PRODUCTION BY STREPTOMYCES PSAMMOTICUS IN SOLID-STATE FERMENTATION

4.1. INTRODUCTION

The application of laccases in biotechnological processes requires the production of high amounts of enzyme at low cost and hence the current focus of laccase research is oriented towards the search for efficient production systems. Production of laccases from microbes has been carried out using submerged as well as solid-state fermentation (SSF) technologies (Rodriguez Couto and Sanroman, 2005; Tong et al., 2007). SSF offers many advantages over submerged fermentation, which include higher product titers, lower wastewater output, reduced energy requirements, simpler fermentation media, etc (Pandey et al., 2001). Moreover, this technique offers the possibility of using by-products and wastes from food and agricultural industries as the raw material for enzyme production, making the process much more efficient from both economical and environmental standpoints. Solid-state fermentation is generally defined as the growth of microorganisms on solid materials in the absence or near absence of free water (Pandey et al., 2000). Most of the work on ligninolytic enzyme production in SSF deal with fungi and large numbers of agro-industrial wastes has been used for ligninolytic enzyme production by different organisms (Rodriguez Couto and Sanroman, 2005).

Any attempt to increase the production of laccases from microbial sources would be of considerable interest in view of its ever-increasing demand. The production of laccases can be considerably enhanced by the addition of inducers, which includes a wide variety of aromatic and phenolic compounds (Leonowicz et al., 2001, De Souza et al., 2004). The use of inducers for laccase production has been widely reported from fungi (Revankar and Lele, 2006). Another valued approach for enhanced enzyme production in SSF is the use of various bioreactors. It includes tray, drum and packed bed reactors. Tray bioreactors tend to be very simple in design, with no forced aeration or mixing for the solid substrate. However, in the tray bioreactors only thin layers can be used, in order to avoid overheating and to maintain aerobic conditions (Tunga et al., 1999). The use of tray fermenters in large-scale production is limited as they require intensive labour, large operational area and high chances of contamination (Pandey et al., 2001). Although the mass heat transfer, aeration and mixing of the substrate are better in rotating drum reactors; the rotation

may cause damage to inoculum that may affect the final product yield. Column fermenters have most commonly been used in laboratory studies for the production of enzymes, organic acids and biologically active metabolites (Pandey et al., 2001). Column fermenters have been reported to be useful for product developments with efficient process controls, particularly for heat removal.

The use of inducers and scale-up processes are two different strategies widely practiced for enhancing laccase yield. The role of inducers in enhanced laccase production in bioreactors using fungal strain has been reported (Meza et al., 2005). However, in such instances it is difficult to determine the contribution of each of these two different strategies towards the enhanced laccase yield. Hence it becomes inevitable to study the effect of each strategy on laccase production independently. However the different enhancement strategies may be combined with an ultimate goal of enhancing the enzyme yield considerably.

4.2. MATERIALS AND METHODS

4.2.1. Substrate preparation for SSF

Five grams of substrate were added to a 250 ml Erlenmeyer flask and was moistened with a salt solution containing (g/L): yeast extract – 1.0, $(NH_4)_2SO_4$ - 0.2, Mg SO_4 - 0.2, $CaCO_3$ - 0.04, Cu SO_4 – 0.002. Three mL of the moistening solution was added to the substrate and the initial moisture level in the substrate was adjusted to 50 % by adding an adequate quantity of distilled water. After sterilization by autoclaving at 121 °C for 45 min, the medium was cooled to room temperature and inoculated with 1 x 10^7 CFU of inoculum and incubated at 30 °C for 96 hours.

4.2.2. Optimization of fermentation process for laccase production under SSF

4.2.2.1. Substrate screening

The use of cheap and easily available agro-industrial residues for the production of value added products is one of the suggested advantages of solid-state fermentation. Different agro-industrial residues were screened to identify the suitable substrate for

laccase production in solid-state fermentation. The substrates used were wheat bran, rice bran, rice straw, coffee pulp, coir pith and sugarcane bagasse.

4.2.2.2. Optimization of particle size and initial moisture content

Process parameters such as particle size of the substrate and initial moisture content were varied at different levels and the effects were studied. The particle size was varied from <300 to >2000μm. Unsieved substrate that contained particles of different sizes were also included in the study. The initial moisture content was varied from 46-75 %.

4.2.2.3. Optimization of pH, temperature and inoculum size

Initial pH of the initial moistening solution was varied from 5–10 and the incubation temperature was studied between 25–40 °C. Inoculum size was varied from 7.5×10^6 – 1.75×10^7 CFU to optimize the laccase production.

4.2.2.4. Effect of carbon and nitrogen supplementation

Supplementation with various organic nitrogen sources (beef extract, yeast extract, peptone, tryptone, and corn steep solid at a concentration of 0.1 % w/v), inorganic nitrogen sources (ammonium sulphate, ammonium chloride, diammonium hydrogen phosphate, potassium nitrate and sodium nitrate in such a way that the medium contained 0.004 % Nitrogen) and additional carbon sources (glucose, galactose, sucrose, starch and xylan at 1 % w/v) were also carried out.

4.2.3. Statistical optimization for laccase production in SSF

Effects of yeast extract, inoculum size and copper sulphate ($CuSO_4$) on laccase production were investigated using central composite design (CCD) in solid-state fermentation. The levels of yeast extract and inoculum level were determined based on our experience from single parameter optimization. The impact of $CuSO_4$, the

widely used laccase inducer, was not studied in the one-factor approach and hence the levels of $CuSO_4$ were set randomly. Central composite designs are response surface designs that can fit a full quadratic model. The effect of each variable on enzyme production was studied at five different levels viz; $-\alpha$, -1, 0, +1, $+\alpha$. A set of 20 experiments was performed in triplicates. All variables were taken at a central coded value considered as zero. The data obtained from RSM on laccase production were subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second-order polynomial equation (1) that represents the behavior of the system

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1 \beta_1 A^2 + \beta_2 \beta_2 B^2 + \beta_3 \beta_3 C^2 + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_2 \beta_3 BC \quad (1)$$

where Y = response variable, β_0 = intercept, β_1, β_2, β_3 = linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$ = squared coefficients, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ = interaction coefficients, and A, B, C, A^2, B^2, C^2, AB, AC, BC = level of independent variables. Analysis of data and generation of response surface graphs were done using the statistical software Design Expert (version 6.0.6, Stat – Ease, Minneapolis, USA).

4.2.4. Effect of inducers

To study the effect of inducers on laccase production, various aromatic compounds such as p-anisidine (p - methoxy aniline), gallic acid (3, 4, 5-trihydroxy benzoic acid), ferulic acid (4 - hydroxy, 3 - methoxy cinnamic acid), guiacol (2 – methoxy phenol), pyrogallol (Benzene – 1, 2, 3 triol), veratryl alcohol (3, 4 dimethoxy benzyl alcohol), catechol (Benzene – 1, 2 diol) and vanillic acid (4 – hydroxy, 3 - methoxy benzoic acid) were incorporated in the fermentation medium. All the inducers were added at a concentration of 1 mM. Catechol and vanillic acid were dissolved in sterile water while all the other aromatic inducers were dissolved in 50 % alcohol. The inducers were added to the flasks just before inoculation.

4.2.5. SSF bioreactor studies

The SSF bioreactor system typically consisted of an aerator pump, air filter, air saturation unit, cylindrical glass column (22 cm x 5 cm) and an air exit unit. A non-aerated column reactor and a 250 mL flask were maintained along with the bioreactor system as control for the experiments. The glass column was placed vertically and packed with 15 g of pre-inoculated rice straw. The cultural conditions optimized for the flask level experiment were adopted for bioreactor studies. Filtered moist air was passed through the bottom of the column. The fermentation was carried out for 72 h at 30 ± 2 °C. The effect of aeration on enzyme production was studied by varying the aeration rate from 0.5-2.0 vvm (vessel volume per minute). To study the effect of inducers in scale-up studies, the aromatic compounds were added to the substrate prior to inoculation. The inducers were prepared in the same manner as described above.

4.2.6. Enzyme extraction

The fermented material was extracted with distilled water to get a final extraction volume of 100 mL. The contents were mixed thoroughly by keeping the flasks on a rotary shaker at 200 rpm for 1 h. After 1 h, the contents of the flask were filtered using muslin cloth. The enzyme extract obtained after filtration was then centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was collected and used for enzyme assay.

4.2.7. Biomass estimation

Biomass estimation was carried out by determining the N-acetyl glucosamine present in the cell wall (Sakurai et al., 1977). It was expressed as mg of glucosamine/g dry fermented matter.

4.3. RESULTS AND DISCUSSION

4.3.1. Single parameter optimization

SSF was carried out for the production of laccases from *S. psammoticus*. The approach of single-parameter optimization was employed to optimize various cultural and nutritional parameters to enhance laccase production and the optimal level of crucial factors were identified using response surface methodology. Selection of an appropriate substrate is a key factor in SSF which determines the success of the process. It has been a practice to use lignocellulosic materials for the production of ligninolytic enzymes such as laccases. In the present study various agro-industrial residues that contain lignin in different proportions were used for laccase production in SSF.

4.3.1.1. Substrate screening

Among the different substrates screened, rice straw was the most suitable substrate for laccase biosynthesis, followed by coffee pulp (Fig. 4.1). The selection of suitable substrate is one of the most important factors that decide the success of solid-state fermentation process. The use of wheat straw for the production of laccases has been reported widely from fungal as well as actinomycetes strains (Rodriguez Couto and Sanroman, 2005; Berrocal et al., 1997). However, the utilization of rice straw which contains 17 % lignin remains to be a less exploited substrate for ligninolytic enzyme production. SSF was usually performed with filamentous fungi due to their ability to penetrate and colonize the solid substrate particles. In the present study, the filamentous nature of *S. psammoticus* was observed as an added advantage which facilitated the penetration of straw particles and utilization of the nutrients; a characteristic usually appreciated in solid-state culture conditions. The present study has proved the utility of rice straw, which is an inexpensive and easily available raw material, as a suitable substrate for laccase production in SSF.

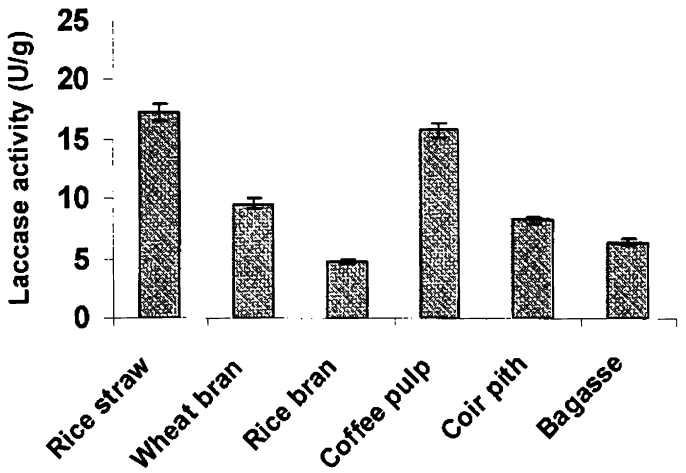


Fig. 4.1. Screening of agro industrial residues for laccase production (▨) in solid-state fermentation

Fermentation conditions include moisture content 50 %, particle size 300–500 μm and incubation temperature 30 °C

The use of rice straw for laccase production by *S. psammoticus* resulted in distortion of the cell wall layers. The difference in the structure of fresh and fermented rice straw is obvious from the Figures 4.2a, 4.2b and 4.2c. Figure 4.2b and 4.2c shows the structural changes that have occurred in rice straw during fermentation with *S. psammoticus*. The channeling and peeling appearance observed on the fermented straw (Fig. 4.2b) was a strong evidence of delignification in the fiber compared to the unfermented straw (Fig. 4.2a). The Figure 4.2c clearly shows that substantial delignification had occurred on the rice straw upon fermentation with *S. psammoticus.*

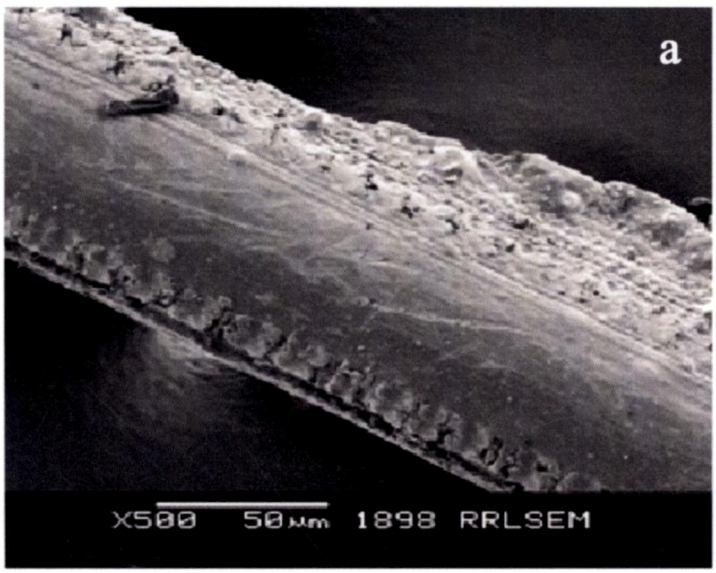
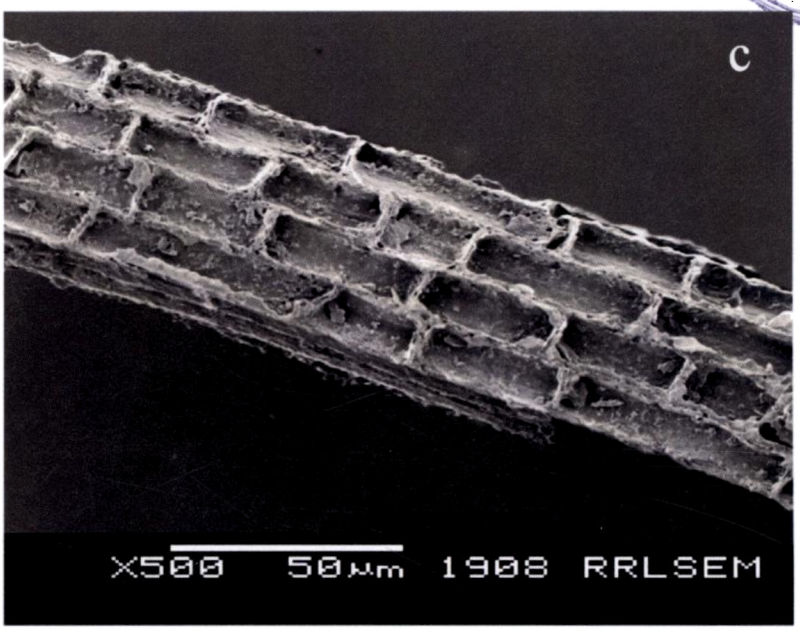


Fig. 4.2. Scanning electron micrographs of rice straw
(a) Surface characteristics of unfermented rice straw

(b) Appearance of rice straw after 48 h of fermentation with *S. psammoticus* under solid–state culture



(c) Appearance of rice straw after 72 h of fermentation with *S. psammoticus*

Lignin present in the lignocellulosic materials not only acts as a barrier to the hydrolytic enzymes like cellulases and hemicellulases in attacking their respective substrates but also reduces the digestibility of the fiber by ruminants. Delignified straw is generally considered as a high-quality fodder with increased nutritive value and digestibility (Bisaria et al., 1997). Upgrading of rice straw for ruminant feed has been studied by several workers (Huttermann et al., 2000). Hence the present work holds better prospects that the rice straw can very well be utilized for laccase production and simultaneously can be upgraded for fodder production with enhanced digestibility and nutritive qualities.

4.3.1.2. Effect of particle size and initial moisture content

The adherence and penetration of microorganisms as well as enzyme action on the substrate clearly depend upon the physical properties of the substrate such as the accessible area, surface area, porosity, particle size, etc of which particle size plays a major role because all the other physical properties of the substrate depends on it. In

the present study particle size in the range of 500-1000 μm was the optimum for laccase production (Fig. 4.3). The enzyme yield was low in the case of substrates with lower and higher particle size, which was in congruence with the general concept that lower particle size results in substrate agglomeration, enhanced channeling problems and decreased heat transfer while larger particles reduce the production due to limited surface area for microbial attack (Pandey et al., 2000). When un-sieved (mixed) substrate which contained different particle sizes was used, the enzyme production was better than that was obtained with lower and higher particle size substrates. This was probably due to the reason that the above mentioned problems were comparatively less experienced with the mixed particle size.

Moisture is another key parameter to control the growth of microorganisms and metabolite production in solid-state fermentation. Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer while decrease in initial moisture level results in reduced solubility and low availability of nutrients to the culture. An initial moisture content of 65 % was the optimum for laccase production by *S. psammoticus* (Fig 4.4). Rice straw is comparatively a dry substrate and hence a low initial moisture level was observed to be inadequate for moistening the substrate evenly. However increasing the initial moisture content above the optimum also resulted in decreased enzyme yield due to the reduction in inter particle space and decreased porosity.

4.3.1.3. Effect of pH, incubation temperature and inoculum size

The optimum pH for maximal laccase production during SSF was pH 8 (26.9 U/g). It has been observed that the laccase production by this organism in submerged fermentation occurred at pH 7.5. The current results confirmed that the enzyme production was favoured by neutral to alkaline pH range, where as the acidic pH decreased the enzyme yield considerably (Fig. 4.5).

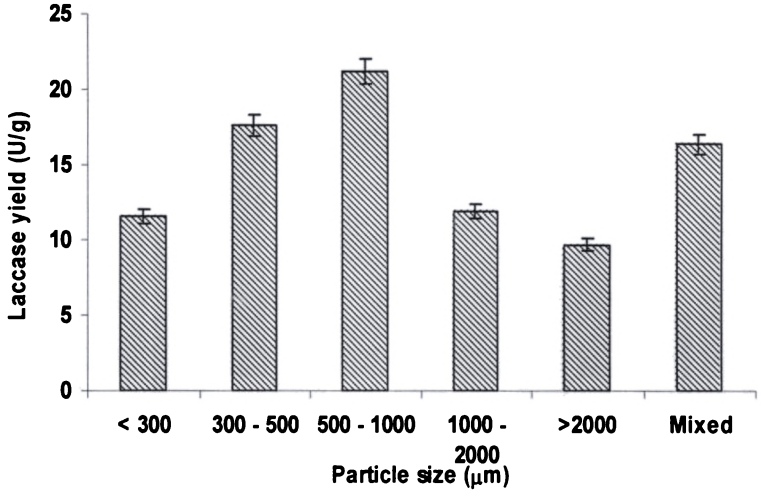


Fig. 4.3. Effect of particle size ( ) on laccase production by *S. psammoticus.*

The moisture content and incubation temperature for the experiment were 50 % and 30 °C respectively. Inoculum size was1 x 10^7 CFU

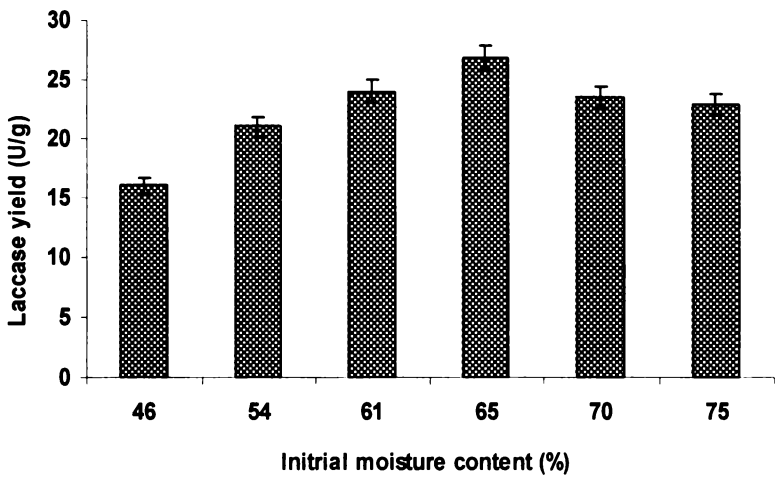


Fig. 4.4. Effect of initial moisture content ( ) on laccase production by *S. psammoticus.*

Particle size of the substrate was 500–1000 µm, incubation temperature was 30 °C and inoculum size was 1 x 10^7 CFU

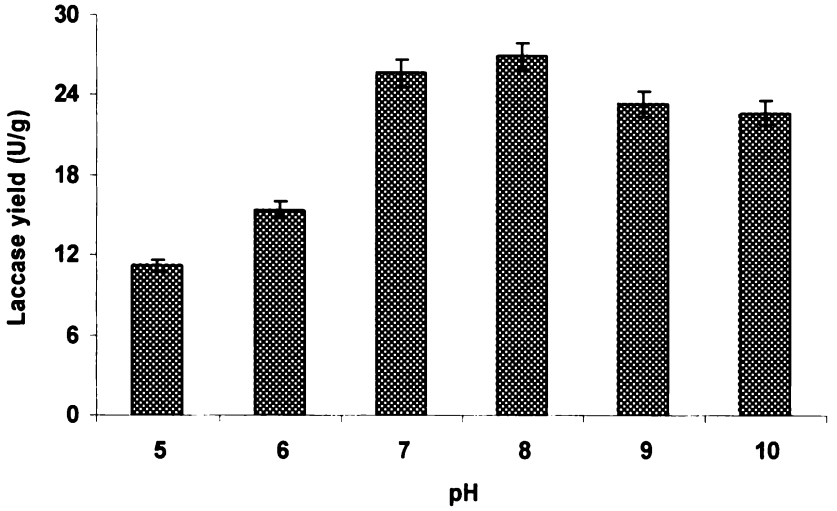


Fig. 4.5. Influence of initial pH (▩) of moistening solution on laccase production under SSF

Culture conditions were particle size 500 – 1000 μm, initial moisture content – 65 %, incubation temperature 30 °C and inoculum level 1 x 10^7 CFU

Temperature is of much significance in the SSF systems because during fermentation there is a general increase in the temperature of the fermenting mass due to respiration (Pandey and Radhakrishnan, 1992). Even though the impact of temperature is more prominent in the scale up processes it remains an inevitable factor in all fermentation systems due to its impact on microbial growth and metabolite production. Results of the present study (Fig. 4.6) suggested that an incubation temperature of 32 °C (27.6 U/g) was the optimum for laccase production and considerable activity was observed also at 30 °C (26.8 U/g). Similar results on the effect of temperature was observed in submerged fermentation also and hence it can be concluded that temperature exerts a similar effect on growth and laccase production irrespective of the mode of fermentation.

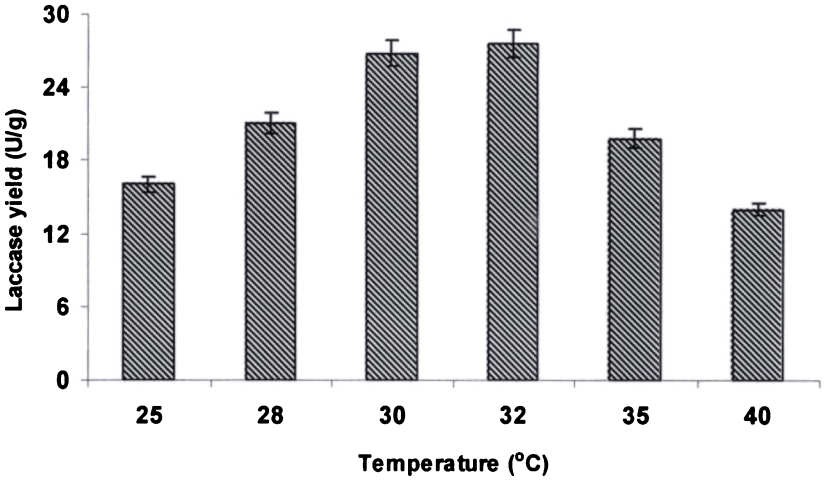


Fig. 4.6. Effect of incubation temperature ( ) on laccase production by *S. psammoticus*.

Culture conditions were particle size 500 – 1000 μm, initial moisture content – 65 %, pH of moistening solution 8.0 and inoculum size 1 x 10^7 CFU

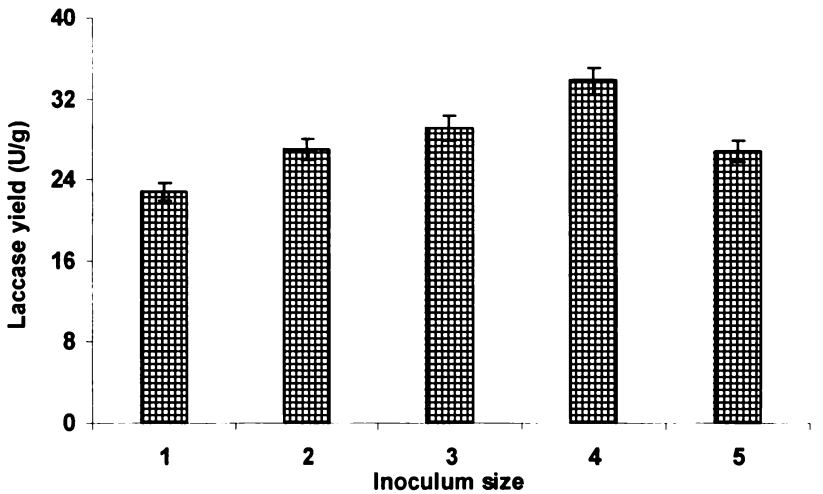


Fig. 4.7. Effect of inoculum size on laccase ( ) production by *S. psammoticus* under SSF

Fermentation conditions include particle size 500–1000 μm, initial moisture content – 65 %, pH of moistening solution 8.0 and incubation temperature 32 °C

The optimization of inoculum size revealed that 1.5 x 10^7 CFU yielded maximum (33.4 U/g) laccase production (Fig. 4.7). The enzyme yield was reduced at lower and higher inoculum levels. A very low inoculum size was found to be inadequate for enzyme production, while the inoculum level above optimum reduced the yield probably due to the competition for nutrients.

4.3.1.4. Effect of carbon and nitrogen supplements

Nature and type of carbon and nitrogen sources are among the most important factors for any fermentation process (Pandey and Radhakrishnan, 1992). The effect of different carbon sources on laccase production has been established in the case of fungal strains (Stajic et al., 2006). In the present study, supplementation of the basal media with different carbon sources failed to exert any positive effect on laccase yield. The yield was reduced invariably by all the carbon sources tried. However, among the different carbon sources used, glucose was comparatively less repressive for laccase production, which yielded 32.1 U/g (Table 4.1), while all the other carbon sources reduced the enzyme yield considerably. This was probably due to the reason that glucose is a readily utilizable substrate which would promote the biomass production. It has already been demonstrated that substrates which are efficiently and rapidly utilized by the organisms result in high levels of laccase activity (Galhaup and Haltrich, 2001). The carbon supplements used in the study might be repressing the genes that are involved in the metabolism of alternative carbon sources and that might be the reason for low laccase yield in the presence of these supplements and hence, no carbon supplemention is needed in the medium for laccase production.

There exist controversial reports on nitrogen requirement by ligninolytic organisms. The ligninolytic enzyme production (lignin peroxidase and manganese peroxidase) by the best-studied fungi *Phanerochaete chrysosporium* was found to be limited by nitrogen sources (Buswell, 1992), where as the same organism has been reported to yield laccase in nitrogen containing media (Srinivasan et al., 1995). The differential effect of the source of nitrogen on laccase production has also been well established. Elisashvili et al (2001) have shown that medium with $(NH_4)_2SO_4$ has given highest levels of laccase activity in *Cerrena unicolor,* while Kaal et al (1995)

have reported the enhanced production of laccase by *Lentinus edodes* and *Pleurotus ostreatus* using peptone as the nitrogen source. Replacement of yeast extract with other organic and inorganic nitrogen sources failed to elicit laccase production by this strain. This confirmed the suitability of yeast extract as the nitrogen source (34.8 U/g) for laccase production by *S. psammmoticus* and similar result has also been reported from a white rot fungal strain (Revankar and Lele, 2006). The results (Table 4.1) also confirmed that the organic sources are better source of nitrogen than inorganic sources for laccase production by this strain.

Organic nitrogen supplements (0.1 % w/v)	Peptone 26.9	Tryptone 25.8	Yeast extract 34.8	Beef extract 27.1	Corn steep solid 23.0
Inorganic nitrogen supplements (0.004 % N)	$(NH_4)_2SO_4$ 22.5	NH_4Cl 18.7	$NaNO_3$ 28.2	KNO_3 23.5	$(NH_4)_2HPO_4$ 16.9
Carbon supplements (1 % w/v)	Glucose 32.1	Galactose 27.0	Sucrose 17.5	Starch 19.4	Xylan 26.5

Table 4.1. Effect of different organic and inorganic nitrogen sources and additional carbon supplements on laccase production by *S. psammoticus* in solid–state fermentation.

4.3.1.5. Time course of laccase production under SSF

The time course of laccase production indicated that the maximum enzyme yield was achieved at 48 h of incubation. The result of time course of laccase production also indicated that the time of maximum laccase production remained the same, irrespective of the mode of fermentation. The enzyme production was observed to be in linear relation with the biomass production as it is presented in Figure 4.8. The production of enzyme at an early incubation time and the linear relation of enzyme

production with biomass were in compliance with the earlier report that ligninolytic enzyme production by actinomycetes is strictly a growth associated primary metabolic activity, while that of the fungi is a secondary metabolic activity (McCarthy, 1987).

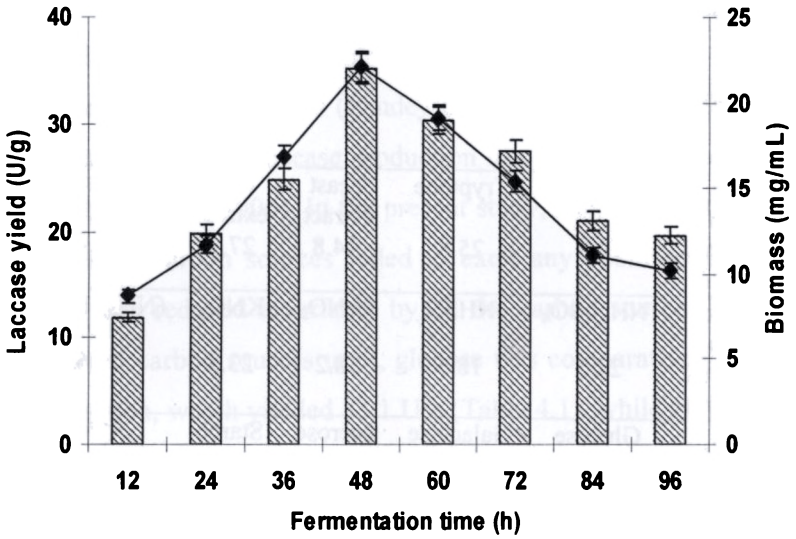


Fig. 4.8. Time course of laccase (▨) and biomass (◆) production by *S. psammoticus* under solid - state fermentation

4.3.2. Statistical optimization

The single parameter optimization indicated that inoculum size had a profound effect on laccase production by this strain and hence it was selected as one of the critical factor for statistical optimization. The replacement of yeast extract with other nitrogen sources reduced the enzyme yield considerably, which necessitated the need to find out the exact level of yeast extract required for enzyme production. Copper sulphate ($CuSO_4$) is one of the widely reported inducer of laccases in many fungi (Palmieri et al., 2000; Galhaup et al., 2002) and hence it was considered logical to

have a better idea on the role of $CuSO_4$ on laccase production by this strain. Based on the above observations, the three parameters viz; yeast extract concentration, inoculum level and $CuSO_4$ concentration were selected for statistical optimization to identify their optimal levels.

A Yeast extract (%)		B Inoculum Size (mL)		C $CuSO_4$ (mM)		Response Laccase yield (U/g)	
A[a]	C[b]	A[a]	C[b]	A[a]	C[b]	A[c]	P[d]
0.20	−1	2.0	−1	2.0	−1	47.0	47.75
0.60	+α	3.5	0	3.0	0	31.3	27.16
0.35	0	3.5	0	3.0	0	43.0	38.15
0.35	0	3.5	0	4.68	+α	35.0	38.15
0.50	+1	5.0	+1	4.0	+1	47.2	45.26
0.35	0	0.98	−α	3.0	0	16.1	17.37
0.35	0	3.5	0	3.0	0	40.0	38.15
0.35	0	3.5	0	3.0	0	35.0	39.65
0.20	−1	5.0	+1	4.0	+1	16.1	14.00
0.20	−1	2.0	−1	4.0	+1	28.0	21.52
0.50	+1	2.0	−1	2.0	−1	24.2	25.11
0.35	0	3.5	0	1.32	−α	52.1	49.12
0.35	0	6.02	+α	3.0	0	15.0	15.41
0.50	+1	2.0	−1	4.0	+1	16.2	17.93
0.35	0	3.5	0	3.0	0	40.0	38.15
0.10	−α	3.5	0	3.0	0	14.1	19.91
0.35	0	3.5	0	3.0	0	33.1	38.15
0.50	+1	5.0	+1	2.0	−1	25.0	30.30
0.20	−1	5.0	+1	2.0	−1	21.0	18.09
0.35	0	3.5	0	3.0	0	38.1	38.15

[a] Actual values
[b] Coded values
[c] Mean of values from three individual experiments
[d] Predicted response

Table 4.2. Experimental plan for central composite design (CCD) performed with *S. psammoticus* under solid-state fermentation for the selected parameters and the actual and predicted responses in terms of laccase yield

The minimum and maximum ranges of variables used and the full experimental plan with respect to their values in actual and coded form are listed in Table 4.2. The results of response surface experiments (CCD), performed for optimizing the levels of yeast extract, inoculum size and copper sulphate are also presented in Table 4.2 in terms of enzyme yield

The analysis of variance (ANOVA) for the selected quadratic model showed that the model was significant with a Model F value of 11.63 and *P*>F value of 0.0003 (Table 4.3). The model terms A^2, B^2, AB, AC and BC exhibited confidence level above 95% (*Prob* > F value less than 0.05). This indicated that the squared effects of yeast extract concentration, inoculum level and the interaction effects of yeast extract and inoculum level, yeast extract and copper sulphate concentration, inoculum level and copper sulphate concentration were significant model terms. The coefficient of determination (R^2) was calculated as 0.9128 for laccase production, indicating that the statistical model can explain 91.28 % of variability in the response. For a good statistical model R^2 value should be close to 1.0 where a value >0.75 indicates the aptness of the model. The model recorded an adequate precision of 10.137 which indicated an adequate signal to navigate the design space. The "Lack of Fit F-value" of 2.65 implied that the "Lack of Fit" was not significant and hence the model was fit.

Source	Sum of squares	DF	Mean square	F value	*Prob*> F
Model	2512.11	9	279.12	11.63	0.0003
Yeast extract · Yeast extract (A^2)	384.74	1	384.74	16.03	0.0025
Inoculum size · Inoculum size (B^2)	853.29	1	853.29	35.55	0.0001
Yeast extract · Inoculum size (AB)	607.26	1	607.26	25.30	0.0005
Yeast extract · $CuSO_4$ (AC)	181.45	1	181.45	7.56	0.0205
Inoculum size · $CuSO_4$ (BC)	245.31	1	245.31	10.22	0.0095
Residual	240.05	10	24.01		
Corrected total	174.27	5			

Table 4.3. Analysis of variance (ANOVA) for the response surface quadratic model

The interaction between yeast extract and inoculum size indicated that at the higher inoculum level, the enzyme yield increased with the increase in yeast extract concentration, while at lower inoculum level there was a drop in enzyme yield with increase in yeast extract concentration (Fig. 4.9). Higher inoculum level might have resulted in rapid depletion of nutrients and hence higher levels of yeast extract were required for maintaining the biomass and enzyme production in such a situation. However, when the inoculum level was low, the increase in yeast extract concentration beyond a particular concentration exerted a negative influence on laccase production.

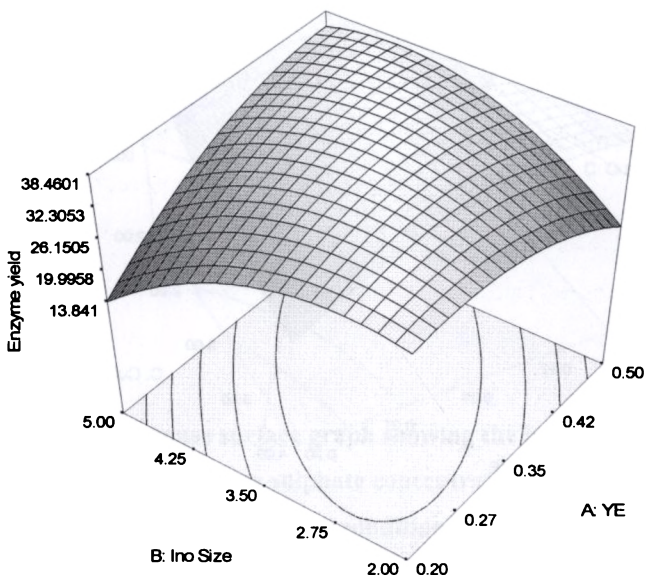


Fig. 4.9. Three dimensional response surface plot for the interaction between yeast extract and inoculum size on laccase production by *S. psammoticus* under conditions optimized by RSM

It was obvious from the 3 D surface curves (Fig. 4.10) that the laccase production increased with increase in yeast extract concentration and decrease in $CuSO_4$ concentration. At higher level of yeast extract (0.5 %), the effect of different levels of $CuSO_4$ concentration on enzyme production was little, while at lower level of yeast extract concentration, decreasing the level of $CuSO_4$ resulted in enhanced enzyme yield. Maximum enzyme yield was obtained when the yeast extract concentration was around 0.3 % and $CuSO_4$ concentration at 2 mM.

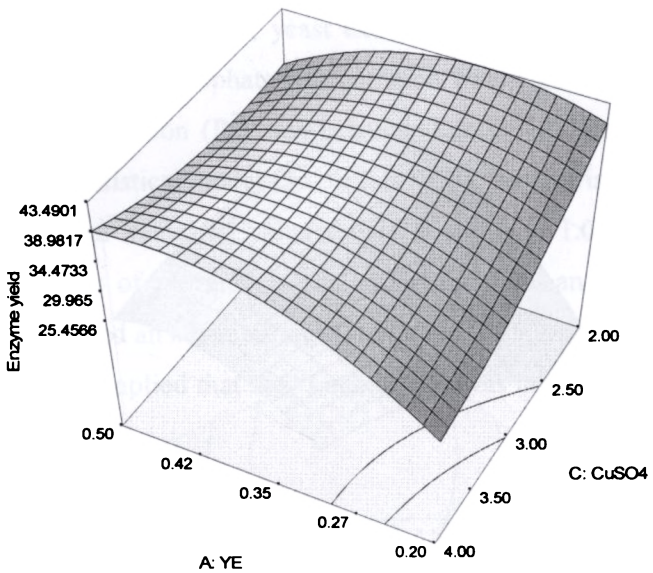


Fig. 4.10. Response surface graph showing the interaction between yeast extract and copper sulphate concentration on laccase production by *S. psammoticus* under conditions optimized by RSM

The interaction between inoculum size and $CuSO_4$ concentration (Fig. 4.11) exhibited almost a similar pattern as the one between yeast extract and $CuSO_4$ concentration. When the copper sulphate concentration was higher, the increase in inoculum size resulted in increased enzyme production. At lower level of $CuSO_4$ (2 mM), increasing the inoculum size beyond a limit reduced the enzyme production

and the maximum laccase production was observed when the inoculum level was around 3.0 mL and the $CuSO_4$ concentration at 2 mM. The interactions of $CuSO_4$ with yeast extract and inoculum size indicated that maintaining a lower level of $CuSO_4$ was favorable for laccase production and this was probably due to the fact that higher levels of copper sulphate are toxic to most of the micro organisms.

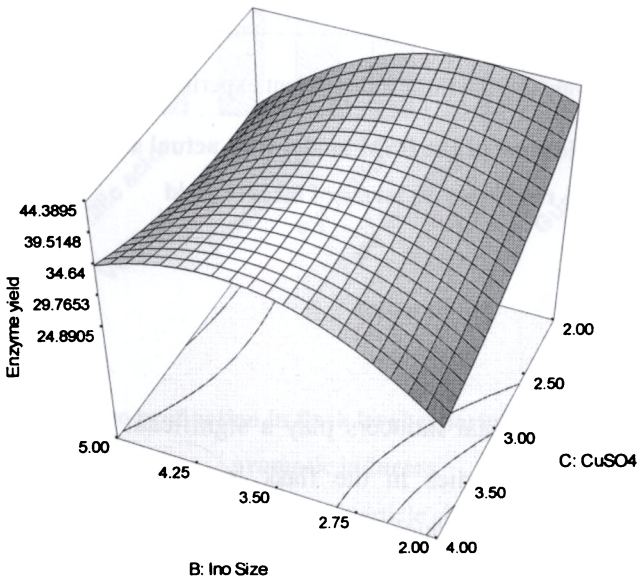


Fig. 4.11. Response surface graph showing the interaction between inoculum size and copper sulphate concentration on laccase production by *S. psammoticus* under conditions optimized by RSM

4.3.2.1. Validation of the model

Model validation was performed with three different solutions suggested by the software (Table 4.4). The results showed good agreement between the predicted and experimental values, which indicated that the model was validated successfully.

Yeast extract (g/L)	Inoculum size (mL)	$CuSO_4$ (mM)	Laccase activity U/g Predicted	Laccase yield U/g Actual[a]
0.20	2.06	2.00	47.7611	55.4
0.20	2.10	2.00	47.7424	54.5
0.50	4.83	4.00	45.3604	49.7

[a] Mean of values from three independent experiments

Table 4.4. Validation of the RSM model with actual and predicted responses in terms of laccase yield

4.3.3. Effect of inducers

Results of our study implied that inducers play a significant role in enhancing the production of laccase. The studies in the flask level experiments revealed that pyrogallol is the most efficient inducer for laccase production by *S. psammoticus*, followed by para anisidine. Pyrogallol enhanced the laccase production by two fold giving a yield of 116.0 U/g against the control (55.4 U/g) while 34 % increase in laccase production was achieved with para anisidine (74.2 U/g). Other inducers like gallic acid, catechol, veratryl alcohol, guaiacol and ferulic acid were also found to be enhancing the laccase yield while vanillic acid was found to be exerting a negative influence on laccase production by this strain (Fig. 4.12). Aromatic inducers and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms (Leonowicz et al., 2001; De Souza et al., 2004) and the nature of the compound that induces laccase production differs greatly with the species. However, it remains a general practice to select the inducers in such a way that they are either polyphenols or lignin related structures.

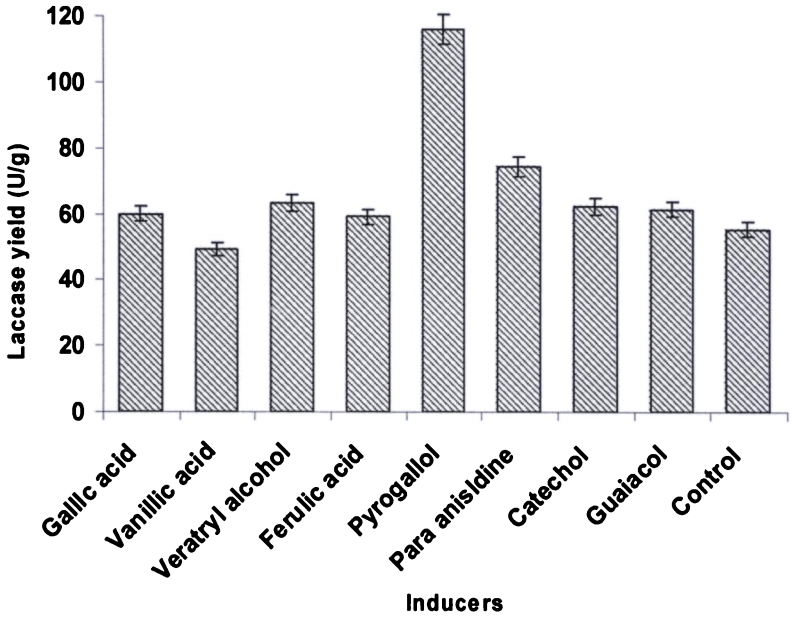


Fig. 4.12. Laccase production in flask level experiment in the presence of aromatic inducers

Fermentation conditions were particle size; 500-1000 μm, moisture content; 65 %, incubation temperature; 32 °C.

4.3.4. Bioreactor studies

The bioreactor studies indicated that laccase production by *S. psammoticus* could be enhanced considerably using packed bed bioreactor, provided, forced aeration is supplied. The packed bed bioreactor system used in the present study is given in Fig. 4.13. The growth and enzyme yield was highest in the upper zone of the packed bed reactor, which was divided into three equal zones. Growth and enzyme production was moderate in the middle zone and lowest in the lower zone of the column, irrespective of the aeration rate provided (Table 4.5). This was probably due to the development of axial gradients of temperature within the packed bed column. Axial temperature gradients are impossible to avoid within packed-bed bioreactors due to the use of convective cooling with unidirectional flow of air (Ashley et al., 1999). Evolution of heat, which is directly related to the metabolic activity of the

microorganism, is one of the well documented characteristics of SSF systems (Trilli, 1986).

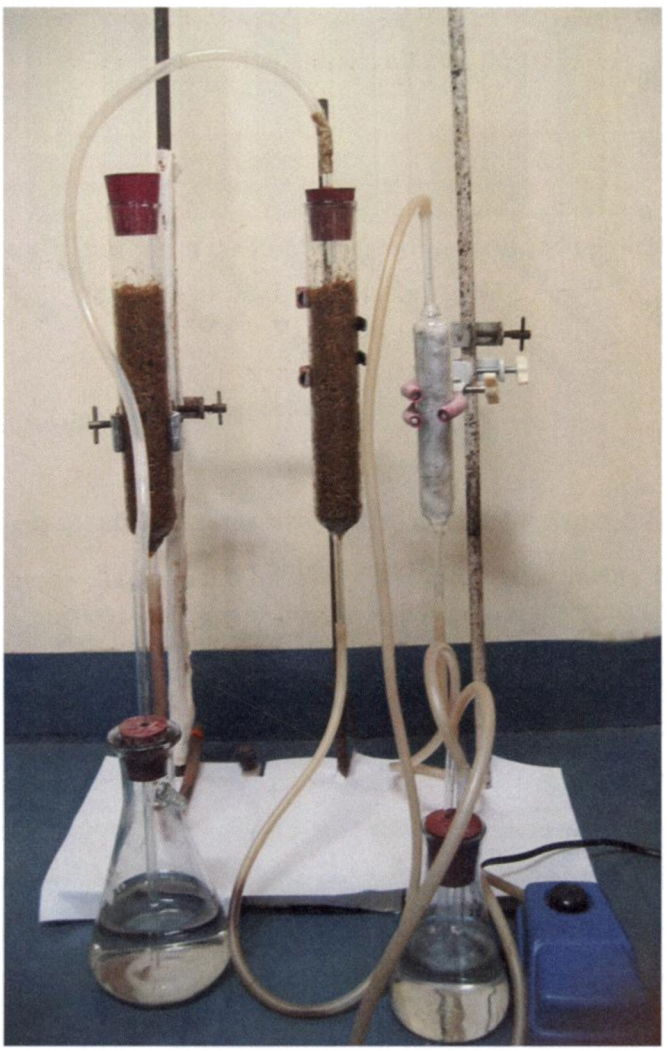


Fig. 4.13. Packed bed bioreactor system used for laccase production in SSF

In the present study, it was observed that forced aeration using humidified air enabled to remove heat from the substrate bed. Similar result on forced aeration has already been reported by Lonsane et al (1992). An aeration rate of 1.5 vvm was the

optimum for laccase production, which resulted in a yield of 75.4 U/g (upper zone), which was 36 % higher as compared to the flasks (55.4 U/g). The yield was reduced when low aeration was provided and the yield was almost negligible in the non-aerated control column. This could be attributed to the inadequate supply of oxygen in the less aerated and non-aerated columns. Oxygen is one of the major influencing factors for the growth and metabolite production by aerobic organisms like *S. psammoticus*.

Aeration (vvm)[a]	Laccase yield (U/g)[b]			Glucosamine (mg/g)[c]		
	Upper zone	Middle zone	Lower Zone	Upper zone	Middle zone	Lower Zone
0.5	59.4	42.3	39.5	22.1	15.4	10.7
1.0	67.7	47.2	42.0	29.4	18.3	14.0
1.5	75.4	56.0	49.5	33.8	21.9	16.2
2.0	70.2	58.1	52.0	31.2	25.0	12.6
Control (without aeration)	9.3	4.9	1.8	5.1	2.2	0.9
Flask		55.4			28.3	

[a] vessel volume per minute
[b, c] values presented are the mean of three independent experiments

Table 4.5. Laccase and glucosamine yields at three equally divided zones of the non-aerated and differentially aerated packed bed column reactor. Values obtained with flask level experiment are given for comparison

The individual experiments on inducers and bioreactor resulted in enhanced laccase production by *S. psammoticus*. Hence, we combined both the strategies with a view to further enhancing the laccase yield. The aeration of the reactor was set at 1.5

vvm and the best laccase inducer; pyrogallol was added to the substrate at a concentration of 1 mM. The result of the time course of laccase production in packed bed bioreactor in the presence of pyrogallol is given in Figure 4.14. The results indicated that there was no change in the hour of maximum laccase production in the reactor. The maximum laccase yield was obtained at 48 h, similar to the result observed in the flask level experiments. The maximum laccase yield obtained in the flask level experiments, in the presence of pyrogallol was 116 U/g. The highest yield obtained in the bioreactor, in the absence of any inducer was 75.4 U/g. The results of the present study showed that the use of inducers is a better strategy for enhancing laccase yield from *S. psammoticus*, as compared to the forcefully aerated bioreactor. However, 3.9 fold increase in laccase yield (215.6 U/g) was obtained by combining both the strategies, which has undoubtedly proved that a combination of these two different strategies is a promising method for enhancing laccase yield by this strain.

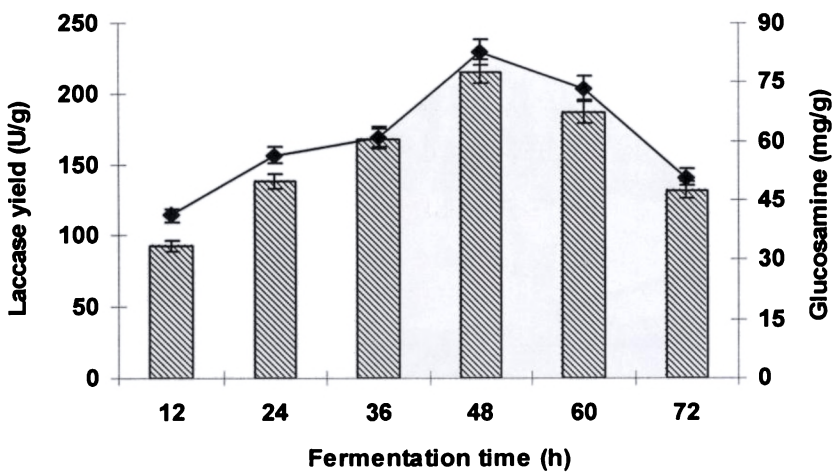


Fig. 4.14. Time course of laccase production in packed bed bioreactor in the presence of pyrogallol

The culture conditions were particle size; 500 -1000 μm, moisture content; 65 %, temperature; 32 °C

4.4. CONCLUSION

Solid-state fermentation is generally regarded as more suitable for the fungal system. The production of 55 U/g of laccase from *Streptomyces* species, using rice straw as the substrate, is a promising result and it suggests that solid-state culture is also functional with actinomycetes. The filamentous nature of these organisms might be favoring their growth on solid substrates. Optimization of the fermentation process by conventional procedures resulted in two-fold increase in laccase production. The enzyme yield was enhanced further by response surface methodology and the model validation performed with RSM suggested that the model was valid with good reproducibility of the results. On the whole, three-fold increase in laccase yield (55.4 U/g) was attained after statistical optimization as compared to the unoptimized medium (17.3 U/g). The study has confirmed the aptness of using statistical methods for enhancing laccase production by this strain and it has also successfully evaluated the utility of rice straw, which is an inexpensive and easily available agro-industrial waste for laccase production under solid-state fermentation. The use of different aromatic inducers served to enhance the laccase yield considerably. The scale-up of laccase production performed in column bioreactor also resulted in the enhancement of production. The scale-up studies carried out in the presence of the best inducer proved to be a highly promising strategy for enhanced enzyme production that yielded 215.6 U/g of laccase.

Chapter 5.

PURIFICATION AND CHARACTERIZATION OF LACCASE

5.1. INTRODUCTION

Laccases have been found commonly in fungi and some higher plants. White rot fungi are the leading laccase producers and the purification of enzyme from this group has been largely reported (Palonen et al., 2003; Zouari-Mechichi et al., 2006; Litthauer et al., 2007). Laccases have also been purified and characterized from certain non-filamentous bacteria like *Azospirillum lipoferum, Marinomonas mediterranea and Bacillus subtilis* (Diamantidis et al., 2000; Fernandez et al., 1999; Martins et al., 2002). Actinomycetes are believed to be the potent producer of laccases, next to fungi. Purification and characterization of laccases from actinomycetes, especially, different *Streptomyces* sp have been accounted. The laccase-like phenol oxidase from *S. griseus* has been reported to have a highly unique homotrimer structure (Endo et al., 2003) while the small laccase (SLAC) from *S. coelicolor* has been drscribed as a dimer, lacking the second domain (Machczynski et al., 2004). Laccase from *S. lavendulae* has been reported as thermostable, being stable at 70 °C (Suzuki et al., 2003). Arias et al (2003) have described a laccase from *S. cyaneus* that was capable of oxidizing non-phenolic compounds in the presence of mediators. The enzymes with remarkable properties are of great interest owing to their significance in various applications.

5.2. MATERIALS AND METHODS

5.2.1. Enzyme production and extraction

Laccase production was carried out by the solid-state cultivation, using rice straw. The substrate was moistened with a salt solution containing (g/L): yeast extract-3.0, $(NH_4)_2SO_4$-0.2, $MgSO_4$-0.2, $CaCO_3$ -0.04, $CuSO_4$-0.5. The initial moisture content and incubation temperature were maintained at 65 % and 32 °C respectively. After 48 h of fermentation, the crude enzyme was extracted from the fermented solid matter using 50 mM sodium phosphate buffer, pH 7.0 (Buffer A), centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant was taken for purification steps.

5.2.2. Purification of laccase

The proteins were precipitated from the supernatant with ammonium sulphate (0-80 % saturation). Laccase activity was detected in 30–60 % saturated fractions. Laccase active fractions were pooled, centrifuged (10000 rpm, 20 min) and the precipitate was dissolved in minimal amount of buffer A and dialyzed against the same buffer overnight at 4 °C. The dialyzate was loaded on to a DEAE-cellulose anion exchange column (10 x 2.8 cm), which was equilibrated and washed with 50 mM sodium phosphate buffer, pH 6.0 (Buffer B). The enzyme was eluted with a linear gradient of NaCl (0 – 1.0 M) at the flow rate of 0.5 ml/min and the eluate was monitored for absorbance at 280 nm, conductivity and laccase activity. Fractions containing laccase activity were then pooled and concentrated with an Amicon ultrafiltration stirred cell containing a 10 kDa molecular mass cutoff membrane (MWCO), and applied to a Biogel P 100 (Bio-Rad) column (30 x 3 cm) that was pre-equilibrated with buffer A. The column was eluted with the same buffer at a flow rate of 0.5 mL/min. The fractions containing laccase activity were pooled and stored at 4 °C until further use.

5.2.3. Enzyme characterization

5.2.3.1. Analytical electrophoresis (Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight of the purified laccase was determined by SDS–PAGE as per the method of Laemmli (1970). The strength of the gel was 12 % (w/v) and the protein bands were stained with coomassie brilliant blue R 250. Medium range (14.3–97.4 kDa) molecular weight markers (Genei) were used to determine the molecular mass of purified laccase. The markers 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).

5.2.3.2. Activity staining

Activity staining of purified laccase was performed using 1, 8 diaminonaphthalene (DAN) as per the method of Hoopes and Dean (2001). Following electrophoresis, gels were incubated in 50 mM sodium phosphate (pH 7.0) containing 1% dimethyl sulfoxide and 2 mM DAN. DAN, from a 1 M stock in 100% dimethyl sulfoxide, was added to the buffer immediately prior to immersion of the gel. The gel was incubated at 40 °C for 30 min. To halt activity staining, the gels were immersed in 50% (v/v) methanol containing 10% (v/v) acetic acid at 50 °C for 10 min. The gel was then immersed in 20 % trichloroacetic acid (TCA) to darken the DAN oxidation product. The activity band was further enhanced by counterstaining the gel with Coomassie brilliant blue dye using protocols developed to detect proteins and subsequently destained in 50% (v/v) methanol, 10% (v/v) acetic acid until the background was clear.

5.2.3.3. Isoelectric focusing

Isoelectric pH of the purified laccase was determined using rotofor (Biorad). Focusing was carried out at a constant power of 12 W for 2 hrs. Twenty samples were collected and analyzed for enzyme activity and pH. The ampholyte used was in the pH range of 3.0-10.0.

5.2.3.4. Effect of pH and temperature on laccase activity and stability

The optimum pH of the enzyme was determined within a pH range of 4-10 using ABTS as substrate in the following buffers: acetate (pH 4-5.5); phosphate (pH 6-8); Tris-HCl (pH 8.5) and glycine-NaOH (pH 9-10) at 30 °C. The effect of temperature on enzyme activity was measured in the range of 25-60 °C at pH 8.5. The effect of pH on enzyme stability was determined by incubating the enzyme in different buffers in the pH range of 6.0 – 10, at optimum temperature. Thermal stability was determined by incubating the enzyme in Tris-HCl buffer, pH 8.5 at different

temperatures. For experiments on pH and thermal stability, the residual activities were measured under the standard assay conditions at frequent time intervals for 90 min.

5.2.3.5. Effect of salt on enzyme activity and stability

Effect of NaCl on purified laccase was determined at different concentrations of NaCl ranging from 0.2-2.0 M. Salt tolerance of the purified laccase was determined by incubating the enzyme at different concentrations of NaCl, up to 2 M for 24 h at 45 °C. The residual activity was calculated under the standard assay conditions at frequent time intervals. Laccase activity in the absence of NaCl was also determined, for comparison.

5.2.3.6. Effect of inhibitors and metal ions

Eight potential laccase inhibitors were selected to evaluate their effect on the purified laccase from *S. psammoticus*. The enzyme was incubated with various inhibitors for 10 min at optimum temperature and the laccase activity was measured with 0.5 mM ABTS. The effect of various metal ions on laccase activity was also evaluated. The final concentration of the inhibitors and metal ions were decided specifically for each of the compound.

5.2.3.7. Total carbohydrate content

The total carbohydrate content of the enzyme was estimated by Dubois method (1956) using glucose as the standard.

5.2.3.8. Copper content

Copper content of the purified protein was determined by biquinoline method (Hanna et al., 1988). The basis for the method is that the copper I ion forms a deep purple

coloured complex with biquinoline, which could be detected spectrophotometrically at 546 nm.

5.2.3.9. Spectral studies

The spectral studies of the purified laccase were carried out using a UV-visible spectrophotometer (Shimadzu UVPC -2401).

5.2.3.10. Substrate specificity

The substrate specificity of purified laccase was tested with a range of substrates that included non-phenolic compounds, substituted phenols and aromatic amines. All the substrates were studied at a final concentration of 1 mM and the oxidation of each of the substrate was measured at its maximum absorbance wavelength and the enzyme activity was calculated using the respective extinction coefficient values.

5.2.3.11. Kinetic studies

The Kinetic constants K_m and V_{max} were determined with substrates such as ABTS, pyrogallol and syringaldazine.

5.3. RESULTS AND DISCUSSION

5.3.1. Purification of laccase

Ammonium sulphate precipitation (30-60 % saturation) increased the specific activity from 1.78 to 7.6 U/mg. A purification fold of 4.3 was achieved by this step. The main increase in specific activity was obtained after the chromatographic step using DEAE cellulose. The specific activity has increased to 20.3 U/mg with a fold purification of 11.4. A linear gradient of 0.04-0.8 M NaCl was used for eluting the fractions. The laccase fractions were eluted with 0.11-0.54 M NaCl gradient. From the ion exchange column, laccase active fractions were obtained between fraction numbers 18-56 (Fig.

5.1.). The anion exchange chromatography helped also to remove the dark colour from the protein solution.

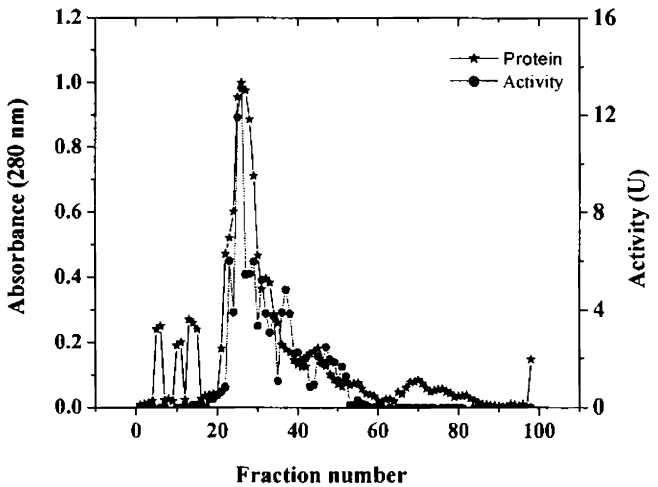


Fig. 5.1. Elution profile of laccase on DEAE-cellulose anion exchange column at an elution gradient of 0.11-0.54 M NaCl

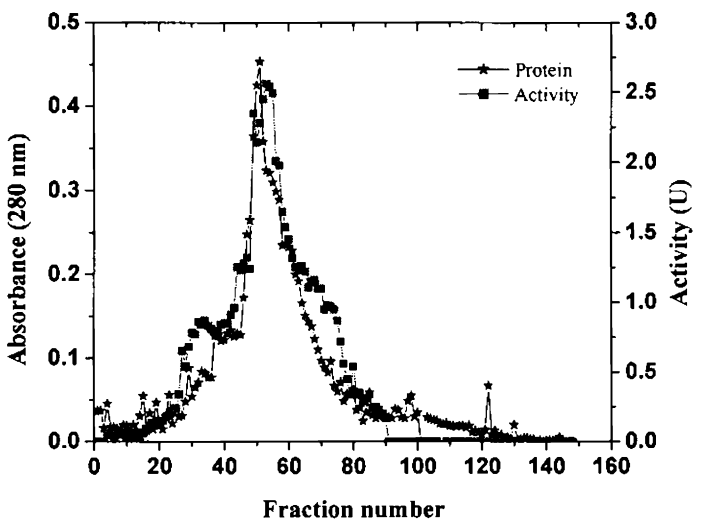


Fig. 5.2. Elution profile of laccase on Biogel-P100 column eluted with 50 mM Sodium phosphate buffer

Subsequent purification by gel filtration using Biogel P 100 served to further increase the fold purification to 12.1, although it was not an effective step as the ion exchange. The elution profile of gel filtration chromatography is shown in Figure 5.2. The laccase fractions were obtained between fraction numbers 14-90 when eluted with 50 mM sodium phosphate buffer. At the end of purification process, laccase was purified to homogeneity with a specific activity of 21.6 U/mg. The purification factor was 12.1 fold, which corresponded to a final yield of 22.1 % (Table 5.1).

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	350.3	625.3	1.78	1	100
(NH4)2SO4 precipitation	58.4	448.8	7.6	4.3	71.8
DEAE cellulose	13.8	280.6	20.3	11.4	44.9
Biogel P-100	6.4	138.2	21.6	12.1	22.1

Table 5.1. Purification of laccase from *S. psammoticus*

5.3.2. Characterization of the purified laccase

5.3.2.1. SDS PAGE, Activity staining and Isoelectric focussing

The homogeneity of the purified laccase was indicated by SDS-PAGE, where one single band could be detected, corresponding to approximately 43 kDa (Fig.5.3a). Lane 1 of Fig 5.3a indicates the ammonium sulphate fraction, Lane 2 - fraction from ion exchange column, Lane 3 – molecular weight markers and Lane 4 – the purified laccase from gel filtration chromatography. The observed molecular mass of the purified laccase was not consistent with most of the *Streptomyces* laccases with

molecular masses ranging between 70-75 kDa (Arias et al., 2003; Suzuki et al., 2003). Activity staining of the purified laccase with 1, 8 diaminonaphthalene resulted in a single band and the result is shown in Fig. 5.3b. The isoelectric point (pI) of the purified laccase was 7.9.

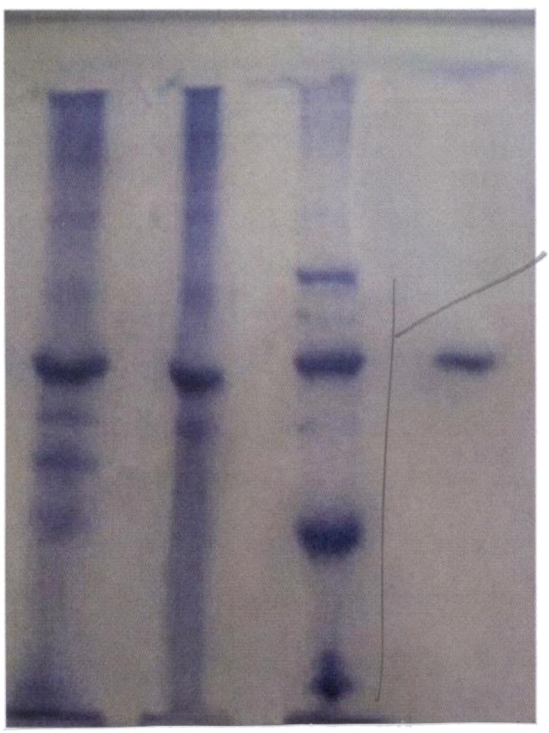


Fig. 5.3a **Fig. 5.3b**

Fig. 5.3a. Molecular mass of purified laccase from *Streptomyces psammoticus* as analyzed by SDS–PAGE stained with Coomassie blue

Lane 1: Fraction from ammonium sulphate precipitation, Lane 2: Ion exchange fraction, Lane 3: molecular weight markers, Lane 4: fraction from gel filtration chromatography

Fig. 5.3b. Activity staining of purified laccase (fraction from gel filtration chromatography) using 1, 8 diaminonaphthalene, counter stained with Coomassie blue

5.3.2.2. pH optima and stability

The optimal pH value for the purified laccase was 8.5 (Tris-HCl) and the enzyme exhibited 97 % activity at pH 9.0. Fig. 5.4 shows that the enzyme activity was higher in the neutral to alkaline pH as compared to the acidic pH. This result was of much importance since the optimum pH of the majority of *Streptomyces* laccases was in the acidic range (Arias et al., 2003; Suzuki et al., 2003) with an exception of laccase from *S. coelicolor* that exhibited a high pH optima (Machczynski et al., 2004). Activity at high pH is generally considered as an appreciable character from the industrial stand point.

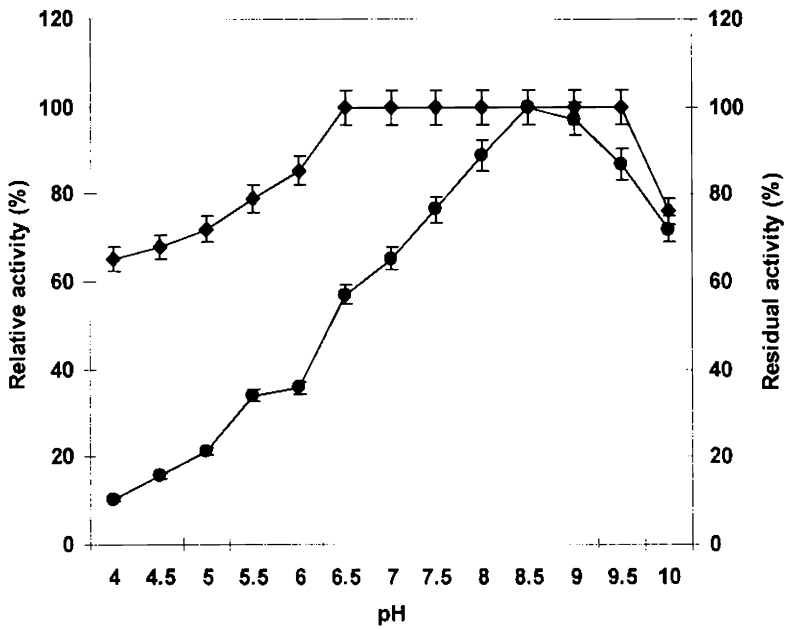


Fig. 5.4. Effect of pH on laccase activity and stability

The purified enzyme was incubated in different buffers (pH 4-10) and relative activity was determined at 30 °C. For stability studies the enzyme was incubated for 90 min (at pH 4-10) and the residual activity was calculated

The stability studies indicated that the enzyme was stable in the pH range of 6.5-9.5 for 90 min at 45 °C. The stability of the enzyme was not affected by the acidic pH

range and the enzyme retained around 65 % residual activity at pH 4, after 90 min. The studies on the effect of pH on the purified laccase clearly indicated that it is an unusual enzyme showing activity and stability in the alkaline pH range. This unusual property makes this enzyme a best candidate for a wide range of applications where alkaline conditions prevail.

5.3.2.3. Temperature optima and stability

Temperature optima of the purified laccase was determined at pH 8.5, using ABTS as the substrate (Fig. 5.5). Maximum activity was observed at 45 °C. Considerable activity was retained also at 50 °C. After an incubation time of 90 min, the enzyme was stable up to 50 °C and at higher temperatures of 55 and 60 °C, the residual activities were reduced to 85 and 69 % respectively. Thermo stability studies showed that purified laccase was not stable at temperatures higher than 50 °C over prolonged incubation.

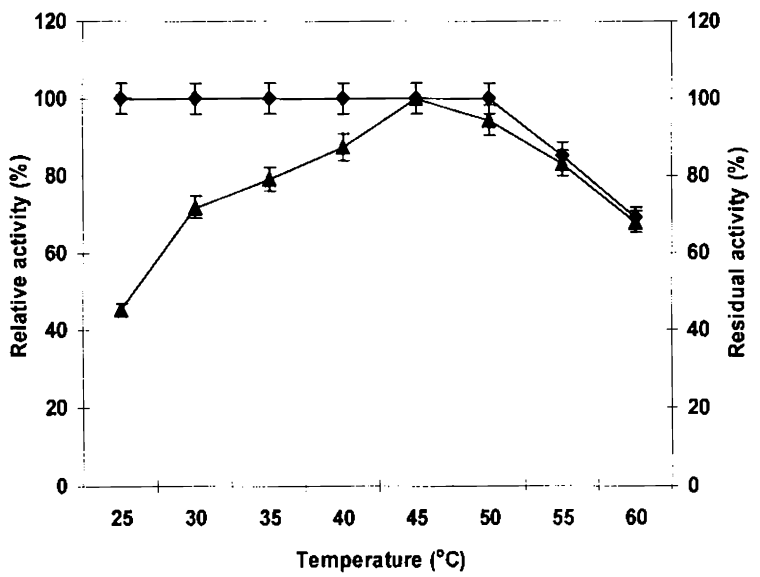


Fig. 5.5. Effect of temperature on laccase activity and stability

The purified enzyme was incubated in different temperatures (25-60 °C) and relative activity was determined at pH 8.5. For stability studies the enzyme was incubated for 90 min (at 25-60 °C) and the residual activity was calculated

5.3.2.4. Effect of salt concentration

The result of halo tolerance studies on the purified laccase is given in Fig.5.6. The enzyme was active at different concentrations of NaCl within the tested range. Activity at low and high concentrations of NaCl indicated that the purified laccase was a halo-tolerant enzyme. Maximum activity was obtained at 0.8 M NaCl concentration (Fig. 5.6). At 0.8 M concentration, the enzyme exhibited 40 % additional activity as compared to the activity in the absence of NaCl (control).

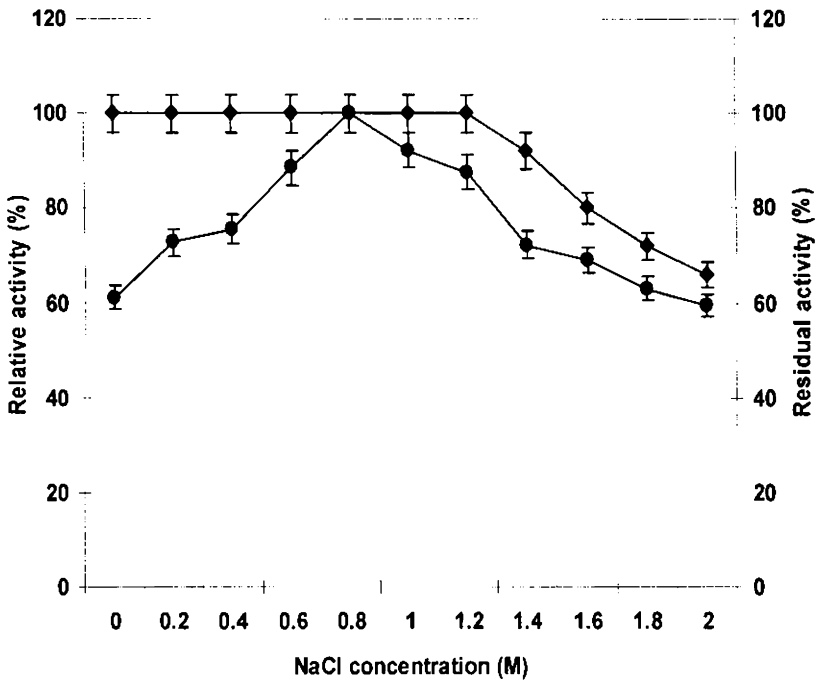


Fig. 5.6. Effect of NaCl on purified laccase

The laccase activity was determined at different concentrations of NaCl. For the stability studies the enzyme was incubated in different concentrations of NaCl for 24 h and the residual activity was calculated under standard assay conditions

The enzyme was found to be stable at NaCl concentrations up to 1.2 M (Fig. 5.6). The stability was gradually decreasing at concentrations higher than 1.2 M NaCl even though 66 % residual activity was retained at 2 M NaCl concentration. It was obvious from the study that there was no change in the stability of enzyme over different

incubation time. The activities retained after 1h, 12 h and 24 h (residual activities) were almost the same, which implied that the reaction of enzyme with salt was relatively rapid and no further loss of activity was possible over longer incubation time. Salt tolerance is one of the preferred characters for enzymes as observed from the application stand point. Laccase is an enzyme used widely for effluent treatments, bleaching etc where there are possibilities for high salt contents. In this aspect the enzyme has better prospects for application purposes. However, similar reports on halotolerant laccases are lacking in the literature even though there are few references on other enzymes (Setyorini et al., 2006; Voget et al., 2006).

5.3.2.5. Effect of inhibitors

Table 5.2 summarizes the effects of putative inhibitors on purified laccase from *S. psammoticus*. The enzyme was inhibited by all the inhibitors that were studied. However, the extent of inhibition varied greatly with the nature and concentration of the tested inhibitors. Complete inhibition of laccase activity was observed with sodium azide and dithiothreitol at the studied concentrations. Sodium azide is a common inhibitor of metalloproteins and laccase inhibition by this compound has been well established. Sodium azide has been reported to prevent the substrate oxidation by laccase (Johannes and Majcherczyk, 2000). EDTA, another well known metal chelating agent fully inhibited the purified laccase at 2 and 5 mM concentrations. Similar results have also been reported from *Streptomyces* cyaneus (Arias et al., 2003).

There are controversial reports about the inhibition of laccases by metal chelators. Saito et al (2003) have reported that sodium azide has exerted little inhibition on laccase from a fungal strain at a concentration of 10 mM while EDTA had no effect on the enzyme even at a very high concentration of 25 mM. L-cysteine and thioglycolic acid also inhibited the enzyme although, complete inhibition by these compounds occurred only at higher concentration. L-cysteine is a sulfhydryl organic compound with a reducing effect on the copper-containing active site of

laccase. Thiourea and kojic acid were found to be comparatively less inhibitory to the enzyme.

Inhibitor	Concentration (mM)[a]	Inhibition (%)[b]
Control	-	0
Sodium azide	0.1	100
	0.1	89
L-Cysteine	1.0	100
	1.0	54
Thiourea	5.0	73
Dithiothreitol	0.1	100
Kojic acid	1.0	47
	0.1	93
Thioglycolic acid	1.0	100
	1.0	87
EDTA	2.0	100

Table 5.2. Effect of inhibitors on purified laccase from *S. psammoticus*

a Final concentration of different inhibitors were decided specifically for each of the compound

b Values represent the means of values from three independent experiments, with a maximal sample mean deviation of ± 5%

5.3.2.6. Effect of metal ions

The effect of various metal ions on the activity of purified laccase is given in Table 5.3. The enzyme activity was enhanced by metal ions such as Fe, Cu, Zn, Na and Mg (each at 2 mM) while the heavy metals like Hg, Cd, Co and Ni reduced the activity considerably, even at half the concentration (1.0 mM) of other metal ions. The most obvious effect on laccase activity was exerted by Fe, which enhanced the activity by twofold. Significant enhancement of enzyme activity was also observed with Na. Cu and Zn was found to enhance the activity almost at the same level. The role of copper in the enhancement of laccase activity has been well demonstrated in both fungi and bacteria (Givaudan et al., 1993). Similar result has been reported from *Streptomyces*

too (Arias et al., 2003). However, the present results on the effect of Fe and Zn on the purified laccase was found to be contradictory to the earlier reports from other *Streptomyces* sp (Arias et al., 2003).

Metal ions	Concentration (mM)[a]	Relative activity (%)[b]
Control	-	100
Cu	2.0	148
Ca	2.0	98
K	2.0	106
Na	2.0	118
Mg	2.0	137
Mn	2.0	79
Fe	2.0	204
Zn	2.0	146
Co	1.0	29
Cd	1.0	21
Hg	1.0	14
Ni	1.0	32

Table 5.3. Effect of metal ions on purified laccase from *S. psammoticus*

a Final concentration of different metal ions were decided specifically for each of the compound

b Values represent the means of values from three independent experiments, with a maximal sample mean deviation of ± 5%

5.3.2.7. Carbohydrate content

The carbohydrate content of laccases usually varies from 1 % to 15 % depending on the source of the enzyme (Fukushima and Kirk, 1995; Munoz et al., 1997). The determination of carbohydrate content revealed that the purified laccase from *S.*

psammoticus was a glycoprotein with a total carbohydrate content of 10 % and the observed result was very much in agreement with the earlier reports on other laccases (Koroljova et al., 1999).

5.3.2.8. Copper content

The copper content of the purified laccase was found to be 3.2 copper ions which was slightly lesser than the expected value of four. There have been reports on laccases with copper content as low as 2.8 (Kim et al., 2002). The existence of apoproteins which are missing copper ions in the active sites and also the uncertainties in determining the copper concentrations have been suggested as the probable reason for the lower copper content detected in laccase (Kim et al., 2002).

5.3.2.9. Spectral Studies

The UV-visible spectrum of the purified laccase showed a peak around 600 nm which was characteristic of the type I copper of typical laccase (Fig. 5.7).

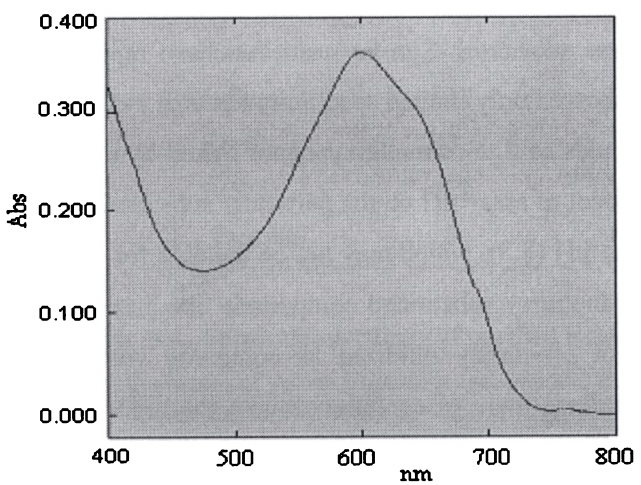


Fig. 5.7. Absorption spectrum of laccase from *S. psammoticus* (2.8 mg/mL) in 50 mM Tris-HCl buffer (pH 8.5) at room temperature

The spectral graph indicated that the purified laccase belongs to the group of well established blue-laccases of fungi and bacteria.

5.3.2.10. Substrate specificity

The substrate specificity studies indicated that pyrogallol is the most suitable substrate for laccase from this strain (Table 5.4). The enzyme oxidized pyrogallol very efficiently than any of the other studied substrates. Pyrogallol has three substituted hydroxyl groups that may be beneficial for the enzyme action, although the specificity towards other compounds with three hydroxyl groups such as gallic acid and tannic acid were much less than that of pyrogallol. This result indicated that not only the number of substitution but also the position of substitution plays a major role in determining the specificity towards a compound. The higher specificity towards pyrogallol has already been reported from *Streptomyces lavendulae* and a fungal strain (Suzuki et al., 2003). However, the relative activity observed with this compound in the present study was far beyond any of the reported values.

Dimethoxy phenol (DMP) was the next preferred substrate for the enzyme. The specificity towards DMP was much higher than ABTS and syringaldazine, the two commonly used laccase substrates. Similar result has been reported by Calvo et al (1998). The enzyme showed only limited specificity towards syringaldazine, which is a dimer of two molecules of 2, 6- dimethoxyphenol linked by an azide bridge. This might be due to the effect of assay pH on the particular substrate. All the assays were carried out at the same pH (8.5), which may not be suitable for some substrates like syringaldazine. Other methoxy substituted compounds like guaicol, ferulic acid and vanillic acid were not efficiently oxidized as compared with the di-substituted compounds. These findings were in congruence with the reports by Palmieri et al (1997).

Among the substituted phenols such as catechol and resorcinol, the enzyme showed more affinity towards the ortho-substituted catechol than the meta-substuted resorcinol. Laccases have been reported to exhibit activity with para and ortho-

diphenols (Xu, 1996) and very less reactivity has been observed generally with the meta-substituted phenols (Jolivalt et el., 1999). The enzyme showed no activity towards tyrosine. The substrate specificity of the purified laccase showed similarity to most of the other reported laccases except the low reactivity towards syringaldazine. The substrate specificity studies indicated that this enzyme could be effectively used for aromatic compounds degradation and treatment of phenol containing effluents.

Substrates (1 mM)	**Relative activity (%)[a]**
ABTS (control)	100
Resorcinol	35.1
Gallic acid	110.8
Tannic acid	81.7
Catechol	58.1
Pyrogallol	475.0
Syringaldazine	45.3
Ferulic acid	62.2
Vanillic acid	75
Guaiacol	70.2
DMP	213.5
DOPA	46.6
L - Tyrosine	0

Table 5.4. Substrate specificity of purified laccase

[a] Values represent the means of values from three independent experiments, with a maximal sample mean deviation of ± 5%

5.3.2.11. Kinetic studies

The kinetic parameter K_m was determined with three different substrates viz; ABTS, pyrogallol and syringaldazine. The enzyme showed greater affinity towards pyrogallol with a K_m value of 0.25 mM (Fig. 5.8a). The K_m value with ABTS and syringaldazine was 0.39 mM and 3.35 mM respectively (Fig 5.8b and 5.8c). Similar result on kinetic studies with ABTS as substrate has been reported from *Streptomyces cyaneus* (Arias et al., 2003). Enzymes from different sources exhibit difference in kinetic parameters too. The K_m value for laccases from the fungi usually ranges from 0.01 mM to 0.6 mM (Galhaup et al., 2002; Saito et al., 2003).

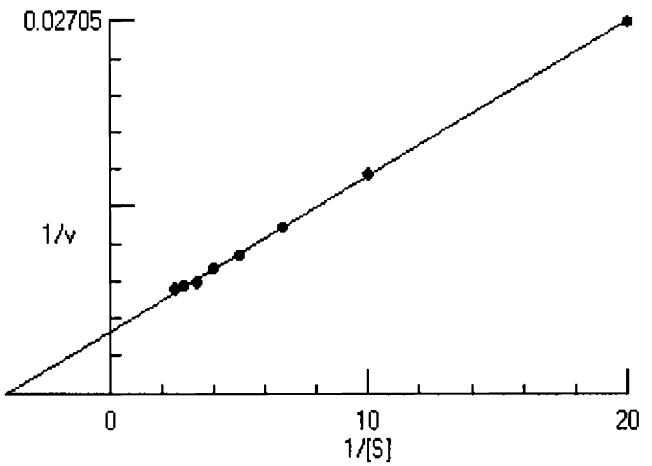


Fig. 5.8a Lineweaver-Burk plot for laccase using pyrogallol as the substrate

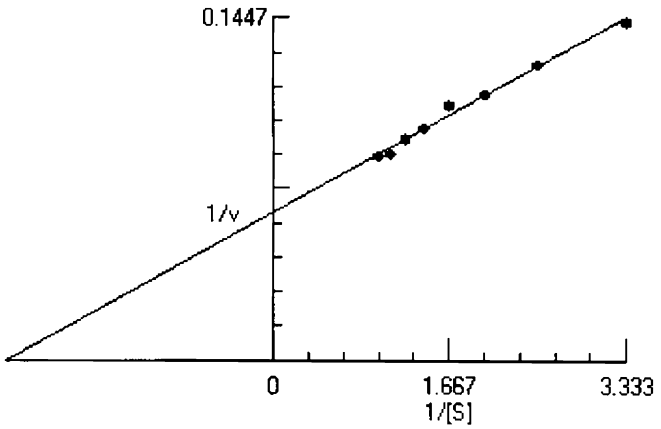


Fig. 5.8b Lineweaver-Burk plot for laccase using ABTS as the substrate

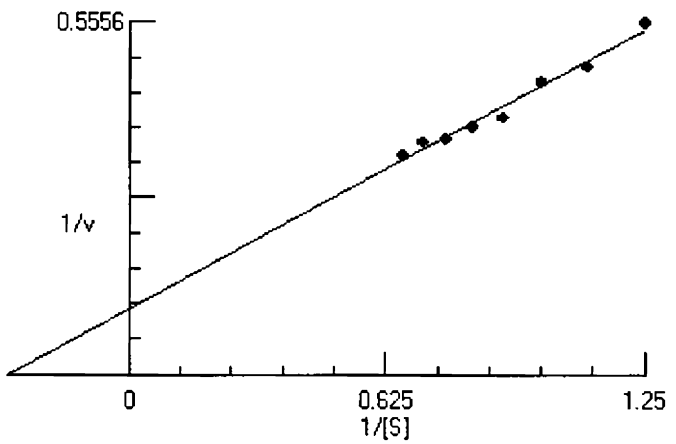


Fig. 5.8c Lineweaver-Burk plot for laccase using syringaldazine as the substrate

The high K_m value obtained for syringaldazine indicated that the enzyme had less affinity towards this substrate. The affinity of the purified enzyme towards different substrates were in the order; pyrogallol > ABTS > syringaldazine and the results of the kinetic analyses were very much in agreement with the substrate specificity studies. The V_{max} value for pyrogallol was 222.8 μmol/min. The V_{max} values corresponding to ABTS and syringaldazine were 15.99 and 9.57 μmol/min respectively. The V_{max} value for ABTS has been reported as 5.55 U mg of protein^{-1} for the laccase from *S. cyaneus* (Arias et al., 2003).

5.4. CONCLUSION

The purified laccase from *S. psammoticus* had a low molecular weight as compared to other *Streptomyces* laccases. It differed from other laccases in a few aspects such as the low specificity towards syringaldazine and activity enhancement by ferrous ion. The enzyme showed very high affinity towards pyrogallol and exhibited wide substrate range. The effect of different inhibitors on the purified enzyme conformed to the general pattern of laccase inhibition. The present study has identified, purified and characterized a halo-tolerant laccase from a mangrove actinomycete. This is the first report on such a novel enzyme from actinomycetes and the present work has made a new attempt for the identification of similar enzymes from this group of organisms. The relatively high activity at alkaline pH and high salt concentrations makes this enzyme different from other reported laccases and also augment its potential for use in diverse industrial applications.

Chapter 6.

APPLICATION STUDIES

6.1. INTRODUCTION

Laccases have attracted increasing scientific attention in the recent years due to their application in diverse industrial sectors such as the paper and pulp industry, where its inclusion in pulping processes has been shown to lower the kappa number and increase the brightness of kraft pulp (Arias et al. 2003). This group of enzymes has been studied for the removal of xenobiotics from aqueous waste streams (Crecchio et al., 1995), stabilization of fruit and vegetable juices (Minussi et al., 2002), oxidation of phenolics in wine (Servili et al., 2000) and denim washing (Pazarlioglu et al., 2005) and textile dye decolourization (Abadulla et al., 2000). Although there are many applications assigned to this valuable enzyme, the intensely studied uses of laccases are its ability to decolourize/degrade industrial dyes and the treatment of phenol containing effluents. Dyes are designed to be resistant to light, water and oxidizing agents and they are difficult to degrade once released into aquatic systems. Moreover it is impossible to degrade or remove all the dyes belonging to different categories using physical or chemical process, and sometimes the degradation products are more toxic (Spadaro et al., 1994). Similarly, the removal of phenols from industrial water effluents is also another important practical problem, since many of the phenolic compounds are toxic in nature.

The growing concern about minimizing the ecological problems has led to a massive change in the scenario of pollution abatement. The current trend is oriented towards utilizing the eco-friendly and inexpensive biological methods instead of the harsh and expensive physico-chemical methods. The biological methods involve the use of either microorganisms as such or enzymes from potent organisms for the treatment of pollutants. The use of free as well as immobilized organisms for dye degradation and phenol removal has been well established (Lacina et al., 2003; Ryan et al., 2007; Kasinath et al., 2003).

There is a growing recognition that enzymes can be used to target specific pollutants for treatment (Karam and Nicell 1997).The lower redox potential of laccases restricts their use in the oxidation of non-phenolic compounds including a wide range of synthetic dyes. However, this problem can easily be surpassed with the use of appropriate mediators. In the presence of a suitable redox mediator laccases

are also able to oxidize non-phenolic structures, (Bourbonnais and Paice, 1990; Call and Mucke, 1997) expanding thus, the range of compounds that can be oxidised by these enzymes. Redox-mediated laccase catalysis has been used in a wide range of applications including dye degradation. Claus et al (2002) have reported the ability of laccase plus mediator system in the decolourization of dyes that were initially resistant to laccase.

Immobilized enzymes are generally preferred over free enzymes for many of the application purposes due to its potential advantages like increased stability and reusability. Enzymes are immobilized on solid matrices by different methods. The chemical immobilization includes attachment of enzyme to matrix by either covalent bonds or cross-linking, while the physical method involves the entrapment of enzyme molecules within different types of matrices (Duran et al., 2002). Treatment of effluents using fungal laccases immobilized on different matrices has been very well established. (Davis and Burns 1990; Hublik and Schinner 2000; Crecchio et al. 1995). However, the fungal laccases, which are mostly acidic in nature, could not be used universally for the treatment of effluents from different industries, largely due to the alkaline nature of the effluents. Under such situations the enzymes from actinomycetes with wide pH range play a better role than the fungal counterparts.

6.2. MATERIALS AND METHODS

6.2.1. Dye decolourization using *S. psammoticus*

6.2.1.1. Dyes

Ten different dyes (each 100 ppm) were screened to assess the ability of the organism to decolourize them. The dyes RBBR, Poly R 478 and Malachite green were purchased from SIGMA chemicals while other dyes such as Rhodamine B, Auromine O, Crystal violet, Methyl orange, Bromophenol blue, Acid orange and Bismarck brown were purchased from a local textile dye industry.

6.2.1.2. Medium, inoculum preparation and cultural conditions

In addition to the dyes, the liquid media used for dye decolourization studies contained $(NH_4)_2SO_4$ - 0.1, $MgSO_4$ - 0.1, $CaCO_3$ - 0.02 and $CuSO_4$ – 0.001 (g/L). The addition of basal salts was required for the growth of the organisms in dye containing media. 48 h grown liquid culture of *S. psammoticus* was used for inoculation. 10 % inoculum was used for the study. The inoculated flasks were kept in a rotary shaker at 150 rpm, 32 °C for 10 days.

6.2.1.3. Decolourization studies

Decolourization of the dyes was determined by monitoring the decrease in absorbance at the peak of maximum visible absorbance (nm) of each dye and expressed as percentage of decolourization. Uninoculated media containing the respective dyes were used as the control. Laccase production was also assessed in the media that contained dyes as the sole carbon source. In the case of dye that showed maximum decolourization, spectrum was recorded (400 – 800 nm) using a Shimadzu UV-VIS spectrophotometer.

6.2.2. Treatment of phenolic solution by immobilized cells

6.2.2.1. Immobilization of cells on polyurethane foam

S. psammoticus was immobilized on (PUF). 5 g of PUF (1 cm^3) was weighed and taken in a 250 mL conical flask and autoclaved for 30 min at 121 °C. 90 mL of the basal medium was added to the flasks and subjected to autoclaving for 15 min. The inoculum for immobilization was the same used for other studies. After cooling, 10 mL of the inoculum was added to the flasks and kept in a rotary shaker at 150 rpm. After 48 h the immobilized PUF was filtered and washed with sterile water and aseptically transferred to 100 mL of the phenol model solution.

6.2.2.2. Sample preparation

The 100 % phenol solution had a total phenolic content of 17.8 mg/mL. The concentration of the phenol model solution was varied by diluting the solution using adequate quantity of distilled water to yield 25, 50 and 75 % phenol solutions. 100 % phenol solution was also included in the study. The phenol degradation by the immobilized cells was monitored for 12 days. Samples were collected at 24 h intervals and subjected to total phenol estimation, colour removal and COD measurements. Flasks containing uninoculated medium of corresponding phenol concentration were used as the controls.

6.2.2.3. Total phenol estimation

Total phenol estimation of the untreated and treated samples was done by the method of Singleton and Rossi (1965). In brief, 0.8 mL of 7.5 % sodium carbonate and 1 mL of Folin-Ciocalteau reagent were added to 0.2 mL of the sample and incubated in dark for 30 min and the absorption at 765 nm was measured. The total phenolic content of the samples was expressed as milligram of gallic acid equivalents (GAE) per milliliter of the sample.

6.2.2.4. Estimation of chemical oxygen demand (COD)

The chemical oxygen demand was determined by the open reflux method. In this method the sample is refluxed in strongly acidic solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced dichromate is titrated with ferrous ammonium sulphate to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter is calculated in terms of oxygen equivalent. Ferroin was used as the indicator.

6.2.2.5. Colour removal

Colour removal from the samples was monitored at 280 nm in a UV-visible spectrophotometer (Shimadzu UV-2401 PC) and compared with the control.

6.2.3. Phenol removal using immobilized laccase

6.2.3.1. Laccase production and partial purification

Laccase production was carried out by the solid-state fermentation of rice straw. The substrate was moistened with a salt solution containing (g/L): yeast extract-3.0, $(NH_4)_2SO_4$-0.2, $MgSO_4$-0.2, $CaCO_3$ -0.04, $CuSO_4$-0.5. The initial moisture content and incubation temperature were maintained at 65 % and 32 °C respectively. After 48 h of fermentation, the crude enzyme was extracted from the fermented solid matter using 50 mM sodium phosphate buffer, pH 7.0 (Buffer A), centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant was collected. The proteins were precipitated from the supernatant with ammonium sulphate. 30-60 % saturated fractions were pooled, centrifuged (10000 rpm, 20 min) and the precipitate was dissolved in minimal amount of buffer A and dialyzed against the same buffer overnight at 4 °C.

6.2.3.2. Immobilization of laccase in alginate beads

Laccase (100 U) was added to 3 % solution of sodium alginate and mixed thoroughly by mild shaking on a rotary shaker. The viscous alginate-enzyme mixture was taken in a syringe fitted with a luer-lock needle and the solution was extruded drop by drop from the syringe into a 0.05 M solution of $CaCl_2$ or $CuSO_4$ under magnetic stirring to produce calcium and copper alginate beads respectively. The beads were kept for hardening at 4 °C for two hours. After two hours, the beads were filtered and washed thoroughly until there was no detectable protein in the wash out solution.

6.2.3.3. Optimization of immobilization conditions

Attempts were made to optimize the conditions for laccase immobilization in alginate beads. The crucial parameters like concentration of sodium alginate, $CaCl_2$ and $CuSO_4$ were varied and subjected to study.

6.2.3.4. Activity measurements

The binding efficiency of the immobilized system was calculated using the following equation:

$$\text{Binding Efficiency (\%)} = \frac{A-B}{A} \times 100$$

where A = Added enzyme (U) and B = Unbound enzyme (U)

The unbound enzyme activity was calculated by measuring the laccase activity in the curing solution and washouts. The immobilized enzyme yield was calculated by dissolving few immobilized beads in 100 mM phosphate buffer (pH 7.5) and the activity was measured using ABTS as the substrate.

6.2.3.5. Reactor studies using immobilized enzyme

Removal of phenols from the sample was carried out by performing batch studies in packed bed column bioreactor. The immobilized beads were loaded in a glass column (50 cm x 2 cm), leaving a headspace of 5 cm. A synthetic phenol sample was prepared by mixing different phenolic compounds such as gallic acid, tannic acid, ferulic acid, resorcinol, guaiacol, catechol, vanillic acid and pyrogallol. The phenolic mixture (hereafter described as sample solution) was passed through the column using a peristaltic pump (Gilson Minipuls 3, France) at a flow rate of 1 mL/min for 6h and the treated sample collected at the outlet was subjected to total phenol estimation at regular intervals and was compared with the phenol content of the sample solution. The reactor studies were done at room temperature (30 ± 2 °C).

6.2.3.6. Thin layer chromatography (TLC)

TLC was performed on silica gel sheets of size10 × 20 cm (Merck, Germany). The phenolic compounds present in the untreated and treated samples were analyzed by comparison with the standards run simultaneously. The standard phenolic compounds used were tannic acid, gallic acid, ferulic acid, resorcinol and pyrogallol (1 mg/mL concentration). The untreated sample was a mixture of above-mentioned phenolic compounds. Chloroform: ethyl acetate: acetic acid (50:50:1) was used as the solvent system. Detection of the compounds was done by keeping the TLC sheet in the chamber pre-equilibrated with iodine or by spraying with 1 % solution of ferric chloride.

6.2.3.7. Nuclear Magnetic Resonance (NMR) spectroscopy

The samples for NMR spectroscopy were evaporated to dryness in a rotavapor (Buchi R-200, Switzerland) and then dissolved in appropriate NMR solvents. Deuterated methanol and CDCl3 were used as the solvents for untreated and treated samples respectively. 1H NMR spectra of the untreated and treated samples were recorded using 300 MHz Bruker Avance DPX Spectrometer. The number of scans for the untreated and treated samples was 16 and 48 respectively.

6.2.4. Mediator-based decolourization of azo dyes by laccase

6.2.4.1. Dyes and mediators

Three azo dyes (Acid orange, Methyl orange and Bismarck brown designated as dye I, dye II and dye III respectively) were selected for dye decolourization studies. The mediators used were 2, 2' – azino – bis – 3 – ethyl benzothiazoline – 6 – sulphonic acid (ABTS), 1-hydroxybenzotriazole (HOBT), aniline and para-hydroxybenzoic acid (pHBA).

6.2.4.2. Decolourization studies

Stock solutions of dyes were diluted in distilled water to a final concentration of 50 ppm. The reaction mixture (1.5 mL) contained 0.5 mL dye solution, 0.5 mL enzyme (58.3 nkat), and 0.5 mL buffer (50 mM sodium phosphate buffer, pH 6.0). Decolorization was determined by monitoring the absorbance decrease at the maximum wavelength in the visible spectrum of each dye (Acid orange-480 nm, methyl orange-466 nm and Bismarck brown-457 nm) and was expressed in terms of percentage decolourization.

For the studies on mediator-based decolourization, the mediator compounds were tested at different concentrations ranging from 0.5-2.0 mM each. The stock solution of HOBT was prepared in 50 % DMSO (dimethyl sulphoxide). The reaction mixture (2 mL) contained 0.5 mL each of dye solution, mediator, buffer and enzyme. In both set of experiments, the reactions were arrested by adding 2 mM sodium azide (0.5 mL). In the controls, the enzyme was replaced with heat-killed enzyme.

6.3. RESULTS AND DISCUSSION

6.3.1. Dye decolourization by *S. psammoticus*

The dye decolourization studies showed that there exists much variation in the ability of *S. psammoticus* to degrade dyes belonging to diverse categories. Laccase production also varied greatly with different dye-containing media and maximum laccase activity was detected on the second day of incubation. Of the ten different dyes used in the study only RBBR showed considerable decolourization (80 % decolourization after 10 days of incubation) and substantial laccase activity was also observed in RBBR containing medium (1.93 U/mL) (Table 6.1). Significant laccase production was also detected in the medium with Poly R 478 (1.03 U/mL) even though the decolourization was not much prominent in Poly R. The poor correlation between decolourization of Poly R 478 and ligninolytic activities of actinomycetes has been established earlier (Ball et al., 1989).

Name of dyes	Classification of dyes	Decolourization of dyes (%)	Laccase (U/mL)
Rhodamine B	Fluorone dye	16	0.68
Bromo phenol blue	Tri phenyl methane	5	NDa
Malachite green	Triphenyl methane	4	0.21
Remazol brilliant blue R	Anthroquinone	80	1.93
Methyl orange	Azo	11	0.29
Bismarck brown	Azo	19	0.55
Acid orange	Azo	16	0.37
Auramine O	Diarylmethane	8	NDa
Crystal violet	Triarylmethane	3	NDa
Poly R 478	Polymeric dye	26	1.03

ND[a] - Not detected

Table 6.1. Decolourization of dyes and corresponding laccase production by *S. psammoticus*

Azo dyes are the most common dyes that were reported to be degraded by different *Streptomyces* sp and approaches to improve the azo dye degradability by *Streptomyces* sp has also been carried out by Paszczynski and his co workers (Paszczynski et al., 1991). However in the present investigation *S. psammoticus* exhibited only limited decolourization towards all the three azo dyes tried. Other dyes (triphenylmethane, di and triphenylaryl dyes) showed very less decolourization even after ten days of incubation and no laccase activity was observed in the corresponding dye based media. Figure 6.1 shows the spectra of RBBR over different incubation periods that corresponded to the degree of decolourization. The decolourization was very obvious in the case of RBBR and similar result has been reported from other *Streptomyces* sp also (Pasti and Crawford, 1991).

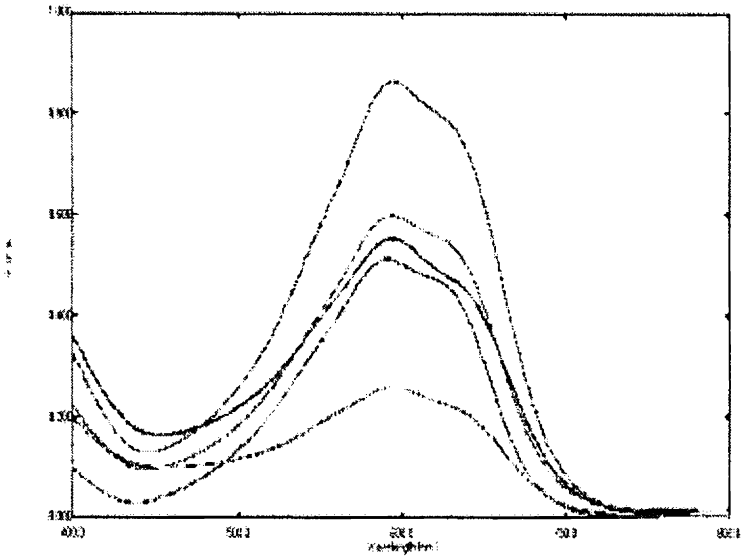


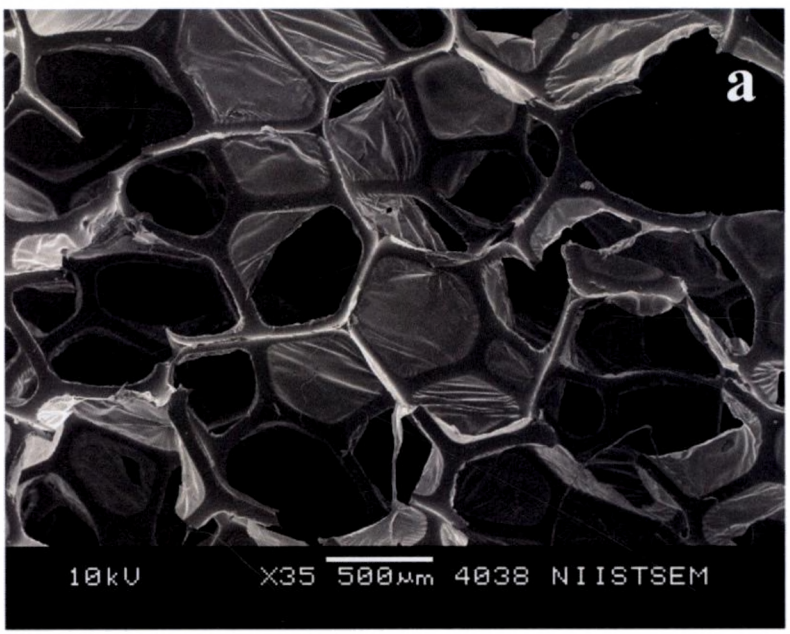
Fig. 6.1. Spectrum showing the decolourization of RBBR over different periods of incubation

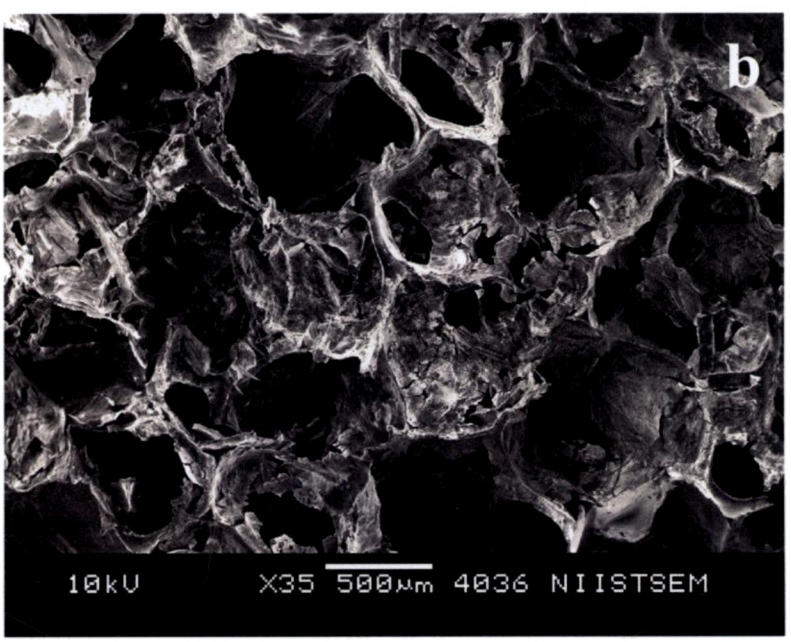
It was observed that almost 25 % of RBBR decolourization occurred within 48 h of incubation. This result was in correlation with the period of maximum enzyme production and it clearly indicated the role of laccase in RBBR decolourization by *S. psammoticus*. After 48 h not much colour reduction was observed and hence the medium was reinoculated (on 120 h) with fresh inoculum that resulted in further decolourization of the dye. Although the same procedure was followed for rest of the dyes the results were not promising as with RBBR. The results of the study indicated that the strain could be hopefully employed for the decolourization of anthroquinone dyes.

6.3.2. Treatment of phenolic solution by immobilized cells

Immobilization of *S. psammoticus* was carried out on poly urethane foam (PUF). Figures 6.2a and 6.2b shows the uninoculated PUF and PUF with the immobilized

cells of *S. psammoticus*. The colonization of PUF with the organism is clearly visible in the Figures 6.2b and 6.2c.





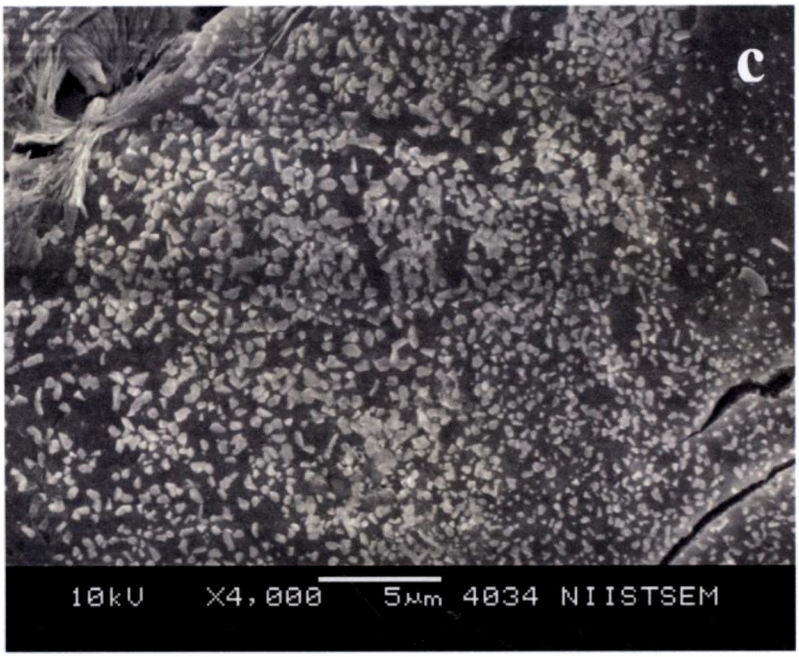


Fig. 6.2. Scanning electron micrographs of polyurethane foam

(a) uninoculated PUF; (b) PUF with immobilized cells; (c) enlarged view of *S. psammoticus* on PUF

The immobilization of *Streptomyces* cells on PUF has already been reported (Beg et al., 2000; Ramesh et al., 2004). The treatment of phenolic sample using immobilized cells was found to be a promising method. The result of phenol removal from the sample using immobilized cells is given in Fig. 6.3. It was observed that the phenol removal was not prominent in the 100 % phenol solution as compared to other concentrations such as 25, 50 and 75 % phenol solutions. This was probably due to that the very high phenol concentration may be inhibitory to the growth of organism. 89.2 % of phenol was removed from 25 and 50 % phenol solution after an incubation period of 12 days while the immobilized system removed only 58 % phenol from the 100 % solution. Considering the very high phenol concentration of the initial sample (untreated) and the observed result of 89.2 % phenol removal, it can be concluded that the immobilized cells of *S. psammoticus* could be used for the treatment of effluents of varying phenol concentrations.

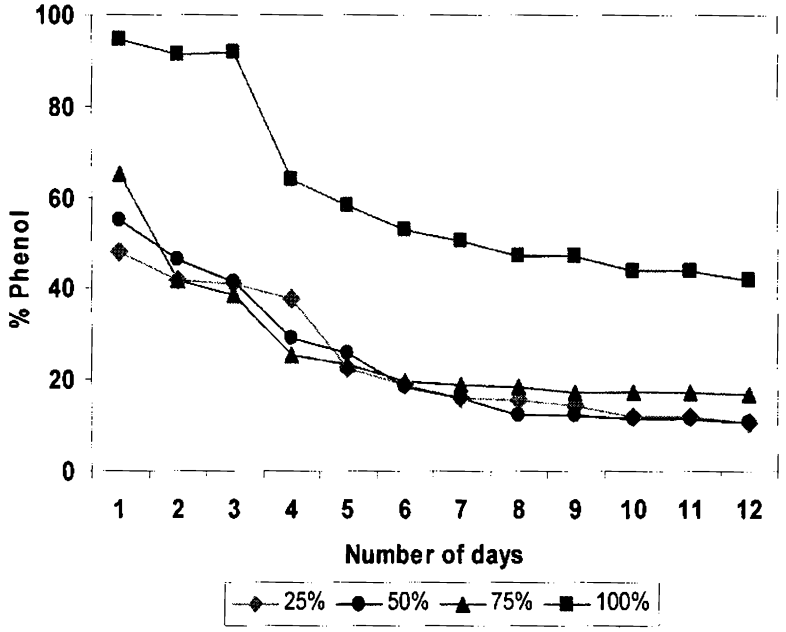


Fig. 6.3. Removal of phenol from the phenolic sample during treatment with immobilized cells

The duration of the treatment was 12 days and the initial phenol concentration of the undiluted sample was 17.8 mg/mL

The result of COD reduction using immobilized cells followed a slightly different pattern from that exhibited by the phenol removal. The reduction in COD was decreasing with the increasing concentration of phenol. The COD reduction was 77.2, 70.3, 64.2 and 54.2 % respectively in the 25, 20, 75 and 100 % phenol solutions (Fig. 6.4). The difference in the rate of phenol removal and COD reduction indicated that the growth of *S. psammoticus* in the phenol solutions resulted in the production of some metabolites or by-products of phenol catabolism that contributed to the remaining COD. These metabolites or by-products may not be susceptible for further oxidation by laccase and hence remained in the solution even after 12 days of incubation. The varying levels of COD reduction from phenolic effluents by laccase producing organisms have been reported. Pedroza et al (2007) has reported 82 % reduction in COD from the effluent of a paper industry after four days of treatment with PUF immobilized *T. versicolor*.

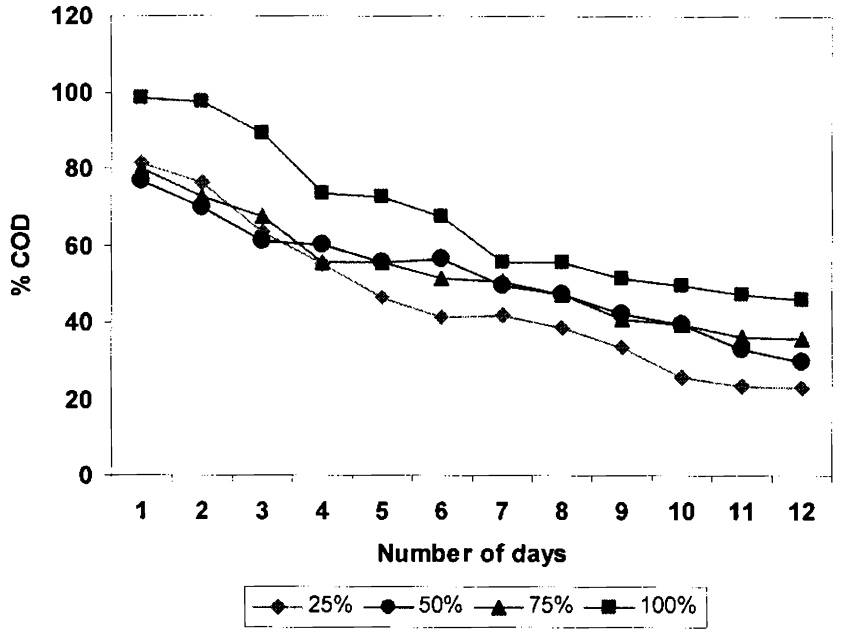


Fig. 6.4. Reduction in COD of the phenolic sample during treatment with immobilized cells

The duration of the treatment was 12 days and the initial COD of the undiluted sample was 8289.2 mg/L

The laccase activity was maximum (1.2 U/mL) in 25 % phenol solution. The activity was much lower (0.71 U/mL) in the 100 % phenol solution and the result suggested that there was a linear correlation between the laccase activity and phenol removal /COD reduction. The initial pH of the differently diluted phenol solutions was in the alkaline range of 8.8-10.2 which was favourable for the growth of the organism and laccase production. The dark colour of the phenol solution was also reduced as the culture grew in the phenol solutions. However, complete decolourization was not observed even at the end of 12 days treatment as it was observed during the spectrophotometric analyses. This might be due to the interference caused by the characteristic dark pigment produced by *S. psammoticus* during growth.

6.3.3. Treatment of phenolic solution using immobilized laccase

6.3.3.1. Enzyme immobilization

Partially purified laccase from *S. psammoticus* was immobilized by physical entrapment in alginate beads. Alginate is a natural polymer and can easily be converted into hydrogels via cross-linking with divalent cations. In the present study two different cations such as calcium and copper were used for the preparation of calcium alginate and copper alginate beads respectively. Calcium alginate is the most commonly used support for enzyme immobilization while copper alginate gels are used specifically for laccases (Palmieri et al., 1994). The formation and the mechanical and structural properties of alginate beads depend upon different parameters such as the alginate concentration, nature of the cations and concentration of the cations (Ouwerx et al. 1998).

6.3.3.2. Optimization of immobilization conditions

Figure 6.5 shows the effect of alginate concentration on the laccase binding efficiency of the immobilised beads. The laccase binding efficiency was maximum when the alginate concentration was 2.5 % for both calcium and copper alginate beads, although the laccase binding efficiencies observed with copper alginate beads were higher as compared to the calcium alginate beads. Low concentration of alginate resulted in beads with reduced strength and increased enzyme leaching. The beads were more transparent and the bead shape was also affected at lower concentration of alginate. The curing time required for the hardening of beads with lower alginate concentration was observed to be 10-12 h while the 2.5 % alginate beads required only 2 h curing time. Increasing the alginate concentration beyond 2.5 % failed to enhance the laccase binding efficiency which indicated that 2.5 % sodium alginate concentration was the optimum for good bead formation.

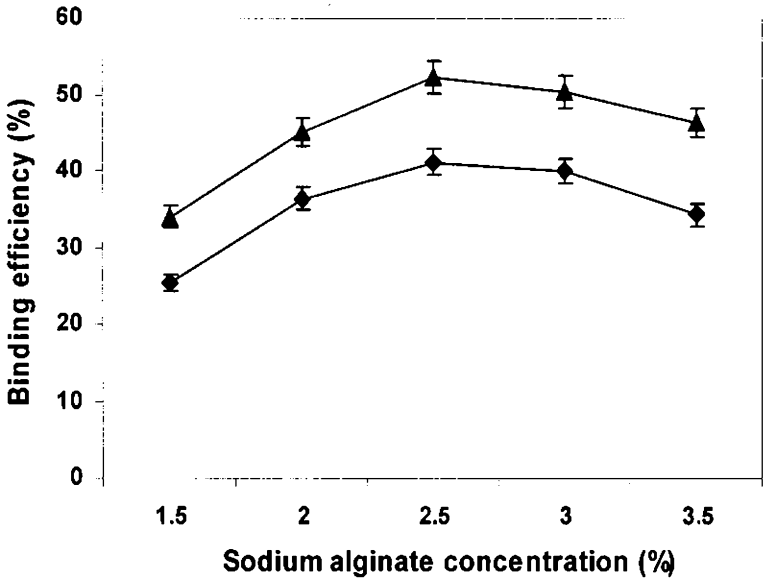


Fig. 6.5. Effect of sodium alginate concentration on laccase binding efficiency of calcium (◆) and copper alginate (▲) beads

Concentration of CaCl2 and CuSO4 was 0.05 M. Concentration of laccase was 100 U.

The nature of the cation is one of the other crucial factors that determines the functional properties of the beads. Extensive studies have been carried out on the type of cations and their interaction with alginate during bead formation(Ouwerx et al. 1998; Rodrigues and Ricardo 2006). It has been reported that the type of the cation has a great influence on the network elasticity (Ouwerx et al., 1998). For the present study, concentrations of both the cations (Ca and Cu) were varied from 0.01 – 0.2 M and the effect was studied. In the case of calcium alginate beads, the maximum laccase binding efficiency was observed at 0.05 M $CaCl_2$ concentration while the copper alginate beads exhibited maximum laccase binding efficiency at 0.1 M $CuSO_4$ concentration (Fig. 6.6).

When the overall efficiency of the two immobilized systems were compared, copper alginate beads proved to be the better support for the immobilization of laccase, exhibiting higher binding efficiency (Fig. 6.7). This was probably because

copper having a high affinity for alginates, was strongly complexed in the polymer network (Ouwerx et al., 1998).

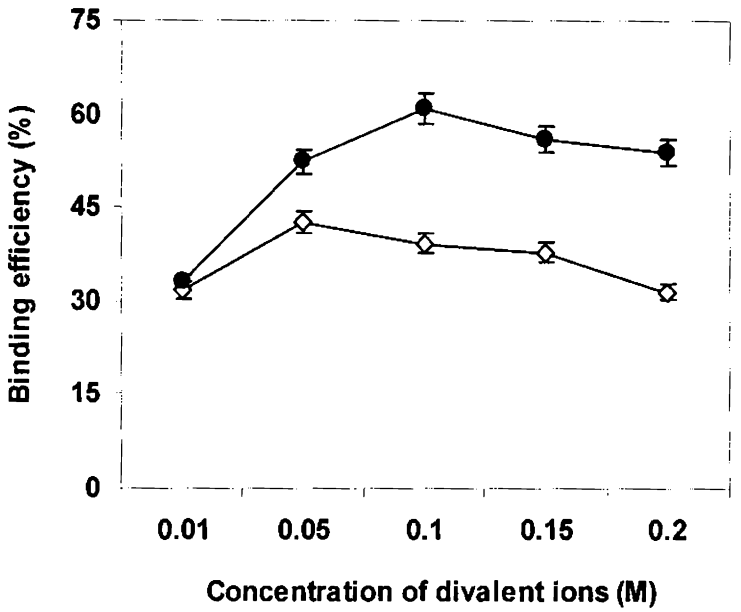


Fig. 6.6. Effect of calcium chloride (◇) and copper sulphate (●) concentrations on laccase binding efficiency of alginate beads.

Concentration of sodium alginate and laccase were 2.5 % and 100 U respectively

It has been reported that Ca alginate beads have higher porosity and lower chemical stability than Cu alginate which, resulted in higher enzyme leakage than Cu alginate beads (Brandi et al., 2006). Also, there are reports that the replacement of Ca2+ with other divalent cations such as Cu2+ might stabilize the alginate beads and reduce alginate porosity (Thu et al., 1996). The pore size distribution of the gel is a critical parameter because it influences the diffusion of molecules such as substrates and products through immobilized biocatalysts. The operational efficiency (efficiency at which the system performs) of any immobilized system depends upon the amount of enzyme retained by the system (laccase binding efficiency). Hence, we chose copper alginate beads for further studies.

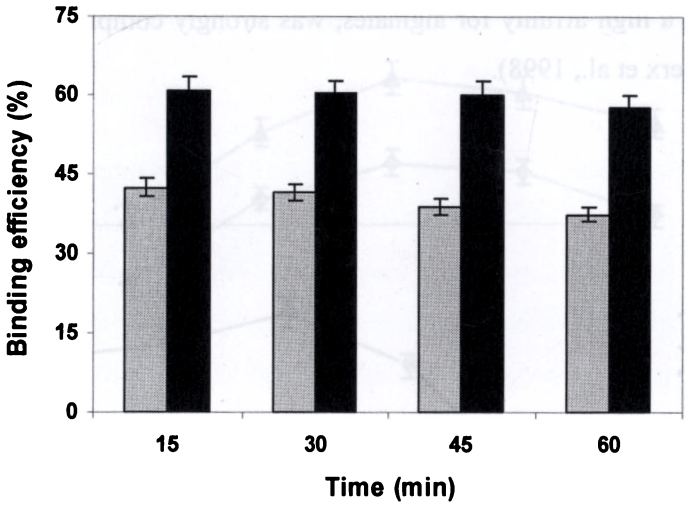


Fig. 6.7. Comparison of calcium alginate ( ) and copper alginate ( ) beads with respect to the laccase binding efficiency

Concentrations of sodium alginate and laccase were 2.5 % and 100 U, respectively. Concentration of CaCl2 and CuSO4 were 0.05 M and 0.1 M, respectively

6.3.3.3. Reactor studies using immobilized enzyme

The packed bed bioreactor system used in the study is shown in Figure 6.8. Batch experiments were performed in the bioreactor that contained laccase-immobilized Cu alginate beads. Figure 6.9 shows the removal of phenols and colour from the phenol model solution in a batch experiment run for 6 h. It was observed that 95 % of the total phenols and 99 % of the colour was removed from the system at the initial stage of 30 min. The extent of phenol removal and colour reduction was maintained almost in the same level up to 180 min. The flow of phenol sample through the immobilized beads resulted in the formation of water insoluble aggregates in the packed bed reactor. Precipitation of phenolic compounds as aggregates on reaction with phenol oxidases is a well-established phenomenon. (Dec and Bollag, 1990). Formation of these polymerization products was an indication of typical laccase activity. These precipitates were easily separable from the beads by filtration after each catalytic cycle. At the end of the first run (6 h), phenol and colour removal was reduced to 70 % and 72 % respectively.

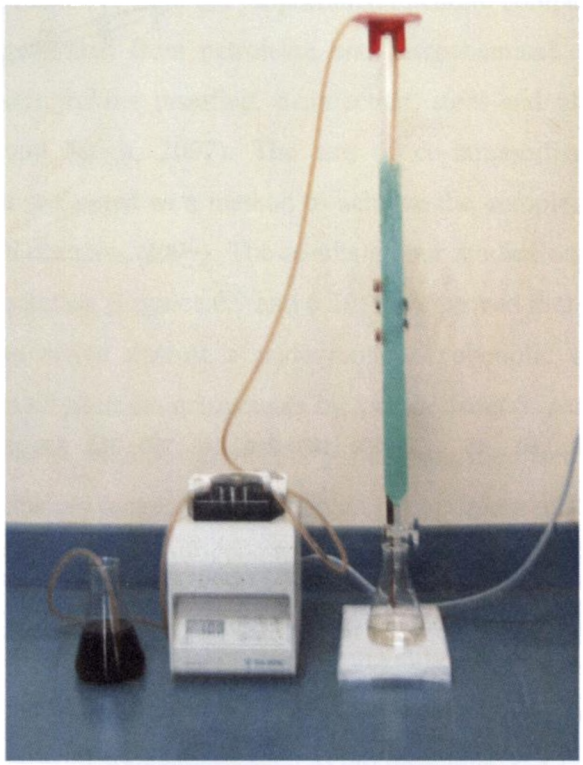


Fig. 6.8. Packed bed bioreactor system for phenol removal using laccase immobilized in copper alginate beads

The operational stability of the immobilized laccase was estimated by reusing the same matrix for successive runs. A total of eight runs, each with duration of six h were performed. The phenol model solution of same composition was used for the successive catalytic cycles and the result of the same is given in Figure 6.10. Enzyme leaching has been reported as the major problem with alginate beads. However, in the present study it was observed that the system maintained 50 % of operational efficiency even after eight successive runs. The result indicated that the enzyme leaching was prominent only during the initial run that reduced the phenol removal from 95 % (at 30 min) to 70 % (at 360 min). After the initial run, the enzyme leaching was relatively low, resulting in 50 % operational efficiency after eighth run (48 h). This was probably due to the fact that the enzyme coated on the surface was leached out easily during the initial run while the enzyme entrapped deep within the alginate beads was retained relatively for long duration.

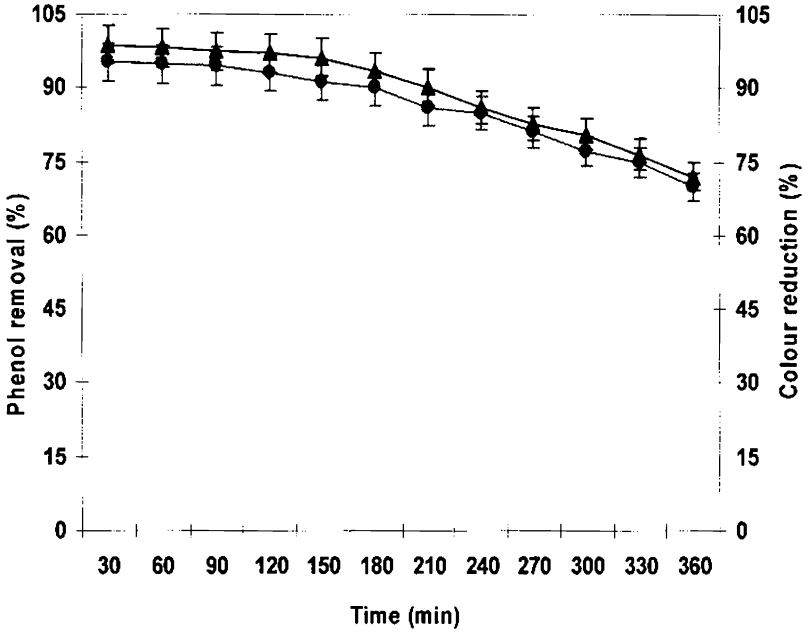


Fig. 6.9. Phenol (●) and colour (▲) removal from the phenol model solution in a batch experiment of 6 h duration

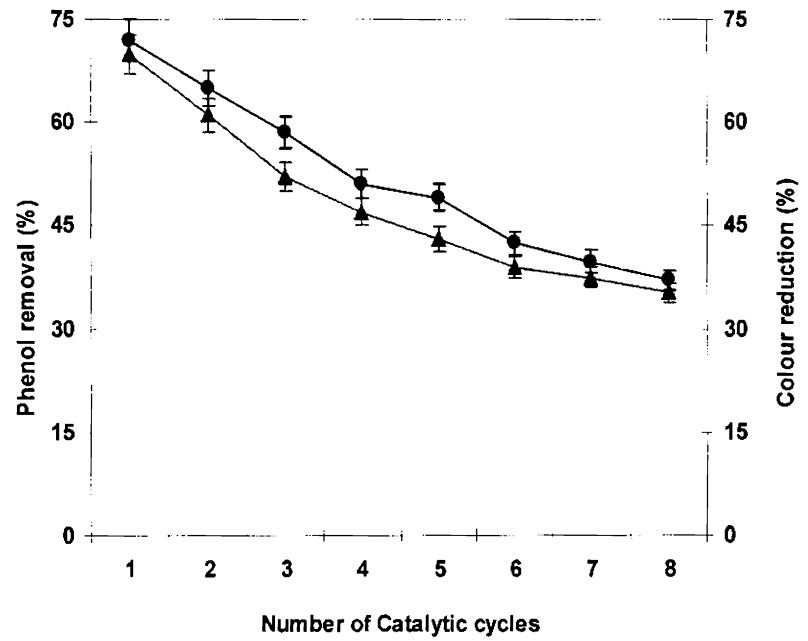


Fig. 6.10. Phenol (▲) and colour (●) removal from the phenol model solution over different catalytic cycles.

The duration of each catalytic cycle was 6 h

Degradation of individual phenolic compounds by laccases has been reported by many authors (Lante et al., 2000). However, the industrial effluents usually contain mixture of phenols in different proportions. Phenolic compounds are present in the wastewater generated from petroleum and petrochemical, coal conversion, pharmaceutical, plastic, rubber proofing, disinfectant, steel and phenol production industries (Nayak and Singh, 2007). The use of co-immobilized laccases and tyrosinases has been suggested as a method to achieve the complete degradation of phenolic mixtures (Krastanov, 2000). The results of our studies on phenol removal from phenol model solution (Figures 6.9 and 6.10) have proved that the laccase from *S. psammoticus* was active against a wide range of phenolic compounds. The effective degradation of phenolic compounds by laccase from *S. psammoticus* could be attributed mainly to its alkaline nature. The partially purified laccase was found to be stable at the alkaline pH range of 6.5 – 9.5 with pH optimum of 8.5, which was in good agreement with the pH of the phenolic solution used in the study (pH 9.2). In this respect the enzyme was different from the fungal laccases, most of which are acidic in nature. The alkaline pH makes the enzyme suitable for use in the treatment of effluents from paper industries, which usually has alkaline pH (Calvo et al., 1998) and also for other industrial applications such as bio bleaching.

6.3.3.4. Thin layer chromatography (TLC)

The authenticity of the results was confirmed by thin layer chromatography technique (TLC). TLC was performed with a phenol model solution that contained five selected phenolic compounds such as pyrogallol, tannic acid, gallic acid, resorcinol and ferulic acid. Figure 6.11 shows the result of TLC. Lane 1 shows the untreated phenolic sample solution. Lane 2-6 corresponds to different phenol standards such as pyrogallol, tannic acid, gallic acid, resorcinol and ferulic acid respectively. The spots of resorcinol and ferulic acid appeared at the same region as it was evident from the figure (Lane 5 and 6) and hence it emerged as a single spot in the sample solution (Lane 1). No mobility was observed for tannic acid with the solvent system used in this study (Lane 3) and similar results using the same mobile phase has already been established (Singh et al., 2001). None of the phenolic compounds in the sample

solution were observed in the treated sample (Lane 7) although; an unknown compound was detected in the treated sample. The disappearance of spots corresponding to phenolic compounds from the treated sample was a clear indication of phenolic compounds degradation. The unknown compound detected in the treated sample might be some breakdown product of phenolic degradation.

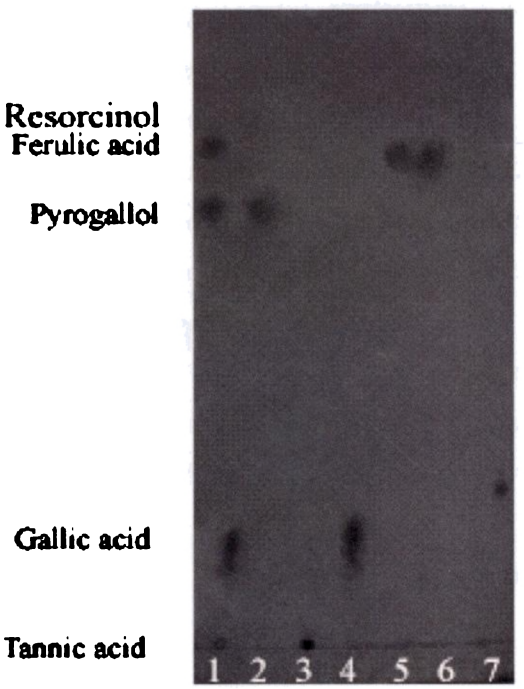


Fig. 6.11. TLC of untreated and treated phenolic solutions

Lane 1 indicates untreated phenolic sample (control), Lane 2-6 corresponds to standard phenolic compounds; pyrogallol, tannic acid, gallic acid, resorcinol and ferulic acid respectively. Lane 7 indicates the treated phenolic sample (test)

6.3.3.5. Nuclear magnetic resonance (NMR)

The results were further confirmed with the help of NMR spectroscopy. The ^{1}H NMR spectrum of the untreated sample solution (Fig 6.12a) showed the presence of different peaks in the aromatic region (6 -7.5 ppm), which corresponded to different

substituted phenols. It was obvious from the ^{1}H NMR spectrum of the treated sample (Fig 6.12b) that the phenols in the sample solution have undergone significant degradation, which resulted in considerable chemical shifts in the aromatic region. The different peaks were visible in the aromatic region of the untreated sample, especially between 6.1 and 6.7 ppm (Fig 6.12c) and these peaks disappeared in the treated sample (Fig 6.12d); a strong indication of aromatic compounds degradation. The massive chemical shifts which occurred in the aromatic region indicated that the enzyme was active against a wide range of phenolic compounds.

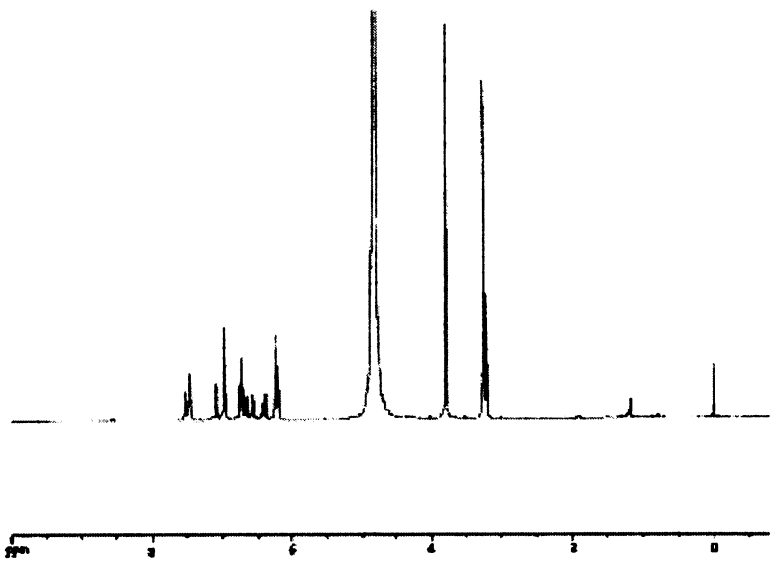
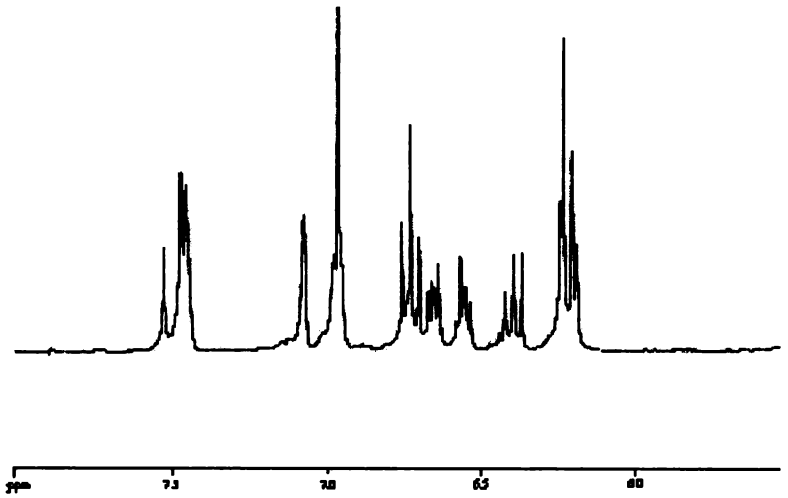
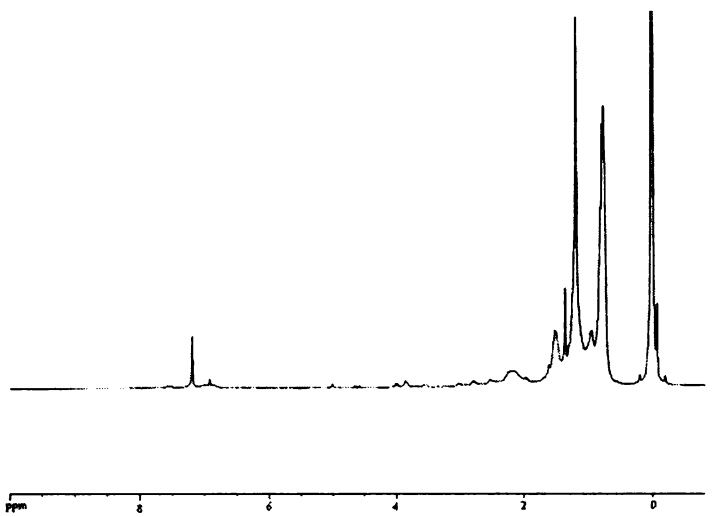


Fig. 6.12. ^{1}H NMR spectra of untreated and treated phenol samples

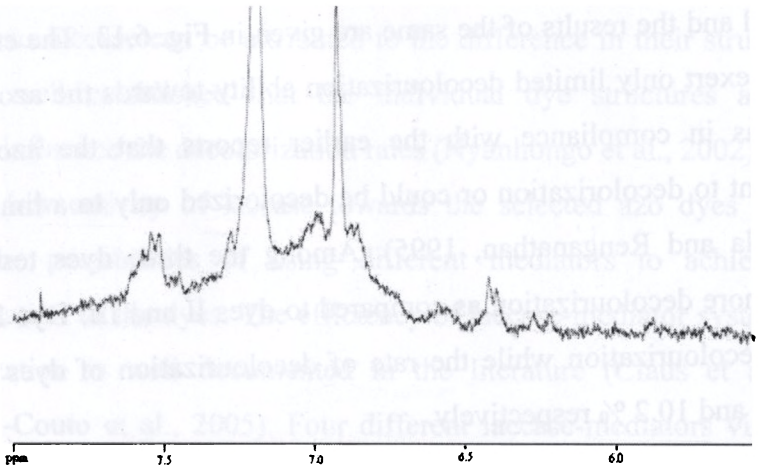
(a) Full ^{1}H NMR spectrum of untreated phenolic sample



c. ^{1}H NMR spectrum showing the aromatic region of untreated phenolic sample



b. Full ^{1}H NMR spectrum of treated phenolic sample



(d) ^{1}H NMR spectrum showing the aromatic region of treated phenolic sample

Another significant difference in the spectra of treated and untreated samples were in the aliphatic region. There were no intensive peaks in the aliphatic region of the untreated sample except the solvent peaks (Fig 6.12a), whereas the treated sample (Fig 6.12b) showed large number of signals between 0 – 5 ppm in addition to the expected solvent peaks. These peaks were characteristics of the aliphatic compounds. In a typical laccase catalyzed reaction, the phenols are converted presumably to quinones, which are reactive and can undergo subsequent conversions to form other intermediates (Lante et al. 2000). The possibility for further degradation of the break down products into aliphatic chains cannot be ruled out. Therefore, the presence of large number of peaks within the aliphatic regions might be due to various degradation products of laccase-catalyzed reactions.

6.3.4. Mediator-based decolourization of azo dyes by laccase

Decolourization of the selected azo dyes by *S. psammoticus* laccase was attempted and the results of the same are given in Fig. 6.13. The enzyme was found to exert only limited decolourization ability towards the azo dyes. The result was in compliance with the earlier reports that the azo dyes are recalcitrant to decolorization or could be decolorized only to a limited extent (Chivukula and Renganathan, 1995). Among the three dyes tested, dye I showed more decolourization as compared to dyes II and III. Dye I exhibited 16.2 % decolourization while the rate of decolourization of dyes II and III were 11.2 and 10.2 % respectively.

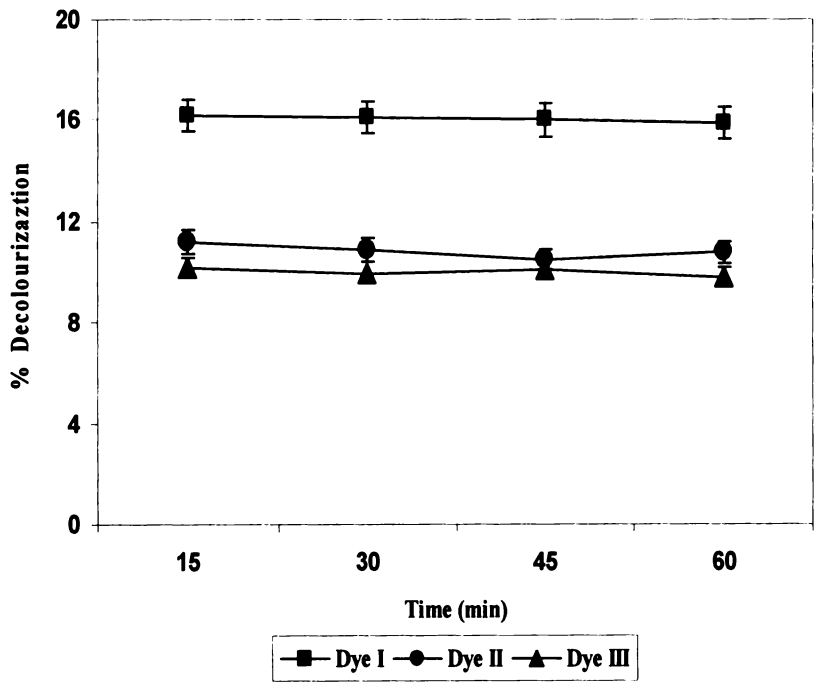


Fig. 6.13. Decolourization of dyes I, II and III by *S. psammoticus* laccase in the absence of mediators, over different incubation time.

The reaction mixture (1.5 mL) contained 0.5 mL each of dye solution, buffer and enzyme (58.3 nkat). The reaction time was 30 min

It was observed that the reaction between the dyes and the enzyme was rapid and no further increase in decolourization was observed with increase in time. The variation in the rate of decolourization of the different azo dyes by *S. psammoticus* laccase can be attributed to the difference in their structures. It has been well established that the individual dye structures and redox potentials influence the decolorization rates (Nyanhongo et al., 2002).

The low activity of laccase towards the selected azo dyes led us to explore the possibilities of using different mediators to achieve better decolourization of the dyes. The efficiency of laccase-mediator system in dye decolourization is well documented in the literature (Claus et al., 2002; Rodriguez-Couto et al., 2005). Four different laccase-mediators viz; ABTS, HOBT, aniline and *p*HBA were used for the study and each of the mediator was tested at four different concentrations to identify the optimum level required for dye decolourization. Figure 6.14 shows the decourization rate (% decolourization) of the dyes at different concentrations of ABTS over an incubation time of 30 min. Maximum decolourization of the dyes was observed at 1.5 mM of ABTS.

It was observed that at higher concentration of ABTS (2 mM), the decolourization of all the dyes was reduced. Increasing the incubation time up to 24 h resulted in increase in the absorbance of reaction mixture. The increase in absorbance was due to the darkening of reaction mixture over prolonged incubation period and the colour change was more distinct at higher concentration of ABTS. The formation of purple colour in a solution of laccase and ABTS in a buffer, after a long storage has been reported (Solis-Oba et al., 2005). In the present study, the colour change was rather intense than the purple colour reported earlier and the probable reason for this may be the auto-oxidation of excess ABTS present in the reaction mixture.

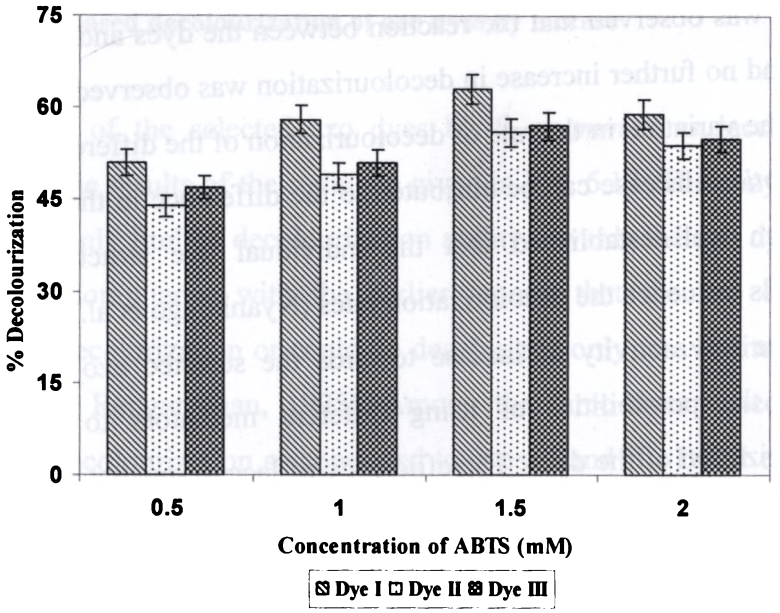


Fig. 6.14. Decolourization of dyes I, II and III by *S. psammoticus* laccase at different concentrations of ABTS

The reaction mixture (2 mL) contained 0.5 mL each of dye solution, mediator, buffer and enzyme (58.3 nkat). The reaction time was 30 min

1-Hydroxybenzotriazole (HOBT) is one of the most intensively researched mediators, which is oxidized to its nitroxide radical by laccase (Bourbonnais et al., 1998; Potthast et al., 1999). The decolourization of dyes by laccase in the presence of HOBT is given in Fig. 6.15. Unlike that of ABTS, the maximum decolourization was observed at 2 mM of HOBT. The results of the present study indicated that HOBT was a better laccase-mediator system than ABTS, for the laccase from *S. psammoticus*. Considerable enhancement in the decolourization of the dyes was observed when laccase-HOBT system was used. 86 % decolourization of dye I was attained with this system. The rate of decolourization for dyes II and III was 71 and 75 % respectively. The improved degradation of the azo dye, methyl orange by laccase-HOBT system has been reported (Zille et al., 2005). In the present

study, it was observed that laccase-HOBT system could decolourize acid orange (dye I) and Bismarck brown (dye III) more efficiently than methyl orange (dye II). The current results on methyl orange decolourization could be explained on the basis of the earlier observation that there exists remarkable difference in the decolourization efficiency of laccases from different sources towards a particular dye (Nyanhongo et al., 2002).

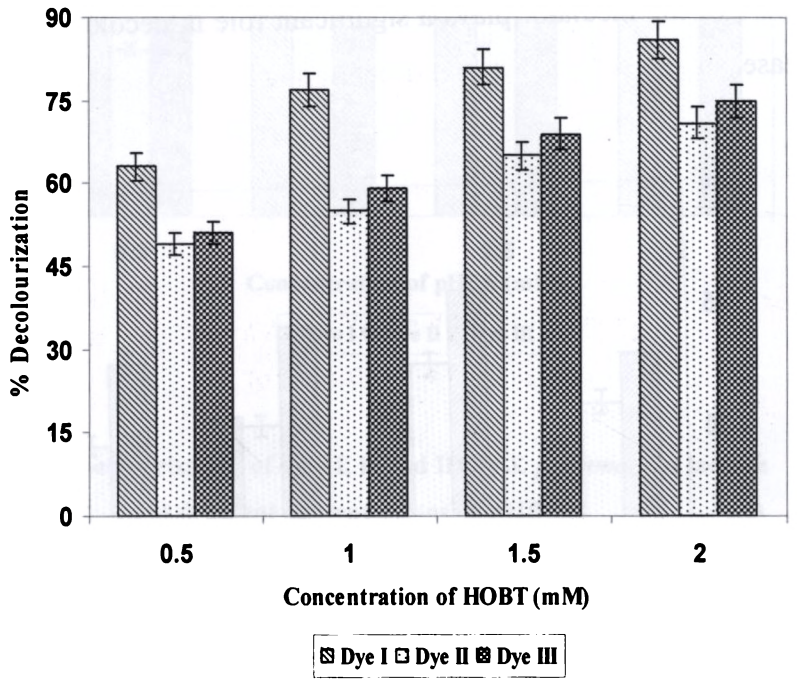


Fig. 6.15. Decolourization of dyes I, II and III by *S. psammoticus* laccase at different concentrations of HOBT

The reaction mixture (2 mL) contained 0.5 mL each of dye solution, mediator, buffer and enzyme (58.3 nkat). The reaction time was 30 min

The efficiency of aniline and PHBA to act as laccase-mediators was also investigated in the study. Both these compounds enhanced the decolourization of dyes in an effective way as compared to the non-mediated system although,

the efficiency of these compounds were less than that of ABTS and HOBT. The rate of decolourization achieved with laccase-aniline system and laccase-PHBA system was given in Fig. 6.16 and Fig. 6.17 respectively. Figure 6.16 shows that 1 mM concentration of aniline resulted in better decolourization of dyes while the concentration of PHBA required for maximum decolourization was 2 mM. PHBA proved to be a better mediator than aniline as it was evident from the decolourization rates achieved (Figure 6.17). The results indicated that nature of the mediator plays a significant role in decolourization of dyes by laccase.

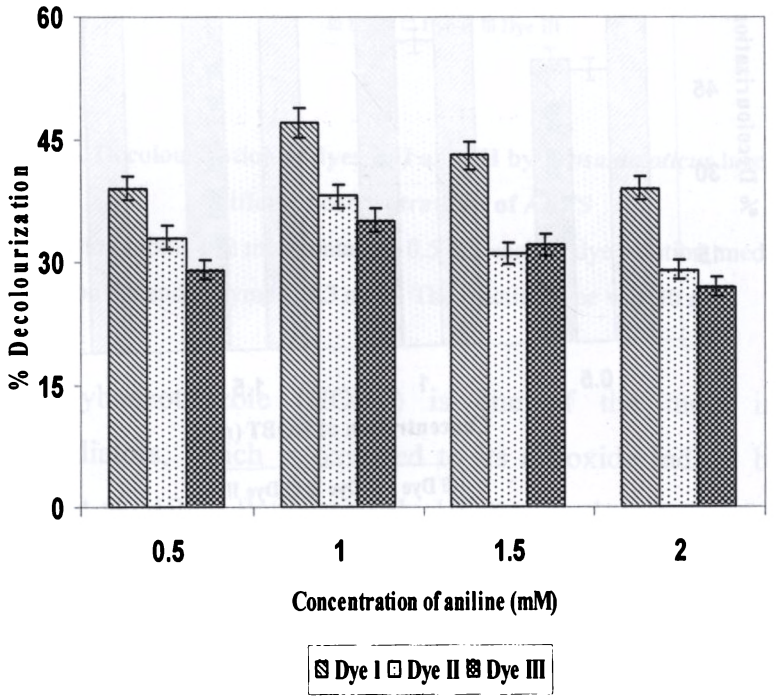


Fig. 6.16. Decolourization of dyes I, II and III by *S. psammoticus* laccase at different concentrations of aniline

The reaction mixture (2 mL) contained 0.5 mL each of dye solution, mediator, buffer and enzyme (58.3 nkat). The reaction time was 30 min

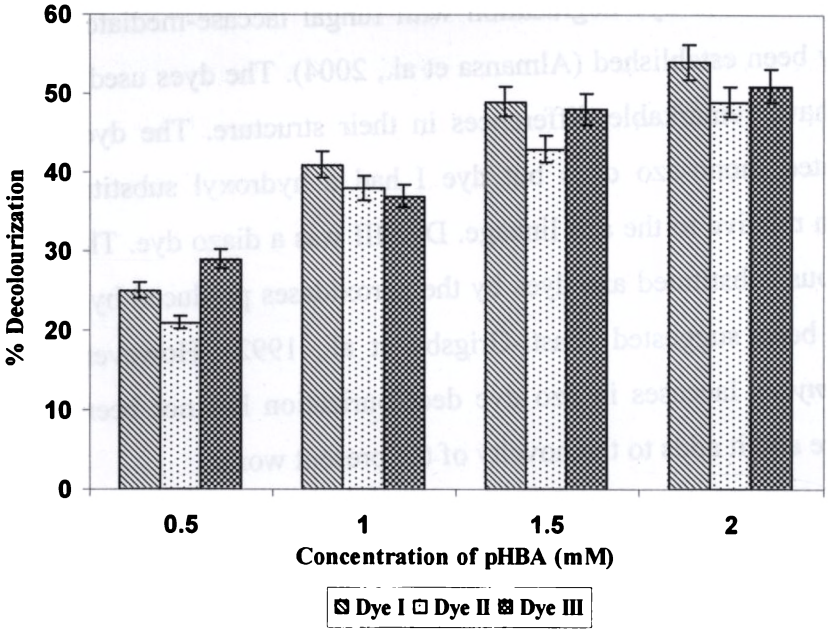


Fig. 6.17. Decolourization of dyes I, II and III by *S. psammoticus* laccase at different concentrations of *p*HBA

The reaction mixture (2 mL) contained 0.5 mL each of dye solution, mediator, buffer and enzyme (58.3 nkat). The reaction time was 30 min

The overall picture of azo dye decolourization in the presence and absence of mediators indicated that the decolourization ability of *S. psammoticus* laccase could be improved significantly with the use of mediators. Five fold increase in the decolourization of dye I was obtained in the presence of the best mediator (HOBT) (Fig. 6.15) as compared to the non-mediated system (Fig. 6.13). Similarly, six fold increase in the decolourization of dye II and seven fold increase in the decolourization of dye III were attained with HOBT (Fig. 6.15) as compared to that obtained in the absence of mediator (Fig. 6.13). Despite the nature of mediator used, the dye I (acid orange) was decolourized more efficiently and the same pattern was observed

even in the absence of mediator. This indicated that the structure of dye is an important factor that determines the rate of decolourization. Influence of structure on azo dye degradation with fungal laccase-mediator systems has already been established (Almansa et al., 2004). The dyes used in the present study have remarkable differences in their structure. The dye I and II are sulfonated mono-azo dyes but dye I had a hydroxyl substitution at ortho position relative to the azo linkage. Dye III was a diazo dye. The degradation of various substituted azo dyes by the peroxidases produced by *Streptomyces* sp has been suggested (Pasti-Grigsby et al., 1992). However, the role of *Streptomyces* laccases in azo dye decolourization has not been cited in the literature and it adds to the novelty of the present work.

6.4. CONCLUSION

The use of microorganisms or their enzyme systems are the two different biological approaches widely practiced for treatment of hazardous chemicals and effluents. Both these strategies have been successfully evaluated during this study. The ability of the *S. psammoticus* in dye and phenol degradation was studied using different dyes and phenolic solution of varying concentrations. Similarly the role of laccase secreted by this organism in pollution abatement was also evaluated in detail by selecting three recalcitrant azo dyes and phenol model solution. The azo dye decolourization was achieved in the presence of different mediator compounds. The present study has successfully evaluated the advantages of using immobilized cells and laccase to degrade selected phenolic compounds. The immobilized enzyme system was successfully reused for the effective degradation of phenolic compounds therby making the entire process inexpensive and reliable.

Chapter 7.

SUMMARY AND CONCLUSION

The thesis includes the experimental methodology and results of optimization and characterization of laccase from an actinomycete strain *Streptomyces psammoticus*. The application of enzyme in pollution abatement was also evaluated with special reference to the degradation of dyes and phenols.

7.1. SUMMARY

- Twenty actinomycete cultures were isolated from the marine and mangrove regions along the west coast of India.
- Six isolates showed positive result in the primary screening and selected for secondary screening by SmF.
- Six isolates produced LiP; four isolates produced laccase and one isolate (NJP 49) produced LiP, MnP and laccase in SmF medium.
- The isolate NJP 49, identified as *Streptomyces psammoticus* was selected as the best strain, based on the enzyme yield.
- Laccase was selected for further work in view of its role in industrial applications.
- Coffee pulp was identified as the best substrate for laccase production in SmF.
- The optimization studies revealed that the laccase production was maximum at pH 7.5 and temperature 32 °C.
- Salinity of the medium was also observed to be influencing the enzyme production. Maximum production was observed in media with 50 % seawater.
- An agitation rate of 175 rpm and 15 % inoculum were the other optimized conditions for maximum laccase yield.
- Pyrogallol and para anisidine proved to be the best inducers for laccase production by this strain.
- Statistical optimization was also done in SmF. Initial screening of production parameters was performed using a Plackett - Burman design.

- The variables with statistically significant effects on laccase production were identified and optimized further by Box-Behnken design.
- The statistical optimization by response surface methodology resulted in a three-fold increase in the production of laccase.
- Optimization of laccase production in SSF was carried out by conventional as well as statistical approaches.
- Rice straw was identified as a suitable substrate for laccase production in SSF, followed by coffee pulp.
- Other optimized conditions under SSF were particle size - 500 – 1000 μm; initial moisture content - 65 %; pH of moistening solution - 8.0; incubation temperature - 32 °C and inoculum size – 1.5 x 10^7 CFU.
- Yeast extract served as the best nitrogen source. No enhancement in enzyme yield was observed with carbon supplementation.
- The level of yeast extract, inoculum size and copper sulphate were optimized statistically using central composite design.
- Statistical optimization resulted in three-fold increase in laccase activity as compared to the unoptimized medium.
- Enhanced production of laccases from *S. psammoticus* in SSF was carried out using two different strategies; laccase inducers and scale-up process.
- Laccase yield was enhanced by a wide range of aromatic inducers. The best inducer was pyrogallol.
- Scale-up studies in packed bed bioreactor was performed at different aeration rates. 1.5 vvm aeration was identified as the optimum condition for laccase production in column bioreactor.
- Fermentation was carried out in bioreactors in the presence of 1 mM pyrogallol, which resulted in 3.9 fold increase in laccase yield.
- Laccase from *S. psammoticus* has been purified to homogeneity through anion exchange and gel filtration chromatography steps with an overall purification fold of 12.1.
- The molecular mass of the purified laccase was about 43 kDa. The pI was 7.9.

- The enzyme was active in the alkaline pH range with pH optima at 8.5 and 97 % activity retention at pH 9.0. The enzyme was tolerant to NaCl concentrations up to 1.2 M.
- The optimum temperature was 45 °C. The enzyme was stable in the pH range 6.5-9.5 and up to 50 °C for 90 min.
- Purified laccase was inhibited by all the putative laccase inhibitors while the enzyme was activated by metal ions like Fe, Zn, Cu, Na and Mg.
- The enzyme showed lowest K_m value with pyrogallol (0.25 mM) followed by ABTS (0.39 mM).
- The purified enzyme was a typical blue laccase with an absorption peak at 600 nm.
- The enzyme was a glycoprotein with a carbohydrate content of 10 %. The enzyme contained 3.2 copper ions per molecule.
- The dye decolourization ability of S. psammoticus was evaluated using ten different dyes. RBBR was effectively degraded by *S. psammoticus*.
- *S. psammoticus* immobilized on PUF removed 89.2 % of total phenolics and 77.2 % COD from the synthetic phenol solution.
- Laccase immobilized on copper alginate beads removed 72 % of the colour and 69.9 % of total phenolics after initial run of 6 h.
- Reusability of the immobilized matrix was studied for up to 8 successive runs, each run with duration of 6 h.
- The degradation of phenolic compounds by immobilized laccase was evaluated and confirmed by thin layer chromatography and nuclear magnetic resonance spectroscopy.
- Four laccase mediators such as ABTS, HOBT, aniline and pHBA were used for mediator based decolourization of azo dyes.
- HOBT was identified as the best mediator for laccase from this strain. Acid orange, Methyl orange and Bismarck brown were decolourized at the rates of 86, 71 and 75 % respectively by HOBT.

7.2. CONCLUSION

The present study has identified an actinomycete culture (*S. psammoticus*) which was capable of producing all the three major ligninolytic enzymes. The study revealed that least explored mangrove regions are potential sources for the isolation of actinomycetes with novel characteristics. The laccase production by the strain in SmF and SSF was found to be much higher than the reported values. The growth of the organism was favoured by alkaline pH and salinity of the medium. The enzyme also exhibited novel characteristics such as activity and stability at alkaline pH and salt tolerance. These two characters are quite significant from the industrial point of view making the enzyme an ideal candidate for industrial applications. Many of the application studies to date are focused on enzymes from fungal sources. However, the fungal laccases, which are mostly acidic in nature, could not be used universally for all application purposes especially, for the treatment of effluents from different industries, largely due to the alkaline nature of the effluents. Under such situations the enzymes from organisms like *S. psammoticus* with wide pH range could play a better role than the fungal counterparts. In the present study, the ability of the isolated strain and laccase in the degradation of dyes and phenolic compounds was successfully proved. The reusability of the immobilized enzyme system made the entire treatment process inexpensive. Thus it can be concluded from the present study that the laccase from this organism could be hopefully employed for the eco-friendly treatment of dye or phenol containing industrial effluents from various sources.

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