STUDIES ON THE MICROBIAL PRODUCTION OF LACCASE BY *ASPERGILLUS FUMIGATUS*

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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JUNE, 2008

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **STUDIES ON THE MICROBIAL PRODUCTION OF LACCASE BY *ASPERGILLUS FUMIGATUS*** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2003 to June 2008 under the supervision of Dr. R. P. Singh, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

The matter embodied in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Vivekanand

(VIVEKANAND)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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Date: 27 June, 2008

The Ph.D. Viva-Voce examination of **Mr. Vivekanand**, Research Scholar, has been held on .....

Signature of Supervisor

Signature of External Examiner

ABSTRACT

The present investigation was undertaken to isolate a potent microorganism with laccase production ability and to develop and economize the process by employing agro-horticultural residual resources.

The persuasive fungal strain secreting laccase was isolated by scrupulous survey of various sites. The strain VkJ was observed to have remarkable levels of laccase production and identified as *Aspergillus fumigatus* from Indian Agricultural Research Institute, New Delhi.

To achieve an improvement in the enzyme production ability of the selected strain, physical, chemical and mixed mutagenesis of the strain was attempted. Double stage mutagenesis by UV irradiation led to the development of strain VkJ2.4.5 having notably higher levels of laccase production. A detectable level of manganese peroxidase production was also observed. Mutagenized strain *A. fumigatus* VkJ2.4.5 had discrete morphological features. The mycelia of the mutant VkJ2.4.5 were loose and dirty white as compared to the wild type VkJ strain which had compact and white mycelia. Surface, submerged and solid-state fermentation systems had been evaluated for laccase production from the mutant VkJ2.4.5 strain that demonstrated that level of enzyme production was higher in solid-state fermentation. Among the six different solid supports viz., wheat straw, wheat bran; banana peel, bagasse, rice bran and poplar leaves; banana peel led to higher levels of enzyme production. In order to further enhance the enzyme production levels; amount of substrate, moisture level, incubation period, aeration volume and additives were evaluated. An attempt to scale up the production was made by designing a column-tray bioreactor using the derived conditions. In successive batches of fermentation same fungal mycelia and additional nutrients were utilized to accomplish maximal production levels in an economic manner. The banana peel employed, was observed to be the best substrate that led to remarkable productivity; since fungi could anchor efficiently and additional nutrients appeared supporting growth and enzyme production.

Biobleaching efficiency of the developed enzyme preparation was evaluated for mixed wood pulp in XCEHH and XODED sequences. Analysis of FTIR spectra of residual lignin revealed characteristic modifications following enzymatic bleaching. Variations in morphology and crystallinity of pulp were evaluated by scanning electron microscopy and X-ray diffraction analysis. The enzyme pretreatment led to decrease in kappa number, yellowness, AOX and COD. Additionally, it improved the mechanical and optical properties of the paper along with reduction in the chlorine consumption which in turn diminish the pollution denoting it therefore to be an ecofriendly and benign bleaching technology.

ACKNOWLEDGMENT

Today, when I am at the verge of attaining another milestone of my life in the form of this thesis, I look back and see a number of helping hands who supported me to reach at this position. I wish to take this opportunity to convey my heartiest gratitude to them for their support, encouragement and cheers.

I am earnestly thankful to my supervisor Prof R P Singh for his decisive guidance, indispensable suggestions, critical analysis of the work and also for providing his valuable time in discussions leading to shape the whole project to its successful completion. As a teacher, I always found him as a source of inspiration and enthusiasm for my entire doctoral work. I am thankful to him for his constant efforts to inculcate the scientific temperament in me for making myself a better researcher. I thank him for providing me a healthy, professional and unconditional support with free atmosphere in the lab for the scientific progress, which is really exceptional. I am grateful to you Sir for everything you did for me.

I pay my regards to Late Prof Ben M J Pereira, Former Head, Department of Biotechnology for providing me necessary research facilities and an excellent working environment in the department. I would also like to thank Prof Ritu Barthwal, Prof G S Randhawa, Prof H S Dhaliwal, Dr R Prasad, Dr V Pruthi, Dr A K Sharma, Dr P Roy, Dr P Kumar, Dr B Chaudhary, Dr S Ghosh, Dr N K Navani and Dr R Pathania for their valuable suggestions and encouragement. I express my gratitude and respect to Dr. Dharm Dutt, Associate Professor, Department of Paper Technology, Saharanpur Campus, IIT Roorkee; who persistently inspired me all along the application work in spite of his busy schedule. I've deep and sincere sense of gratitude towards Dr. J S Upadhyaya, Head, DPT, for his constant encouragement and providing all in-house research facilities and excellent working environment. I also extend my sincere thanks to Mr Ratnesh Kumar, Mr Arora, Mrs Bhatjiwal, Mr Harpal and Mr Rakesh Kumar for their constant support at DPT, Saharanpur Campus.

I was blessed with my seniors and wish to pay my regards to, Dr Ruma Ganguly, Dr Nidhi Sabharwal, Dr Amit Sharma, Dr Nand, Manish Rana and Sujeet Kumar (JNU New Delhi) for training with techniques required for the experiments and for improving my experimental skills. I can never forget the moments I spent with them during my initial days of stay at IIT Roorkee.

I wish to convey my special thanks to Pallavi and Nidhi for being so nice to me. I wish to thank them for being caring, supportive and always accommodating with me. Because of them only I could spend a

very cheering and encouraging time at IIT Roorkee. I am grateful to them for always being with me and providing me the confidence to go ahead in my lean times. I also thank Durga Prasad, Anindita, Seema, Alok, Swarnima, Shalini, Harjeet, Mohan lal and Rajni for always making me cheer.

I would like to convey my sincere thanks to Mr. Arora, Mr. Lokesh, Mr. Anand, Mrs. Surita, Mrs. Shashi, Mr. Saini, Riayaz ji and Giri ji for their assistance throughout the research period.

I would like to extend my sincere thanks to friends for being with me. Their indirect support has made this journey so congenial and easy. I thank Sandeep, Vishal, Saurav and my TT group for always being in touch with me. I am thanking them for their memorable concern, affection and constant care. I convey my thanks to Sangam, Vipin, Govind and Amit for being so close and helpful to me.

I can never be able to express the gratefulness which I wish to pay for my respected papa and amma for their cheerful, enthusiastic and dedicated efforts to engage myself in higher pursuits. Their constant care and support has shaped me to become eligible for reaching at this position. I regard them as my Ideal and Strength. I wish to extend my thanks to my brother and sisters for making my life so lively and happy. Finally, there is no word to express my feelings for a special person sitting miles apart from me who had always with me in my pinks and blues to support and encourage me during the needy periods of this work, thanks Manisha for your cooperation, inspiration and affection that made me able to achieve this task.

A word of special thanks to Ma'am, Mrs. Pratima Singh for her kind affection and encouraging thoughts to achieve this destination. I never missed my home during my stay at Roorkee. The words will never be enough to convey my gratitude for every immense help provided to me in all my needs.

I would like to thank Almighty God for blessing me the company of such nice people around me and for giving all the strength and patience to face various colors of life. Also, I sincerely apologize before them who have helped me reaching this stage but I forgot to mention their contribution. I believe, they will be always with me as they were there in the time of need.

Last but not the least; I am thankful to IIT Roorkee for providing me MHRD Institute Assistantship and AICTE New Delhi for awarding the National Doctoral Fellowship for my entire doctoral work.

Vivekanand

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ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt
ANOVA	Analysis of Variance
APPL	Acid Precipitable Polyphenolic Lignin
AOX	Adsorbable Organic Halide
BOD	Biological Oxygen Demand
bp	Base pair
b-θ	Number of block
COD	Chemical Oxygen Demand
^{o}C	Degree Celsius
Cl	Chlorine
df	Degree of freedom
DMAB	3-dimethylaminobenzoic acid
DNA	Deoxyribonucleic acid
DNS	Dinitro Salicylic Acid
DTT	Dithiothreitol
ECF	Elemental Chlorine Free
FTIR	Fourior Tranform Infrared Spectroscopy
g	Gram
h	Hour
HBT	N-hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
IAA	Iodoacetamide
IU	International unit
ISO	International Organization for Standardization

K-θ	Number of Treatment
kb	Kilobase
l	Litre
MALDI ToF/ToF	Matrix Assisted Laser Desoption/Ionisation Time of Flight
MBTH	3-methyl-2-benzothiazolinone hydrazone hydrocholoride
MEA	Malt Extract Agar
µg	Microgram
min	Minute
ml	Millilitre
mm	Millimeter
N	Number of replicates
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PDA	Potato Dextrose Agar
rpm	Revolution per minute
SEM	Scanning Electron Microscope
SS	Sum of Square
SSE	Error sum of square
SST	Treatment sum of square
TNT	Trinitrotoluene
TPA	Tons per annum
TCF	Total Chlorine Free
TLC	Thin Layer Chromatography
TOX	Total Organic Halide
UV	Ultra Violet
v/v	Volume per Volume
w/v	Weight per Volume

w/w	weight per weight
XRD	X-ray Diffraction

Chapter I

Introduction

INTRODUCTION

The pulp and paper industries play significant role for the development of modern civilization. Pulp and paper production has increased globally and will continue to do so in near future. Today, the paper industry accounts for about 2.5% of world's total production and to 2% of world trade. The overall paper consumption in India has touched to about 4.2 million tons in the year 2000, thus making India a bigger market for industries to flourish in near future. As on date, there are more than 600 pulp and paper industries in India manufacturing nearly 5.5 million tons of paper and paper board and about 0.68 million tons of newsprint. The projected demand for paper, paper board and newsprint is expected to go upto 8.3 million tons by the year 2010 that may be fulfilled by utilization of different raw materials such as hard and soft wood, sugarcane bagasse, cereal straws (wheat and rice), grasses and cotton stalks etc.

Pulping is an essential step during paper manufacture which involves rupturing of bonds within the wood structures to separate the fibres. There are many processes such as mechanical, chemical, thermomechanical and chemimechanical for pulping and nowadays up to 70% of the total pulping is carried out by chemical means where wood chips are cooked in an aqueous chemical solution (NaOH, Na_2S, Ca $(OH)_2$, $MgSO_4$) at high temperature and pressure. To get whitened pulp for manufacturing of high quality paper, pulping should be followed by bleaching which is also a chemical process applied to lignocellulosic materials for achieving the desired level of brightness. Lignin, responsible for colour of pulp and also for holding cellulose fibers together is attacked and dissolved during bleaching. The removal of lignin and hemicellulosic components is crucial as these are major chromophoric materials present in plant cell wall and their elimination is essential for defibrillation of pulp. Lignin, responsible for maintaining the structural integrity of wood is dissolved and removed by exposing it to harsh physiochemical treatments during multistage bleaching sequences using the combination of chlorination and alkaline-extraction steps. This results into the release of a range of chloro-aromatic derivatives

from the lignocellulosic biomass that is ecotoxic and cause irreparable damage to the environment. Apart from this, pulp and paper industries also generate large volumes of effluents rich in organochlorine compounds (Adsorbable Organic Halide - AOX) which are ecotoxic, mutagenic, persistent, bioaccumulating and with multiple adverse impacts to the biological systems. The ever increasing pressure from environmental protection authorities as well as stringent laws imposed by the government, therefore, has forced the pulp and paper industries to seek an environmentally benign bleaching technology.

Vigorous efforts are in progress to minimize the use of chlorine during bleaching which has compelled industries to explore and develop alternative bleaching technologies that may be less polluting and eco-friendly. Alternative bleaching technologies have been developed involving replacement of elemental chlorine with chlorine dioxide, leading to elemental chlorine free bleaching (ECF) and with complete removal of chlorine, termed as total chlorine free (TCF) bleaching. These processes use oxygen, ozone and peroxide etc., but these seem economically unviable, since these require numerous modifications in the infrastructure and hence are viable particularly to large paper mills.

Biobleaching is a promising alternative for eliminating chlorine based chemicals from the bleaching process. Biobleaching involves the use of microorganisms and/or ligninolytic and hemicellulolytic enzyme systems. Discovery of oxidative enzymes of ligninolytic system provide a biological substitute to conventional bleaching which is technically attractive and less intrusive to the environment. Three major extracellular oxidative enzymes that are implicated in lignin degradation are laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP). The perception of lignin degradation was changed from an oxidative depolymerisation process caused by a single enzyme, to a process of intensive oxidative and reductive conversions in which different enzymes of ligninolytic family can participate. Laccase has been identified as one of the key enzyme that plays a major role in lignin degradation. It catalyzes monoelectronic oxidation of substrates at the expense of molecular oxygen and produces water as the only by-

product. Laccase attacks only phenolic subunits of lignin, but its substrate range can be extended to non-phenolic subunits by inclusion of a mediator (Laccase-mediator system). The oxidation of phenolic compounds result into generation of phenoxy radicals while non-phenolic compounds are oxidized into their corresponding cationic radicals. Laccases are mostly extracellular glycoproteins that contain four copper atoms in their structure. Expanding knowledge of laccase has enabled the attempts to incorporate this enzyme for use in other industrial sectors. Along with laccase, MnP has also been observed to be involved in lignin degradation during biobleaching. Furthermore, it has been found that laccase and MnP may act synergistically to affect an increasing and accountable level of lignin degradation.

Various reports are available for the production of laccase mainly from brown and white rot fungi as well as from bacteria. Furthermore, there has been a growing interest in analyzing the lignin modifying enzymes from wide array of fungi, not only because of comparative biology reasons but also to look for economical and superior lignin degrading system for employing in various biotechnological applications. Many fungi from ascomycetes group have been reported to secrete ligninolytic enzymes. The isolated strain *Aspergillus fumigatus* was found to be capable of producing notable levels of laccase and at the same time generated MnP which are of prime importance for biobleaching. A synergistic action of both laccase and MnP may lead to the effective degradation of lignin, therefore, microorganisms possessing both the activities may be promising for achieving the same. Keeping in view of the above, the present study was undertaken to critically review the parameters that are crucial for the production of the secondary metabolite mainly laccase. Attempt was made to make the production economical by evaluating the fermentation systems and low cost substrates employed for the process of production. The major outlines of the work are defined as below:

a. To isolate the microbial strains from different natural sites; screening and evaluation of strains for laccase production.

b. Attempting an enhancement in laccase production of the wild type isolate by

physical, chemical and mixed mutagenic treatments.

c. Analysis of the fermentation systems mainly surface, submerged and solid-state systems for production; evaluation of critical parameters of the selected system for achieving maximum levels of production.

d. Biochemical characterization and evaluation of the enzyme preparation for bleaching applications in the pulp and paper industry.

e. Designing of a process for scaled up production of the enzyme.

Chapter II

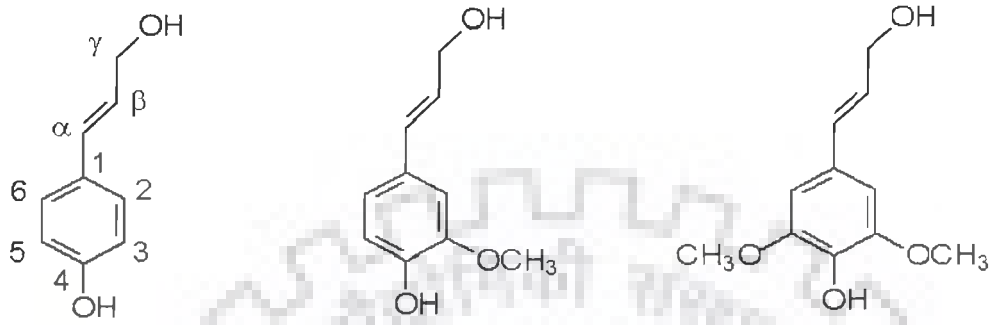
Literature Review

LITERATURE REVIEW

Lignocellulosic biomass from plants, which primarily consists of cellulose, hemicellulose and lignin, is a renewable source for food, energy and chemicals. It accounts for the major end product of photosynthetically fixed carbon. Lignin component of the lignocellulosic biomass is considered among the highly recalcitrant natural products in the biosphere and thus its bioconversion is among the most important and intricate processes occurring in the carbon cycle (Thompson *et al.*, 1998; Brumer *et al.*, 1999). Lignocellulosic material is produced in huge amounts by several agricultural practices, forestry and in the naturally occuring vegetation. Lignin content of angiosperms (hardwood) varies between 20% and 25% and in gymnosperms (softwood) between 28% and 32%. It is found in higher concentrations in middle lamella and also an essential part of the plant cell wall, imparting rigidity and protecting the easily degradable cellulose from the attack of pathogens. Lignin is an aromatic and amorphous polymer which is synthesized from phenyl propanoid precursors by polymerization in higher plants. There are three monomers that make up almost all lignin found in nature. The precursors of lignin viz. *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol consists of an aromatic ring and a 3-carbon side chain (Fig. 1), *p*-coumaryl alcohol is a minor component of grass and forage based lignins. Coniferyl alcohol is the predominant lignin monomer found in softwoods. Both coniferyl and sinapyl alcohols are the building blocks of hardwood lignin. The structure and chemical composition of wood greatly influences its degradation by microorganisms. The accessibility of enzymes to fibers is limited due to numerous factors such as adsorption to surface area and low fiber porosity (Eriksson *et al.*, 1990; Kuhad *et al.*, 1997; Eriksson 2000). In addition, molecular organization of different components also limits the accessibility of microorganisms and their enzymes to wood and its fiber components and hence the degradation of lignin is a significant step in the global carbon cycle (Crawford and Crawford, 1980).

2.1 Lignin : structure, biosynthesis and function

Lignin is a polymer of phenylpropanoid units synthesized from three precursor alcohols (Fig.1). These are *p*-coumaryl alcohol, which gives rise to *p*-hydroxy phenyl units in the polymer; coniferyl alcohol, the guaicyl units; and sinapyl alcohol, the syrigyl units.



***p*-Coumaryl alcohol** **Coniferyl alcohol** **Sinapyl alcohol**

Fig. 1 Monomers of lignin

These alcohols are products of a long biosynthetic pathway starting from photosynthesis and leading via the shikimic acid pathway to L-phenylalanine, then by the elimination of ammonia, transcinnamic acid is formed, which is then converted to *p*-coumaric, ferulic and sinapic acids via successive hydroxylation and methylation reactions (E'riksson *et al.,* 1990; Thurston, 1994; Eriksson, 2000). During cell wall thickening, free radical condensation of these precursors is initiated by plant cell wall peroxidases and results in the formation of a heterogeneous, amorphous, optically inactive, random and highly branched polymer-lignin (Fig. 2).

Lignin has several important functions in the cell wall matrix of plants. It strengthens the cell wall structure, lending therefore the mechanical support to the plant. It also serves as a barrier to protect the plant against microbial attack. The low permeation property of lignin plays an important role in the internal transport of water, nutrients and metabolites in the plant (Eriksson *et al.,* 1990; Kuhad *et al.,* 1997, Eriksson, 2000).

However, lignin that contributes strength, stability, and hydrophobicity to lower plants and trees imposes challenge to mankind and environment in many ways; (i) in the

bleaching of wood pulp, residual lignin is removed from the polysaccharide components of wood using chlorine and chlorine derivatives. This results in the generation of ecotoxic constituents creating environmental hazards; (ii) lignin is also an obstacle for efficient bioconversion of cell-wall polysaccharides to simple sugars and limits its digestibility by ruminants (Eriksson, 2000). The structural features of lignin dictate unusual constraints on biodegradative system responsible for initial attack. Thus, for its degradation, ligninolytic enzyme systems must be extracellular and nonspecific (Eriksson *et al.*, 1990; Thurston, 1994; Eriksson, 2000; Saparrat *et al.*, 2002).

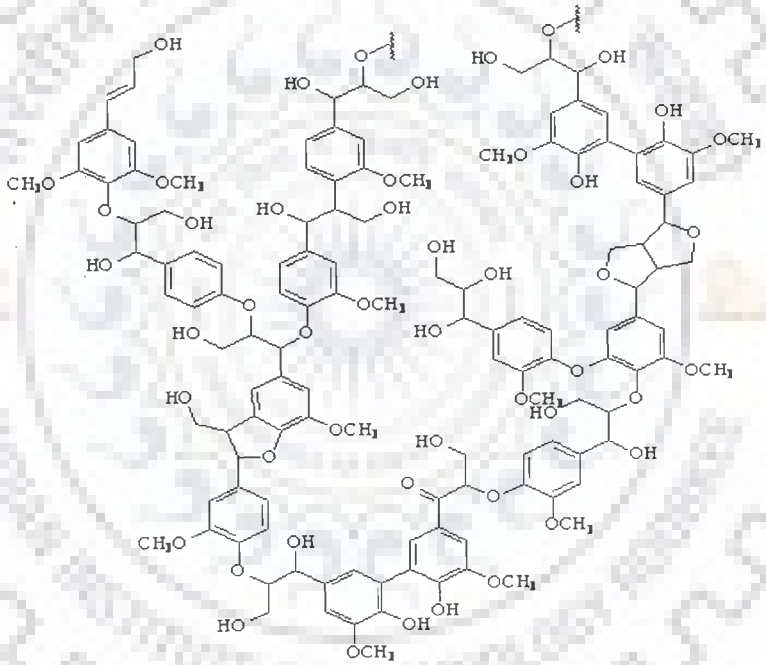


Fig. 2 Structure of lignin

2.2 Microbial and enzymological aspects of lignin degradation

2.2.1 Lignin degrading microbes

There is a variety of organisms which are involved in lignin degradation. These include bacteria, actinomycetes and fungi. Each lignin degrading microbial strain attacks lignin in its own specific way, although it is known that patterns of attack on the lignin structures are often

similar within a specific group of decaying organisms (Crawford and Crawford, 1980; Eriksson *et al.,* 1990; Kuhad *et al.,* 1997; Eriksson, 2000).

2.2.1.1 Lignin degradation by bacteria

Majority of reports on lignin degradation by bacteria are based on dimeric compounds as substrates (Gurujeyalakshmi and Mahadevan, 1987; Ruttimann *et al.,* 1987; Vicuna *et al.,* 1993) and evidence supporting mineralization has not been reported (Vicuna *et al.*, 1993). Cell suspensions of *Methylosinus trichosporium* have been reported to oxidize the aromatic alcohols; benzyl alcohol, vanillyl alcohol, veratryl alcohol to their corresponding aldehydes and with the exception of vanillyl alcohol, the aldehydes were further oxidized to the corresponding aromatic acids (Mountfort, 1990). The ability of *Serratia marcescens,* isolated from a compost heap, attacking kraft lignin as well as natural and synthetic radiolabeled lignin was examined by Perestelo *et al.,* (1989). *S. marcescens* was found to degrade upto 15% lignin when incubated with glucose. The utilization of various lignin, indulin and lignin-model substances was also reported by *Acinetobacter sp.* (Vasudevan and Mahadevan, 1992). *Alcaligenes paradoxus*, isolated from a bacterial consortium, was capable of degrading the lignocellulose complex and was also able to grow on veratric and vanillic acids. A strain of *Bacillus* sp. isolated from decaying coir, utilized up to 0.25% indulin as the sole carbon source in 24 h (Uma *et al.,* 1994).

Laccase activity was also been detected in the *Azospirillum lipoferum* and *Bacillus subtilis* (Givaudan *et al.,* 1993; Hirose *et al.,* 2003; Durao *et al.*, 2006). The bacterial laccase from *Azospirillum lipoferum* was purified and characterized (Diamantidis *et al.,* 2000). More recently, CotA protein of *Bacillus subtilis* was demonstrated to be a laccase (Hullo *et al.,* 2001). Chandra *et al.,* (2007) had reported that three potential bacterial strains, *Paenibacillus* sp, *Aneurinibacillus aneurinilyticus* and *Bacillus* sp that were capable of effectively degrading the kraft lignin, a major byproduct of the chemical pulping process and major contributor to the colour and toxicity of effluent. Yang *et al.,* (2007) had observed biodegradation of three

different wood chips by *Pseudomonas* sp PKE117 and appear to be a potential bioagent for biopulping.

2.2.1.2 Lignin degradation by actinomycetes

The main impact of actinomycetes on lignin is its solubilization, whereas mineralization to carbon dioxide was much lesser (Eriksson *et al.,* 1990). Wang *et al.,* (1990) had reported that *Streptomyces lividdns* TK23-3651, a genetic variant, had enhanced extracellular peroxidase and hydrogen production. Actinomycetes degrade lignin and released lignin-rich water soluble fragments called acid precipitable polyphenolic lignin (APPL). APPL is not further metabolized by actinomycetes and APPL reduction seems to be a unique property of strains belonging to *Streptomyces* sp and *Thermomonospora* sp (Eriksson *et al.,* 1990; Vicuna *et al.,* 1993; Kuhad *et al.,* 1997). Recently, ability of three *Streptomyces* strains for degrading alkali-lignin, produced from the treatment of wheat straw, was examined by Hernandez *et al.,* (2001). The degradation rate of lignin and growth of an alkalophilic ligninolytic strain was also studied. The highest activities of laccase and MnP obtained were 2915.37 Ul^{-1} (4 day) and 1152.88 Ul^{-1} (8 day), 49.84% of lignin of wheat straw was degraded following ten days of cultivation (Zhang *et al.,* 2002). Recently, degradation products from the addition of extracellular enzymes from *Thermomonospora* BD25 to ball-milled wheat straw, oat spelt xylan and solubilized kraft pulps were characterized by HPLC and TLC. The results highlighted the ability of culture supernatant from *Thermomonospora fusca* BD25 to release both simple sugars and aromatic compounds from lignocellulosic substrates and suggested a role for this organism in the biobleaching of pulp (Tuncer and Ball, 2002). *Thermobifida cellulolytica* has been found to decompose lignocellulose (Kukolyav *et al.,* 2002). Kraft pulp biobleaching by *Streptomyces cyaneus* CECT 3335 laccase mediated oxidation of nonphenolic substrates was studied by Arias *et al.,* (2003).

2.2.1.3 Lignin degrading fungi

Fungi are the potential degraders of lignin. Among the wood degrading fungi; soft-rot, brown-rot and white-rot fungi along with ascomycetes have been reported which may be able to degrade the wood.

2.2.1.3.1 Soft-rot fungi

Soft-rot fungi are more conspicuous on the surface of wood and degrade lignin quite slowly and incompletely. The soft-rot fungi have also been reported to attack aromatic moieties of lignin (Haider and Trajonowski, 1975). However, soft-rot fungi mainly cause demethylation of lignin and degrade the side chains and aromatic rings to a lesser extent. The rate and extent of decay by soft-rot fungi depends on the type of substrate they attack. Lignocellulose decay of a saltmarsh grass by ascomycetous fungi was studied by Newell *et al.*, (1996). *Phaeosphaeria spartinicola, Buergenerula spartinae, Phaeosphaeria spartinae* and *Passeriniella obiones* are the predominant soft rot fungi isolated and have role in lignin degradation.

2.2.1.3.2 Brown-rot fungi

Brown-rot fungi prefer coniferous wood. These are mainly humefiers, causing only limited changes in lignin. They decompose wood by removing the carbohydrates extensively and leaving lignin in a modified but undegraded state. They do not cleave lignin aromatic rings efficiently or if they do open the rings, they are unable to make significant decomposition in the resulting lignin fragments (Crawford and Crawford, 1980). The most studied brown-rot fungi are *Lenzites, Piptoporus betulinus, Trametes quercina, Poria placenta* and *Fomitopsis pinicola.* In advanced stages of degradation, the residual material left is brown mass, which mainly consists of lignin. The limited changes in lignin by brown-rot fungi involve demethoxylation, aromatic hydroxylation and limited side-chain oxidation (Kuhad *et al*, 1997). Delignification of eucalyptus hardwood by brown-rot fungi has been studied by Ferraz *et al.,* (2000). Ring-U-^{14}C-labelled benzaldehyde and benzoic acid were effectively mineralized by *Tyromyces palustris* and *G. trabeum,* thus indicating that the brown-rot basidiomycetes are capable of

metabolizing certain aromatic compounds to CO_2 and H_2O, despite the fact that brown-rot fungi cannot degrade polymeric lignin (Kamada *et al.*, 2002).

2.2.1.3.3 White-rot fungi

White-rot fungi are capable of effectively degrading all the major components of wood to carbon dioxide and water (Eriksson, 1993; Kuhad *et al.*, 1997; D' Souza *et al.*, 1999; Saparrat *et al.*, 2002). These fungi grow on diferent types of hard and softwoods and degrade lignin to varying extents. Fungi such as *Phanerocheate chrysosporium* and *Trametes versicolor* have a non selective or simultaneous mode of wood degradation i.e., they degrade lignin, cellulose and hemicellulose simultaneously while others such as *Cyathus stercoreus* and *Pycnoporus cinnabarinus* degrade lignin components more selectively (Sethuraman *et al.*, 1999; Geng and Li, 2002). Different white-rot fungi which have shown potential for lignin degradation include *Phlebia radiata* (Niku-Paavola *et al.*, 1988), *C. stercoreus* (Sethuraman *et al.*, 1999), *Cythus bulleri* (Vasdev *et al.*, 1995; Dhawan and Kuhad, 2002), *P. cinnabarinus* (Eggert *et al.*, 1996, 1997), *Cythus stercoreus* (Sethuraman *et al.*, 1999), *Ganoderma lucidum* (D'Souza *et al.*, 1999), *Flavodon flavus* (Raghukumar and Rivonkar, 2001) and *C. rigida* (Saparrat *et al.*, 2002).) Production of lignocellulose degrading enzymes and degradation of *Quercus petraea* (oak) leaf litter by saprotrophic basidiomycetes has been studied (Steffen *et al.*, 2007; Valaskova *et al.*, 2007). Biological pretreatment with white-rot fungi has shown potential for improving enzymatic hydrolysis of wood and grass. Zhang *et al.*, (2007) had evaluated the biological pretreatment of bamboo culms (*Phyllostachys pubescence*) with white-rot fungi *Echinodontium taxodii* 2538 and *Trametes versicolor* G20. Jonathan *et al.*, (2008) had studied biodegradation of four economically important Nigerian tree wood wastes by *Pleurotus tuber-regium* in solid-state fermentation. Reduction in lignin was observed in wood with the increase in period of incubation.

2.2.2 Enzymology of lignin degradation

Although the recalcitrant nature of the lignin impedes its easy conversion, environmental factors and biological systems can transform lignin to varying levels. Four main enzymes have been implicated in the degradation of lignin: laccase, lignin peroxidase (LiP), managanese peroxidase (MnP) and H_2O_2 producing enzymes.

2.2.2.1 Lignin peroxidase (LiP, E.C. 1.11.14)

Lignin peroxidase is a heme containing glycoprotein differentiated from the other ligninolytic enzymes by its ability to oxidize veratryl alcohol as a substrate (Linko, 1992). Producton of lignin peroxidase has been observed from *Bjerkandra* sp strain BOS55 (Mester and Field, 1997); *Phanerochaete chrysosporium* (Zacchi *et al.*, 2000; Fujian *et al.*, 2001); *Irpex lacteus* (Rothschild *et al.*, 2002) and *Phanerochaete sordida* YK-624 (Sugiura *et al.*, 2003). LiP oxidizes its substrates by two consecutive one-electron oxidation steps. Cation radicals are intermediates in these reactions. Compared to other phenol oxidases and peroxidases, LiP has an unusually high redox potential and can oxidize not only phenolic but also non-phenolic, methoxy-substitutcd lignin subunits (Fig.3). The LiP family contains multiple isozymes with a molecular weight range of 38-43 kDa. LiPs are glycoprotein of the oligomannose type with a number of possible O-glycosylation sites and also with one or more N-glycosylation sites (Thurston 1994, Cullen 1997, Conesa *et al.*, 2002). One of the major problems of wild-type lignin peroxidase (LiP) is its inactivity in the presence of excess hydrogen peroxide and high concentration of aromatic compounds.

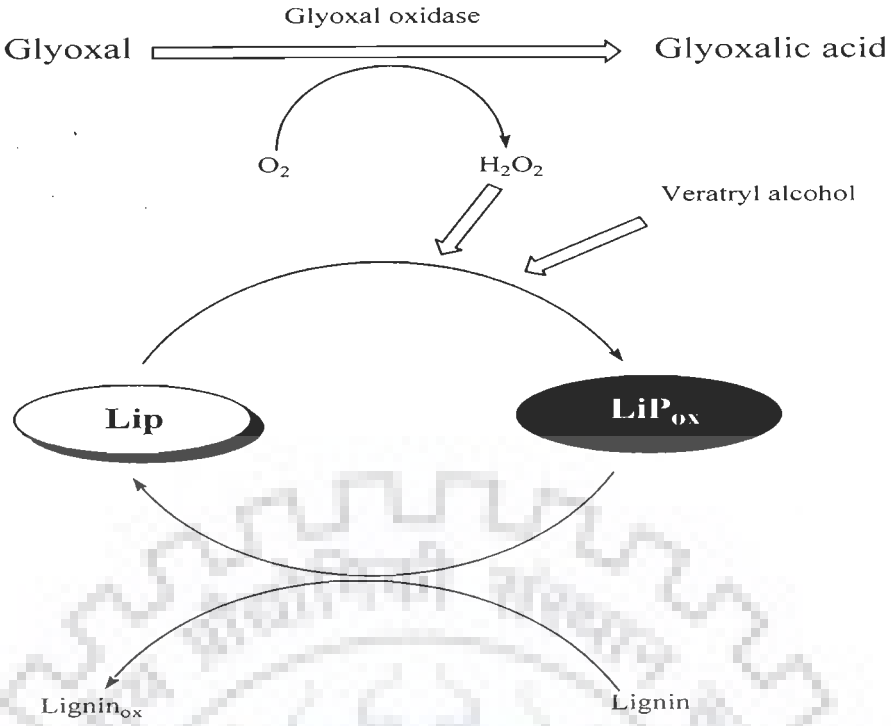


Fig. 3 The catalytic cycle of LiP

2.2.2.2 Manganese peroxidase (MnP, E.C. 1.11.13)

Manganese peroxidases, a common peroxidase among white-rot fungi requires co-substrates hydrogen peroxide and Mn^{2+} for the enzyme activity. It has been proposed that this enzyme uses a similar method for the degradation of lignin as laccase, but instead of using a phenolic substrate to oxidize lignin, it oxidizes Mn^{2+} to Mn^{3+} which in turn oxidizes lignin (Wariishi *et al.*, 1992). The presence of similar peroxidases have also been reported that do not require Mn^{2+} as a co-substrate and these enzymes have been termed as manganese-independent peroxidases (MiP) (Keharia and Madamwar, 2002). Manganese peroxidase (MnP) is heme-containing extracellular fungal peroxidase. It was first identified in ligninolytic cultures of *Phanerochaete chrysosporium* as a Mn^{2+} and H_2O_2 dependent oxidase (Kuwahara *et al.*, 1984) and has also been purified and characterized from other white-rot fungi mainly from *Trametes versicolor* (Paice *et al.*, 1993); *Phlebia radiate* (Lundell and Hataka, 1994); *Bjerkandera* sp strain BOS55 (Moreira *et al.*, 2001); *Phanerochaete sordida* YK-624 (Hirai *et al.*, 2002) and *Physisporinus rivulosus* (Hakala *et al.*, 2005). It oxidizes Mn^{2+} to Mn^{3+} in the presence of a proper chelating agent and Mn^{3+} then should form a complex with the chelator for the oxidation

of phenolic substrates. Organic acids like oxalic, malonic, pyruvic and malic acids are suitable chelators. As depicted in fig. 4 Mn^{3+}/oxalate and Mn^{3+}/malonate form stable chelators. A Mn^{3+} complex can oxidize phenolic lignin substructures by acting as a mediator between the enzyme and the polymer that leads to the formation of phenoxy radicals as intermediates (Kuhad *et al.*, 1997; Call and Mucke, 1997).

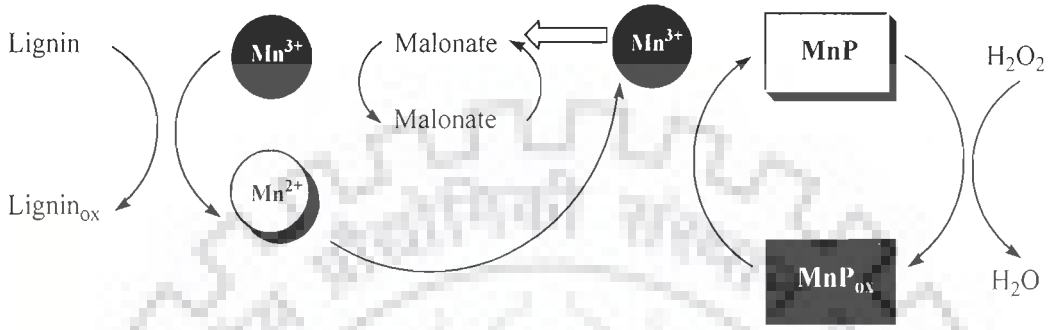


Fig. 4 The catalytic cycle of MnP

Several other extracellular fungal enzymes are produced simultaneously with MnP and appear to work in synergism with this enzyme. Laccase, which coexists in the cultures of few ligninolytic fungi, was shown to work in conjunction with MnP in the degradation of lignosulfonates and solubilization of lignins. The highest degradation rates were obtained when the enzymes (laccase and MnP) work together (Shoemaker and Leisola, 1990). Manganese peroxidase oxidises as well as reduces the xenobiotic compounds involving redox mediators such as Mn^{2+} (Christian *et al.*, 2005).

2.2.2.3 H_2O_2 producing enzymes

Aryl alcohol oxidase has been reported in the cultures of *T. versicolor*, *P. sajar-caju* and *B. adusta* (Muheim *et al.*, 1990; De Jong *et al.*, 1994) and *Pleurotus eryngii* (Varela *et al.*, 1999). The enzyme oxidizes aromatic alcohols to aldehydes and reduces O_2 to H_2O_2. Southern blot screening for aryl alcohol oxidase genes in 30 fungal species by Varela *et al.*, (2000) revealed the presence of the gene in *Pleurotus* sp and *B. adusta*. The aryl alcohol gene from *Pleurotus eryngii* has been cloned and exprcssed in *A. nidulans* (Varela *et al.*, 1999, 2001). Other H_2O_2

producing enzymes include veratryl alcohol oxidase produced by *B. adusta* UAMH 8258 (Pickard *et al.,* 1999 b).

2.2.2.4 Laccase (E.C. 1.10.3.2)

Laccases (E.C. 1.10.3.2 p-benzenediol: phenol oxidase) belongs to a family of multi copper oxidases. Laccases are widely distributed in higher plants, fungi, bacteria and also in some insects. Certain plants such as *Rhus vernicifera* have also been reported to produce laccase (Nitta *et al.*, 2002). *Arabidopsis* has also been reported to produce laccase by Pourcel *et al.*, (2005). Laccases are multinuclear copper-containing glycoproteins belonging to the family of enzymes known as phenol oxidases, more specifically "blue laccases" (Leontievsky *et al.,* 1997). It is a polyphenol oxidase, since it can oxidize phenolic substrates that in turn can initiate the polymerization reaction. Laccases from a range of sources vary greatly with respect to their degree of glycosylation, molecular weight and kinetic properties (Gianfreda *et al.,* 1999). An important feature of fungal laccase is the presence of a covalently linked carbohydrate moiety (10-45%) of the total mass of entire molecule. The four copper binding sites for the catalytic activity per unit of laccases are however conserved.

The role of laccases in lignin degradation has been demonstrated by laccase less mutants of *Pycnoporus cinnabarinus* which had a greatly reduced ability for degrading the lignin (Eggert *et al.,* 1996). It has been observed that laccase activity increased during the formation of fruiting bodies, which indicates a possible function of laccase in their development (Sanchez *et al.,* 2004). Laccase has also been proposed to be involved in sclerotization in insects (Suderman *et al.,* 2006), enzymatic browning (Dijkstra and Walker, 1991), and the formation of humus (Stevenson, 1994, Avallone *et al.,* 2003, Blackwood *et al.,* 2007). Laccase-like enzymes have been reported in bacteria *Azospirillum lipoferum* (Claus, 2003), *Aquificales* and proteobacteria (Alexandre and Zhulin, 2000). Laccases are characterized by low substrate specificity and oxidation of a wide range of phenolic substances, including diphenols, polyphenols, different substituted phenols and aromatic amines. Most laccases reported so far are extracellular

enzymes and differ markedly in their primary sequence, reaction mechanism and in their biochemical characteristics (Thurston, 1994; Mayer and Staples, 2002; Record *et al.*, 2002; Claus, 2003). The discovery of low molecular weight organic compounds acting as mediators (Bourbonnais and Paice, 1990) has significantly expanded the role of laccase in industrial applications.

2.3 Structure and reaction mechanism of laccase

Laccases are extracellular glycoproteins containing four copper atoms that are distributed into three sites- T1, T2, T3. The T1 site have type-1 copper which is coordinated via two His and one Cys residues as ligands. This centre is responsible for substrate oxidation by single-electron step and also for the blue colour of the enzyme. The amino acid histidine (H458) which coordinates the copper in T1, functions as the primary electron acceptor. Type-2 copper is coordinated by trinuclear cluster and involved in the reoxidation of T1 copper. Type 3 copper forms a trinuclear centre coordinated by eight His residues and regulates oxygen reduction by enzyme oxidation by four electron transfer. Laccases occur widely in fungi as constitutive and inducible forms. Recently, the laccase from *Trametes versicolor* and *Pycnoporus cinnabarinus* have been crystallized (Antorini *et al.*, 2002; Bertrand *et al.*, 2002; Claus, 2003, 2004; Mougin *et al.*, 2003).

The reaction mechanism of laccase may be described in terms of the reduction of molecular oxygen. Laccases exhibit high affinity for oxygen as their electron acceptor, however they display a little affinity for their reducing substrates also. Laccases catalyze the oxidation of mono and polyphenolic substrates and aromatic amines by the removal of a hydrogen atom from their hydroxyl group or removal of a single electron, to form a free radical. The radicals are susceptible to further oxidation by laccase or they may undergo non-enzymatic reactions such as hydration or polymerization. Although the exact mechanism of laccase activity has not been elucidated (Burke and Cairney, 2002) but it is believed to comprise of three major steps; in the first step, binding of reducing substrate to the active site and its transformation into free radical

form takes place, then extraction of electrons from the reducing substrates by mononuclear T1 site is done followed by their transfer to the trinuclear T2/T3 site and eventaually reduction of oxygen to water at T2/T3 site takes place and substrate is released. Finally, the radical compounds formed from the substrate are released which may undergo non-enzymatic coupling reactions. They are very reactive and able to interact with themselves or with other compounds present in the vicinity, thus inducing numerous reactions (Claus, 2003, 2004; Mougin *et al.,* 2003). The catalytic mechanism of laccase has recently received considerable attention and several researchers are currently investigating the interaction of laccase with its substrates through crystallography and X-ray diffraction studies (Antorini *et al.,* 2002; Ducros *et al.,* 1998; Piontek *et al.,* 2002).

2.3.1 Mediators

The oxidative action of laccase may be extended to a range of substrates by the addition of low molecular weight chemicals, termed as mediators (Johannes and Majcherczyk, 2000; Fernández-Sánchez *et al.,* 2002). These mediators improve the pertinency of enzyme for bioremediation and several other biotechnological applications. Numerous naturally occurring mediators that are often produced by fungi have been identified. These include phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol (Johannes and Majcherczyk, 2000). The commonly used mediators of laccase for their industrial applications are ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] and HBT [1-hydroxybenzotriazole] (Johannes and Majcherczyk, 2000). Call and Mucke (1997) had reported effective laccase mediator, HBT which is suitable for its applicability for bleaching of pulps at pilot plant scale. Bourbonnais and Paice (1990) discovered that laccase in presence of ABTS, not only degraded phenolic and non-phenolic lignin model compounds but also decreased pulp kappa number and caused an increase in the release of methanol from lignin methoxy groups during bleaching. The structure of ABTS and HBT is shown in fig. 5. The possible mechanism of delignification by laccase mediator system lies in the redox cycles in which oxygen oxidizes the reduced form of laccase to the native laccase, which in turn oxidizes a mediator to produce oxidized mediator species and

the original laccase. Lignin moieties in the residual lignin in pulp then undergo oxidation by the mediator oxidized species, resulting in degradation and dehydrogenative polymerization and reduction of mediator (Fig. 6) (Call and Mucke, 1997).

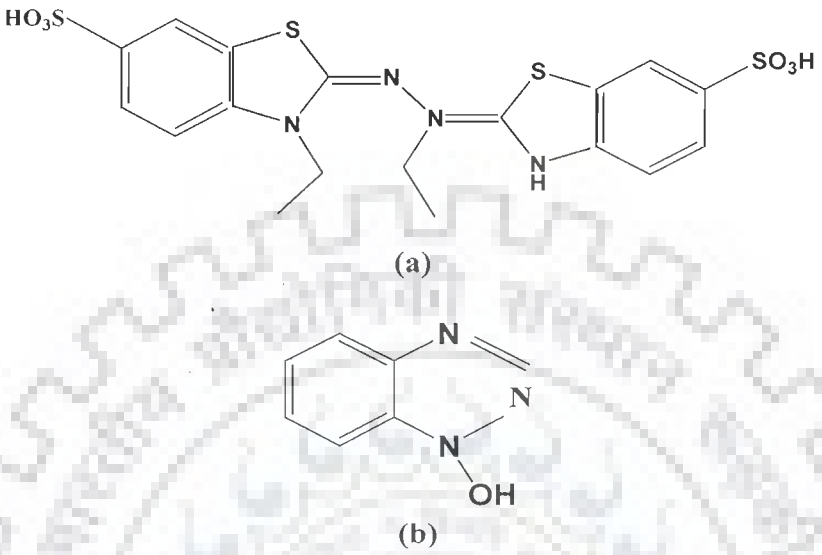


Fig. 5 Laccase mediators (a) 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); (b) N-hydroxybenzotriazole (HBT)

A naturally occurring mediator, 3-hydroxyanthranillic acid (3-HAA) has been found in the white-rot fungus *P. cinnabarinus* (Eggert *et al.*, 1996). Pulp delignificatin has been investigated using natural organic mediators (acetosyringone, syringaldehyde and *p*-coumaric acid) from plants (Camarero *et al.*, 2007). Laccase system utilizing polyoxoetalate as inorganic mediator has been evaluated for biobleaching of oxygen delignified kraft pulp (Tavares *et al.*, 2004)

2.3.2 Molecular oxygen as a regulatory agent

Chemical and spectroscopic studies of degraded lignin have shown that lignin degradation was largely oxidative. Crestini *et al.*, (2003); Palonen and Viikari (2004) postulated that oxygen is required for biological decomposition of model lignins and soluble aromatic compounds that contain either ether linkage or oxygen in their molecular structure. Enhancement in lignin metabolism because of increased oxygen pressure was also observed in

white-rot fungi such as *Lentinula edodes* (Leatham and Kirk, 1983) and *Trametes versicolor* (Kawai *et al.*, 1987).

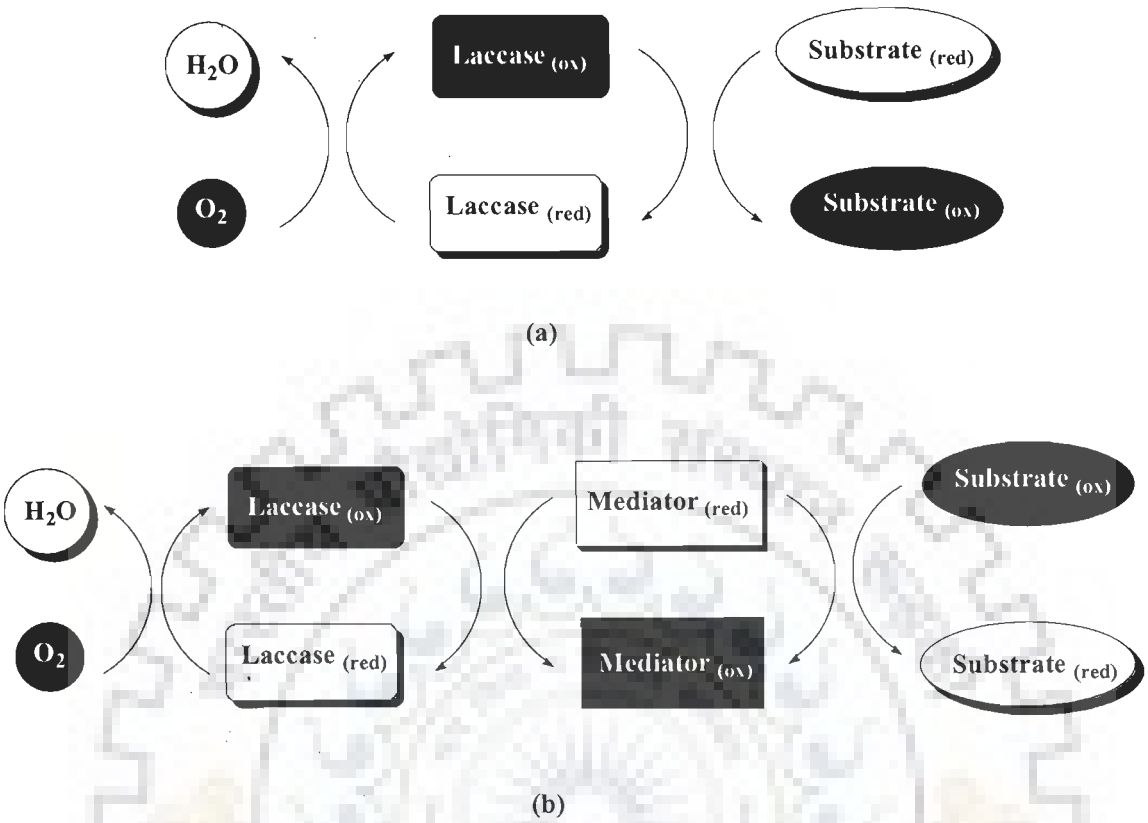


Fig. 6 Proposed mechanisms for the oxidation of substrates by LMS. (a) in the absence and (b) in the presence of mediator respectively.

Oxygen appears to have a significant influence on the laccase activity of *P. chrysosporium* (Srinivasan *et al.*, 1995). Ozonolysis of lignin containing technical pulp aimed at its bleaching results in the oxidative destruction of the residual lignin, which is accompanied by oxidation and degradation of polysaccharides and cellulose (Roncero *et al.*, 2000). Recently higher levels of laccase production have been reported from *C. rigida* when grown under agitated cultivation conditions (Saparrat *et al.*, 2002). Crestini *et al.*, (2006) had observed that immobilized heterogeneous catalytic systems are able to extensively oxidize both phenolic and non-phenolic, monomeric and dimeric, lignin model compounds. Ferraroni *et al.*, (2007) had detected the two intermediates in the molecular oxygen reduction and splitting. Several phenolic compounds were subjected to oxygen-alkali oxidations under oxygen delignification conditions and their degradations were examined in detail. It has been detected that about 95% of the compounds were degraded by molecular oxygen which supports that the molecular oxygen has a

key role to play in lignin degaradtion (Yokoyama *et al.*, 2007). Characterization and degradation of lignin from steam explosion of pine and corn stalk of lignin had been performed and the role of superoxide ion and ozone was studied by Bonini *et al.*, (2008).

2.3.3 Laccase Isozymes

Several laccase isozymes are reported and can be detected in the same organism, these may differ in their amino acid sequence and display unusual kinetic properties towards standard laccase substrates. Four different laccase isozymes have been detected in the basidiomycete *Rhizoctonia solani* (Wahleithner *et al.*, 1996) and in the ascomycete *Fusarium proliferatum* (Kwon and Anderson, 2001). Fungi may produce several isozymes of laccase that differ with respect to the degree of glycosylation and carbohydrate moieties present in the sugar chain. *Trametes versicolor*, is an example of this, which was found to produce isozymes differing in the carbohydrate content (Brown *et al.*, 2002). Several isoforms of laccases have been reported in single species (*lcc1*-5). Five isozymes of laccase are produced by *Trametes sanguinea* (Hoshida *et al.*, 2001), *Trametes villosa* (Yaver and Golightly, 1996) and *T. versicolor* (Jonsson *et al.*, (1995), while four isoforms of laccase are reported in *Trametes* sp Strain C30 by Klonowskaa *et al.* (2005).

2.4 Molecular genetics of laccase

Laccases appeared to be encoded by complex family of structurally related genes. Multiplicity is common among laccases. Almost all basidiomycetes produce more than one type of laccase. The saprophytic fungi are more diverse in their laccase gene than the mycorrhizal fungi. All of these isoforms are supposed to be encoded by a multigene family.

2.4.1 Cloning of laccase gene

Cloning and sequencing of at least 17 fungal laccase genes have been completed. These include four from *Rhizoctonia solani* (Wahleithmer *et al.*, 1996), five from *T. villosa* (Yaver and Golightly, 1996; Yaver *et al.*, 1996), two from *Agaricus bisporus* (Perry *et al.*, 1993) and a single gene from *Coriolus hirsutus* (Kojima *et al.*, 1990), *Cryphonectria parasitica* (Choi *et al.*, 1992) and *Pleurotus ostreatus* (Giardina *et al.*, 1995). Table 1 shows the diversity among laccase genes from various organisms. A gene coding for the multi-copper phenol oxidase has

been isolated and characterized from the white rot basidiomycetes *T. versicolor* (Jonsson *et al.*, 1995). The gene is preceded by a putative TATA box which is approximately 130 bp upstream of the start codon. The gene is predicted to have 10 introns, ranging from 51-60 bp. Five laccase genes have been isolated, cloned and characterized from *T. sanguinea* M 85-2 by PCR screening with degenerate primers that were designed based on the conserved copper-binding sequences of laccase (Hoshida *et al.*, 2001). A successful cloning of a laccase gene (*clac2*) from *Coprinus congregatus* have been attempted (Kim *et al.* 2001). The cloned cDNA consisted of 1817 bp and its open reading frame consisted of 1086 bp corresponding to 362 amino acids with a signal sequence of 16 amino acids. A unique feature with this gene is that it contains only two copper binding regions while other fungal laccases usually have four such regions. Three laccase genes (*LAC1, LAC2* and *LAC3*) in the plant pathogenic ascomycetes fungus *Gaeumannomyces graminis* var. tritici (Litvintseva and Henson, 2002) were identified. The demonstration of laccase in *A. lipoferum* (Givaudan *et al.*,1993) and identification of putative laccase genes in several completely and partially sequenced genomes of proteobacteria (Alexandre and Zhulin, 2000) has stimulated research into cloning of ligninase genes from bacteria. The identified putative microbial laccases include some defined proteins *PcoA* from *E. coli, CopA* from *P. syringae* and *Xanthomonas campestris,* involved in copper resistance while *CumA* from *P. putida* was involved in manganese oxidation (Alexandre and Zhulin, 2000). Recently, *Marinomonas mediterranea* has been shown to possess multipotent polyphenol oxidase, tyrosinase and laccase activities and the gene *PpoA* has been cloned and expressed in *Escherichia coli* (Sanchez-Amat *et al.*, 2001).

Table 1. Diversity profile of laccase genes in various fungi

S.No.	Organism/Gene	Size of gene (bp)	Signal sequence (aa)	Mature protein (aa)	Introns	References
1.	*Trametes villosa*					
	lcc 3	3016	21	491	12	Yaver and
	lcc 4	2906	22	498	10	Golightly,
	lcc 5	2812	23	504	11	(1996)
2.	*T. versicolor*					
	Lcc I	1932	20	519	10	Ong *et al.*, (1997)
	Lcc II	1560	21	499	-	Cassland and
	Lcc III	1563	-	-	-	Jonsson, 1999
	Lcc IV	2561	23	527	-	Ong *et al.*, (1997)
3.	*Coriolus versicolor*					
	CVLG1	3099	23	503	11	Mikuni and Morohoshi, (1997)
	CVL3	2684	21	520	08	Iimura *et al.*, (1992)
4.	*Pycnoporus cinnabarinus*					
	lcc3-1	2629	21	497	10	Eggert *et al.*, (1998)
	lcc3-2	2840	23	498	10	Temp *et al.*, (1999)
5.	*Coprinus congregatus*					
	clac2	1815	16	362	-	Kim *et al.*, (2001)

2.4.2 Regulation and expression of laccase gene

Cloning and expression of laccase gene in heterologous host is a suitable method to understand its regulation at molecular level. The yield of the purified enzyme achieved in these systems are still far from what is expected for a pilot scale up.

The level of *lcc1* RNA was induced by about 17 fold by the addition of 2, 5-xylidine to *T. versicolor* culture while the levels of *lcc2* RNA was not significantly affected (Yaver *et al.*, 1996). Similarly, the transcription of two laccase genes (*lcc3-1* and *lcc3-2*) from *P. cinnabarinus* was studied using competitive RT-PCR (Temp *et al.*, 1999). A new laccase isozyme *lac3* from *Trametes* sp strain C30 has been expressed in *S. cerevisiae* (Klonowskaa *et al.*, 2005). Hoshida *et al.* (2001) reported the heterologous expression of *lcc1* cDNA from the white rot fungus *T.*

sanguinea into *S. cerevisiae* and *Pichia pastoris*. pRCUP2 was the vector used for the expression in *S. cerevisiae* and the expression was controlled by copper concentration. pPIC3.5 and pPIC9 were used for the expression in *Pichia pastoris* and the expression was induced by methanol. Transcription of laccase genes in *G. graminis* var. tritici were induced in different conditions. *LAC1* was transcribed constituitively in all conditions analysed. In contrast, transcription of *LAC2* was copper inducible (Litvintseva and Henson, 2002). Copper mediated induction was detected for the *P. anserine* (Fernadez-Larrea and Stahl, 1996), *T. versicolor* (Jonsson *et al.*, 1995) and *P. ostreatus* (Palmieri *et al.*, 2000) laccase genes. Laccase IV gene from *Trametes versicolor* has been cloned and expressed in *A. niger* C28B25. Wastewaters, containing melanoidin has been found to induce laccase gene expression in the white-rot fungus *Trametes* sp. I-62 (Gonzalez *et al.*, 2008). Table 2 depicts heterogenous expresssion of laccase in yeast and filamentous fungi. In order to improve the yield of heterologous proteins by fungi, different approaches have been reported such as increase of gene number, use of strong promoters, appropriate signal sequences and protein fusions. An interesting alternative could be the use of culture conditions such as solid-state fermentation which have been shown to improve enzyme levels in a wide range of fungal strains (Pandey, 2003; Viniegra-Gonzalez *et al.*, 2003). Heterologous production of laccase was studied under different fermentation conditions mainly submerged and solid-state fermentation condition (Tellez-Jurado *et al.*, 2006).

Table 2. Heterologous expression of laccase in yeast and filamentous fungi

White-rot fungi	Expression Vector/ Promoter	Host
C. hirsutus (Kojimia *et al.*, 1990)	YEp5l / GATAO	*S. cerevisiae*
T. villosa (Yaver *et al.*, 1996)	pDSY2 / ά amylase	*A. oryzae*
T. versicolor (Jonsson *et al.*, 1997, Cassland and Jonsson, 1999)	pHIL-D2 / AOX 1; pAJ401/ PGK	*P. pastoris* *S. cerevisiae*
Schizophyllum commune (Hatamoto *et al.*, 1999)	pTPT / tannase	*A. sojae*
Coprinus cinereus (Yaver *et al.*, 1999)	pDSY67 / ά amylase	*A. oryzae*
P. cinnabarinus (Otterbein *et al.*, 2000, Record *et al.*, 2002)	*pPICZa B, pP1CZB AOX 1;* gpdA pLac 1, 2	*P. pastoris* *A. niger*
T. versicolor (T´ellez-Jurado *et al.*, 2006)	gene IV	*A. niger*

2.5 Production of laccase

The production of ligninolytic enzymes from fungi is highly regulated by nutrients constituting the derived medium and also implicating the alternative nutrient sources is a useful tool for improving the production (Miura *et al.*, 1997). The industrial importance of laccases has necessitated to enhance the laccase producing ability of the fungi (Lorenzo *et al.*, 2002). The enzyme is produced in relatively low quantities from the host organism and improvements using inducers is often not satisfactory. These factors have contributed to continuous emphasis being placed on alternative production strategies including cloning and heterologous expression of laccase genes. Submerged fermentation process was mainly utilized for the production of laccase (Stajic *et al.*, 2004; Sharma *et al.*, 2005; Songulashvili *et al.*, 2006; Niladevi and Prema, 2007; Elisashvili *et al.*, 2008) and attempts have also recently been made to produce laccase by

solid-state fermentation (Gomez *et al.,* 2005; Couto *et al.,* 2006; Eichlerova *et al.,* 2007; Locci *et al.,* 2008). In addition to the components of the culture medium a wide range of environmental parameters affect the production level of laccase by fungi.

2.5.1 Submerged Fermentation

It has been many years since submerged fermentation is in practice for the production of value added products. The advantage of the submerged fermentation is that it can be modified for continuous operation. The process is well defined where homogenous conditions may be maintained and various parameters such as pH, temperature and dissolved oxygen may also be easily controlled. It is a well-defined system for the production of ligninolytic enzymes. A large group of investigators have summarized the production of laccase in submerged fermentation (Scherer and Fischer, 1998; Koroleva *et al.*, 2002; Lorenzo *et al.*, 2002; Palonen *et al.*, 2003; Stajic *et al.,* 2004; Sharma *et al.,* 2005; Songulashvili *et al.,* 2006; Niladevi and Prema, 2007; Elisashvili *et al.,* 2008). It is however an energy intensive process that involves high capital investment and also generates large volume of liquid wastes. Also, in this system, maintenance of adequate levels of oxygen may often be critical to the success of the manufacturing process and oxygen may have toxic effects on anerobically growing microorganisms, mainly due to the threat arising from reactive oxygen species.

Several inexpensive natural substrates, mainly insoluble lignocellulosic material such as; baggase, barley husk, corn cobs, wheat straw and wheat bran were identified, evaluated and are considered as pragmatic alternatives to expensive carbon sources for large scale production of laccase. Other synthetic sources that also have been studied are glucose, maltose, mannitol, cellulose, fructose, galactose, sucrose etc (Da Cunha *et al.,* 2003; Stajic *et al.,* 2006).

2.5.1 Solid-state fermentation

Solid-state fermentation (SSF) is a process carried out with microbes growing on nutrient impregnated solid substrate with little or no free water. SSF can be directly carried out with abundantly available low-cost biomaterials (cellulose, lignin, starch, hemicellulose, chitin, etc.)

with minimal or no pretreatment and thus, is relatively simple, uses less energy than submerged fermentation (SmF) and can provide unique microenvironments conducive to microbial growth and metabolic activities. Currently, SSF is undergoing a renewed surge of interest, primarily because of the opportunities that it offers for increased productivity and product concentration as compared to SmF and also the prospect of using a wide range of agro-industrial commodities and waste streams as solid substrates. Large amounts of excess plant biomass are produced by the agro-industries and it may be worthwhile to use these as a renewable resource for sustainable production of industrially important products via microbial cultivation. If these are left unused the biomass would remain in waste streams and require expensive disposal or treatments. However, with the increased interest in SSF with an aim of developing industrially applicable SSF systems, research and development in SSF system have actively been taken up (Pandey *et al.,* 1999; Ooijkaas *et al.,* 2000; Robinson *et al.,* 2001; Pandey, 2003). In recent years, SSF has been exploited for the production of a spectra of value added products e.g. enzymes, organic acids, antibiotics, alkaloids, plant growth factors, biofuel and aroma compounds. Filamentous fungi are the most appropriate group of microorganisms for this system, due to their hyphal mode of growth and also due to their physiological capabilities. Further, many fungi grow on solid materials such as wood and leaves in nature and typically produce higher enzyme yields in natural growth mode. Filamentous fungi are well adapted to spread over and penetrate into solid substrate. In SSF, the colonies arising from the adjacent spores soon merge and then the hyphal density within the mycelium increases. Hyphal growth also gives the fungus a much higher penetration than unicellular microorganisms and fungi also have enzymatic machinery to aid their penetration in the solid matrix. Among the fungi, *Coriolopsis rigida, T. hirsuta, Cyathus* sp., *Dichomitus squalens, P. chrysosporium* and *Pleurotus* sp. have extensively been studied (Gomez *et al.,* 2005; Couto *et al.,* 2006; Eichlerova *et al.,* 2007; Locci *et al.,* 2008).

Generally, solid supports utilized for SSF are of agricultural origin. In recent years, there has been a spurt in the activities towards utilization of agro-industrial residues such as cassava

bagasse (Pandey *et al.,* 2000), sugar cane bagasse (Pandey *et al.,* 2000; Kumar *et al.,* 2003), wheat and rice straw etc. However some groups had also employed residual horticultural materials e. g. kiwi fruite waste and mandarin peels for enzyme production (Roukas, 2000; Gomez *et al.,* 2005; Couto *et al.,* 2006; Eichlerova *et al.,* 2007; Locci *et al.,* 2008). Recently, coconut oil cake, chest nut shell, barley bran, groundnut shells and seeds, coconut flesh and grape seeds have been utilized under solid-state fermenattaion for production of laccase (Ramachandran *et al.,* 2004; Rosales *et al.,* 2005; Gomez *et al.,* 2005; Couto and Sanoraman, 2006; Couto *et al.,* 2006). Various processes have been investigated for the utilization of lignocellulosic substrates such as wheat straw, corn and rice straw, wheat bran, sugar beet pulps and wood.

2.6 Scale up strategies and bioreactor

Solid-state fermentation is a process for production of enzymes and other products of industrial applications with several advantages over other fermentation systems. Most studies dealing with ligninolytic enzymes have been performed in submerged conditions despite these fungi grow in nature in solid-state conditions. Solid-state fermentation was chosen here for the production of laccase because it mimics the natural environment of the fungi. In addition, this technique allows the utilisation of diverse agro-horticultural residues as a substrate, making the process ecologically and economically promising. SSF may be conveniently operated in batch, fed-batch or continuous modes, although batch processes are most common (Raghavarao *et al.,* 2003). Use of anticorrosive and non-toxic material is necessity for developing a biorector. It should also be of lower cost. Other important aspects to be considered during the construction of a bioreactor are the effective regulation of aeration and heat removal which may be achieved by providing filters on the air inlet and outlet ports. This could help in the problems of ineffective heat removal, evaporative loss of water from the substrate bed and thermal gradients, which affect the production and quality of the desired product (Mitchell *et al.,* 2000).

The major bioreactor types that have normally been evaluated for the production of enzymes and industrially significant products are given as below.

2.6.1 Solid-state fermentation bioreactor

Despite the numerous processing and biological advantages that SSF offers over SmF, fewer types of bioreactors have been designed for SSF. Bioreactors based on SSF process can be divided in various categories on the basis of mixing and aeration i.e. tray bioreactor, packed beds, discontinuous and continuously rotating drums and bioreactors with continuous and forced aeration. Couto and Sanroman, (2005) had studied different natural solid substrates, especially lignocellulosic agricultural residues, as growth substrates for fungi for laccase production, which act as inducers. Moreover, most of them are rich in sugars and are easily metabolised by microorganisms. This makes the whole process much more economical and natural.

2.6.1.1 Tray bioreactor

It consists of flat trays, where bioparticle system is placed forming a thin layer of about 1.5 or 2 cm of thickness and kept in a chamber at constant temperature with passive aeration and usually has an open top. Tray bioreactors have no forced aeration or mixing of the substrate (Mitchell *et al.*, 2000). It has a chamber in which air with relative humidity and controlled temperature is circulated in and around. As only thin layers of substrate can be utilized to maintain the aerobic and proper metabolic conditions, such bioreactors are restrictive in the amount of substrate that can be fermented. Three bioreactor configurations (immersion, expanded-bed and tray) with different agitation systems for laccase production by *T. versicolor* under SSF conditions using an inert (nylon sponge) and a non-inert (barley bran) support had been evaluataed by Couto *et al.*, (2003) and the tray configuration led to the highest laccase activities.

2.6.1.2 Packed-beds

A packed-bed bioreactor consists of a static bed on top of a perforated plate through which conditioned air is blown. One interesting variation is the introduction of air through a perforated rod that is inserted into the center of the bed for air circulation (Ashley *et al.*, 1999).

Packed-bed bioreactors were used for the production of protease enzyme by *Aspergillus niger* under solid-state fermentation (Sahir *et al.*, 2007).

2.6.1.3 Continuously rotating drums

In this bioreactor, the substrate bed is held within a horizontal or near horizontal drum, which may or may not have baffles and is continuously rotated. Air is not blown forcefully through the bed itself, but rather across the top of the substrate bed through headspace. Dominguez *et al.*, (2001) had designed a new rotating drum bioreactor for ligninolytic enzyme production by *Phanerochaete chrysosporium* grown on an inert support. Performance of an intermittent agitating rotating drum type bioreactor for solid-state fermentation of wheat straw was studied by Kalogeris *et al.*, (2003).

2.7 Approaches for improvement in laccase activity

Due to variety of laccase applications, there is a potentially large demand for enzyme displaying desired characteristics. Process improvement, economization and engineering of laccases may aid in overcoming the shortfalls mainly that of currently available laccases.

2.7.1 Mutagenesis

Production of enzyme levels may be enhanced by different approaches notably by employing mutagenesis and genetic modifications and by designing and defining the process and the critical parameters regulating the production levels. Due to relatively later developments in genetic modifications in filamentous fungi, studies were directed towards mutagenesis and specifying the fermentation parameters. The induced mutagenesis and strain selection is widely practiced for strain improvement. Mutant *Penicillium variabile* M-80.10 developed by physical mutation (UV irradiation) was observed to be an overproducer of glucose oxidase (127% higher) than wild type *Penicillium variabile* P16 (Petruccioli *et al.*, 1995). *Candida* sp 99-125 developed following the series of mutagenic treatments increased production of lipase by ten folds as compared to wild type strain (Tan *et al.*, 2003). Chand *et al.*, (2005) has reported increased cellulase production in mutant *Aspergillus* CMV5-A10 than wild type *Aspergillus* CMV5

2.9 Pulp Bleaching: Chemical Process vs Biobleaching

Pulping and bleaching are two essential steps in paper manufacturing. Pulping is process in which lignocellulosic materials consisting of cellulose fibers together with lignin is converted into a fibrous mass known as pulp, is used primarily for paper making. Residual lignin which imparts brown colour to the pulp along with extractives and degraded carbohydrates is left behind during pulping. Lignin remains in the pulp because of its high molecular weight, hydrophobic nature and strong affinity with carbohydrates, is removed in subsequent bleaching stages. Delignification of pulp is performed by multi-step bleaching procedure as all the chromophoric materials may not be eliminated by single step.

Bleaching is the removal or alteration of the chromophoric substances from the pulp to achieve desired levels of brightness, while preserving the strength and integrity of the same. Bleaching chemicals are oxidizing agents that break up the lignin by introducing the solubilizing groups and disrupt lignin-carbohydrate linkages allowing the same to get dissolved. In conventional bleaching stages, chlorine (C) is mostly used to chlorinate lignin followed by alkaline extraction (E) by treating with NaOH. The remaining lignin is oxidized either by hypochlorite (H) or chlorine dioxide (D). Little doses of hydrogen peroxide (P) in alkaline extraction stage or in final bleaching stage are also employed.

Most of the pollutants associated with pulp and paper industries are released during pulping and bleaching stages where majority of chemical inputs are performed. The bleaching of pulp with chlorine and chlorine based chemicals generate effluents containing various chlorinated compounds which include chlorinated phenolics and resins, dioxins and furans. The recommended levels of AOX, COD and BOD are affected as these compounds also get into the air, water, soil and ultimately to food chain causing imbalance to the ecosystem. Air emissions from pulping processes and power generation facilities may release odours, particulates, or other pollutants leading to environmental deterioration. These complex compounds are persistent with multiple adverse effects to the environment and could be mutagenic, toxic as well as carcinogenic.

Biotechnology in pulp bleaching has attracted considerable attention and achieved interesting results. In recent years, intensive studies have been performed to develop enzymatic, environmentally benign bleaching technologies (Crestini and Argyropoulos, 1998; Bermek *et al.*, 2002). The aim of the enzymatic treatment depends on the actual mill conditions and may be related to environmental demands, reduction of chemical costs and maintenance or even improvement of product quality. The use of oxidative enzymes from fungi that may directly attack lignin, is a second-generation approach, which could produce larger chemical savings. Certain fungi can delignify kraft pulps by increasing their brightness and responsiveness to brightening chemical agents. The enzyme laccase can also delignify pulps and enzymatic processes are likely to be easier to derive and apply than the fungal treatments. The use of laccase-mediator system has shown potential for the biobleaching of pulp reducing the kappa number of pulp and improving the paper properties (Call and Mucke 1997; Bajpai, 1999; Wong *et al.*, 2000). Two different approaches using enzymes have generally been attempted to accomplish this goal. The process involving use of hemicellulases (specifically xylanases) for enhancing the chemical removal of lignin in multi-stage bleaching sequences and an alternative approach, i.e., direct delignification using enzymes called ligninases, which directly attacks and depolymerizes lignin in pulp, is still in the exploratory stage and awaits possible future commercialization. However, in both cases the aim is to enhance delignification and therefore facilitates the subsequent bleaching of pulp by applying reduced amounts of bleaching chemicals, especially chlorine and chlorine-containing compounds. White-rot fungi initially have been identified as producers of lignolytic enzymes. These enzymes, unlike hemicellulases attack lignin directly and hence appear to be more effective for biobleaching. These fungi secrete a number of oxidative enzymes and some unknown substances (mediators) into their surroundings, which together bring about the slow but continuous degradation of lignin. The most important degrading enzymes are laccase, lignin peroxidase and manganese peroxidase.

Attempts of employing these enzymes have suggested that these could prove useful in bleaching of the kraft pulp (Tavares *et al.,* 2004; Sigoillot *et al.,* 2005).

Recently, environmental concerns and increasingly stringent emission standards have led the pulp and paper industry to devise ways to decrease the level of chlorinated lignin residues in its effluents through both production process changes and improved treatment technologies that have led to the development of alternative bleaching agents including chlorine dioxide, ozone and hydrogen peroxide. The enormous potential of applying biological treatments to displace chemical bleaching stages has not been ignored and several research groups have been active in this field. Application of laccase and N-hydroxybenzotriazole as an efficient delignification system for chemical pulps has significantly advanced the potential for biological bleaching technologies(Call and Mucke, 1997; Camarero *et* al., 2004, 2007; Ibarra *et al.*, 2006). The role of laccase in biological systems to catalyze the polymerization and depolymerization of lignin has been actively investigated. Various findings have shown that laccases are consistently more effective than other enzymes, particularly for the pretreatment of pulp (Tavares *et al.,* 2004; Sigoillot *et al.,* 2005). Enzymatic processes have been developed as one of alternative bleaching technologies. Enzymes provide a simpler and cost-effective approach to cut the use of chlorine, chlorine compounds and other bleaching chemicals. Enzymes also offer a simpler approach for accomplishing the higher brightness to be attended. Attempts to enhance the application of laccase have focused on the use of chemical mediators that are believed to be oxidized by the enzyme and then undergo oxidative reactions with lignin in the pulp fiber. ABTS was one of the first reported mediators for laccase; its application has been extensively studied. HBT has been reported to be a more effective mediator for laccase assisted delignification of chemical pulps. Future improvements in laccase assisted delignification of kraft pulps would be enhanced if the fundamental chemical reactions contributing to this process were understood.

2.10 Other applications

Synthetic dyes released into the environment in wastewater streams from textile and dye manufacturing industries are considerably recalcitrant, mutagenic and/or carcinogenic and thus pose a serious environmental hazards. Current technologies for dye-decolourization are chemically derived that include adsorption, chemical transformation and incineration. It has been suggested that enhanced microbial decolourization may provide a less expensive and more environmentally acceptable alternative to chemical treatment (Selvam *et al.,* 2003). Dye degradation by laccases is a well-studied phenomenon and the application of laccases for decolourization of these industrial textile dyes, including triarylmethane, indigoid, azo and anthraquinonic dyes has been suggested (Abadulla *et al.,* 2000; Campos *et al.,* 2001; Claus *et al.,* 2002; Martins *et al.,* 2003; Zilly *et al.,* 2002). Differential induction of ligninolytic enzymes were studied for the degradation of olive mill wastewaters (Jaouani *et al.,* 2003, 2006). The ability of the polyphenol oxidase enzymes such as laccases, to oxidize polycyclic aromatic hydrocarbons was demonstrated using mediators and the natural laccase from *Trametes versicolor,* found to be effective in bioremediation (Johannes and Majcherczyk, 2000). Water and soil bioremediation from *Irpex lacteus* has been studied by Novotny *et al.,* (2000). The role of laccases from *Coriolopsis gallica* (Calvo *et al.,* 1998), *Aspergillus* 2BNL1 (Souza *et al.,* 2004) and *Aspergillus fumigatus* (Sahoo and Gupta, 2005) have been implicated in the decolourisation of alkaline effluents such as the effluent from the pulp and paper industry. Lignin-degrading basidiomycetous fungi have been studied for their role in bioremediation of lignosulphonates (Eugenio *et al.,* 2008).

Degradation of more than 70% of benz[α]anthracene by ligninolytic fungus *Irpex lacteus* in submerged culture has been reported (Cajthaml *et al.,* 2006). The use of laccase in biosensor technology is mainly attributed to its broad substrate range allowing it for the detection of a large array of phenolics (Kuznetsov *et al.,* 2001). Biosensors, that utilize laccase, include an electrode that may be used for the detection of phenols, such as catechols in tea. Laccase from

Aspergillus oryzae was developed for the determination of l-cysteine in pharmaceutical formulations, which is an important thio-containing compound and involved in a variety of important cellular functions (Ghindilis *et al.,* 1992; Santhiago and Vieira, 2007).

Organic synthesis of chemicals suffers from several drawbacks, including the high cost of chemicals, cumbersome multi-step processes and toxicity of reagents. Enzymatic polymerization has drawn considerable attention recently since it is capable of generating polymers that are impossible to produce through conventional chemical synthesis (Aktas and Tanyolac, 2003). The application of laccase in organic synthesis has arisen due to its broad substrate range, and the conversion of substrates to unstable free radicals that may undergo further non-enzymatic reactions such as polymerization or hydration. The choice of mediator has also been known to affect the final product of organic synthesis (d'Acunzo *et al.,* 2002). Examples of the potential application of laccases for organic synthesis include manufacture of anti-cancer drugs and cosmetics ingredients (Couto and Toca-Herrera, 2006). Laccase also has important role in the food and beverage industry. These may prevent undesirable changes such as discoloration, clouding, haze or flavour changes in beer, fruit juices and wine and also improves their shelf life by removing phenols such as coumaric acids and anthocyanins (Gianfreda *et al.,*1999; Mayer and Staples 2002). Rodriguez *et al.,* (2008) evaluated the potential of ligninolytic enzymatic complexes (where laccase activity was predominant), obtained from the *Trametes versicolor*, *Bjerkandera adusta* and *Fomes fomentarius* in the degradation of cell wall components of wheat straw.

2.11 COMMERCIAL LACCASES

Various companies producing laccase have reported it's utilization in paper and textile industry, waste water treatment and bioremediation. Commercial laccases available are summarized as below (Table 3).

Table 3. List of commercially available laccases

Supplier/Firm	Commercial laccase	Application
Novo Nordisk A/S, Denmark	NS51001 & NS51002	Pulp and paper industry
GmbH, Germany	Lignozym	Pulp and paper industry
Iogen Corp, Canada	BioBrite®	Pulp and paper industry
Sigma-Aldrich, USA	*Rhus vernicifera* laccase	Pulp and paper industry
AETL, India	EcowashBB	Textile Processing
Beldem-Andenne, Belgium	Frimapec W70	Color extraction
Novo Nordisk A/S, Denmark	DeniLite™,	Bleaching of dyed textile
Amfep Brussels, Brazil	Laccase	Feed industry
Jena Bioscience GmbH, Germany	L pack	Decomposition of phenolic compounds
ASA Spezialenzyme, Germany	Laccase C	Waste detoxification
United States Biological, USA	L0850	Detoxification of lignocellulosic hydrolysates

2.12 PATENTS ON LACCASES

Various patents on the production and application of the laccase are listed (Table 4).

Table 4. Patents for laccase production and application

Patent No.	Date	Title
US7175673	02/13/2007	Composition for the oxidation dyeing of keratinous fibers containing a laccase and dyeing method using this composition
US7183090	02/27/2007	Laccase enzyme and the gene encoding the enzyme
US7169965	01/30/2007	Transgenic plants expressing secretory laccase and use thereof
US7071384	07/04/2006	Methods for commercial production of heterologous laccase in plant tissue and extraction of the laccase from plant seed
US6893470	05/17/2005	Keratinous fibre oxidation dyeing composition containing a laccase and dyeing method using same
US6872388	03/29/2005	Degradation of cercosporin by laccase
US6840964	01/11/2005	Oxidation dyeing method using N-acetylcysteine as a reducing agent and laccase as an oxidizing agent
US6800792	10/05/2004	Commercial production of laccase in plants
US6799585	10/05/2004	Use of solutions containing enzymes for cleaning fermentation or storage tanks

Chapter III

Materials and Methods

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Various chemicals used for the study were of highest purity, quality and analytical grade and procured from different national and international manufacturers. N-hydroxybenzotriazole (HBT), 3,4-dimethoxybenzyl alcohol, dimethyl succinate, 2,6-dimethoxyphenol, 3-methyl-2-benzothiazolinone hydrazone hydrocholoride (MBTH) and 3-dimethylaminobenzoic acid (DMAB) were procured from Alfa Aesar, London; 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS) and guaiacol were purchased from Fluka, USA. Syringaldazine, N-methyl-N′-nitro-N-nitrosoguanidine (NTG), ethidium bromide and xylan were purchased from Sigma-Aldrich, USA. Potato dextrose agar (PDA) and other routine chemicals were purchased from HiMedia, India. Banana peel, wheat and rice straw, wheat and rice bran and bagasse were procured from local market.

3.1.2 Microorganisms

Mutant *Aspergillus fumigatus* VkJ2.4.5 strain, was developed in the laboratory by successive double stage mutagenesis of the wild type *A. fumigatus* VkJ strain ITCC 6035 (isolated from sugarcane industry waste, M/s Triveni Sugar Mills Ltd., Deoband, Uttar Pradesh, India) and was identified by Indian Agricultural Research Institute, New Delhi. The strain was maintained on PDA (2% dextrose, 2% agar, 20% potato extract) slants, stored at 4°C and sub-cultured periodically.

3.2 METHODS

3.2.1 Strain selection

A total of thirty two fungal strains were isolated from the soil samples of sugarcane industry waste, decomposing wood, manure and paper industry waste sites. Samples from sites were collected, mixed with two parts of double distilled water and centrifuged (5000 g, 20 min)

at room temperature. The clear supernatant obtained was subjected to serial dilutions and plated on PDA followed by incubation at 30°C for 5-7 days. Distinct colonies obtained were picked up and restreaked on PDA plates to select the respective single and pure colonies. These were further purified, and isolated pure cultures were maintained on PDA slants incubated at 30°C for 5 days. Among 32 isolates, five strains were selected for further studies on the basis of the of zone formation as observed on the screening plates. Selected strains were maintained on PDA slants and stored at 4°C for further investigation.

3.2.2 Screening of laccase producing strains

Primary screening of the selected strains for the laccase production ability was performed by using malt extract agar containing gallic acid (0.5%, w/v) (Bavendamm, 1928). The medium containing malt extract (15 gl^{-1}) and agar (20 gl^{-1}) was prepared in distilled water and autoclaved (121°C, 15 min). Cell free culture supernatant (100 µl) was placed into wells (4 mm diameter) and plates were incubated at 30°C for 4-5 days. Enzymatic hydrolysis of the surrounding gallic acid resulted into diffused brown coloured zones around the wells. Controls with heat killed supernatant (140°C, 20 min) did not result into any coloured zones.

3.3 MUTAGENESIS AND SCREENING

Improvement in the laccase production ability of wild type *A. fumigatus* VkJ strain was attempted by physical, chemical and mixed mutagenesis. The schematic representation of the step-wise mutagenesis and screening is shown in fig. 8.

3.3.1 Single-Step Mutagenesis

3.3.1.1 Physical mutagenesis

Physical mutagenesis employing UV irradiation was performed according to the method of Petruccioli *et al.*, (1995). Spores from 4 day old wild type VkJ strain were suspended in sterile saline (0.85 %) containing 0.1% Tween-80. Aliquots (3 ml) of this suspension (~120x10^6 spores ml^{-1}) were then transferred to the sterile petridishes (9 cm diameter) and irradiated with

UV light (2.5 $Jm^{-2}s^{-1}$, distance 0.45m) for different time periods (1-10 min). To avoid photorepair, plates were stored in the dark (4°C, 24 h). It was followed by serial dilutions on PDA medium to check survivability. A parallel control was also maintained where spores were treated identically except that UV exposure was not performed.

3.3.1.2 Chemical mutagenesis

3.3.1.2.1 *N-methyl-n'-nitro-N-nitrosoguanidine treatment*

Mutagenesis of *A. fumigatus* VkJ was also carried out using N-methyl-n'-nitro-N-nitrosoguanidine (NTG) as described (Yukioka *et al.*, 1997). Briefly, a spore suspension of *A. fumigatus* VkJ was prepared in saline solution (0.85%). The spores (~$120x10^6$ spores ml^{-1}) were treated with 100 µg ml^{-1} of NTG in saline solution for different durations (10-120 min). Following the treatment, spores were washed twice, plated on PDA medium and incubated at 30°C for 4 days. Survivability and frequency of mutants were determined on PDA. Control spore suspension was also treated identically excluding the N-methyl-n'-nitro-N-nitrosoguanidine.

3.3.1.2.2 *Ethidium Bromide (EtBr) treatment*

Ethidium bromide treatment was performed as described (Chand *et al.*, 2005). For mutagenesis, fungal spores (~$120x10^6$ spores ml^{-1}) obtained from 4 days old culture, were suspended in sterile saline (0.85%) and treated with EtBr for different time period (200 µg ml^{-1}, 10-120 min). Following the treatment, spores were washed twice, plated on PDA medium and incubated (30°C, 4 day). Survivability and frequency of mutants were determined on PDA. Control spore suspension was also treated identically excluding ethidium bromide.

3.3.2 Second Stage mutagenesis

A mutant developed by first stage of mutagenesis with higher laccase activity was further subjected to second stage of mutagenesis employing both, physical and chemical methods; using UV irradiation, NTG and EtBr respectively (Fig.7).

3.3.3 Mutant isolation and screening

Rapid screening of mutants for their enhanced laccase production ability was performed on MEA medium with gallic acid as described earlier. The colonies showing higher zone diameter on screening plates were picked up and stored for future investigation.

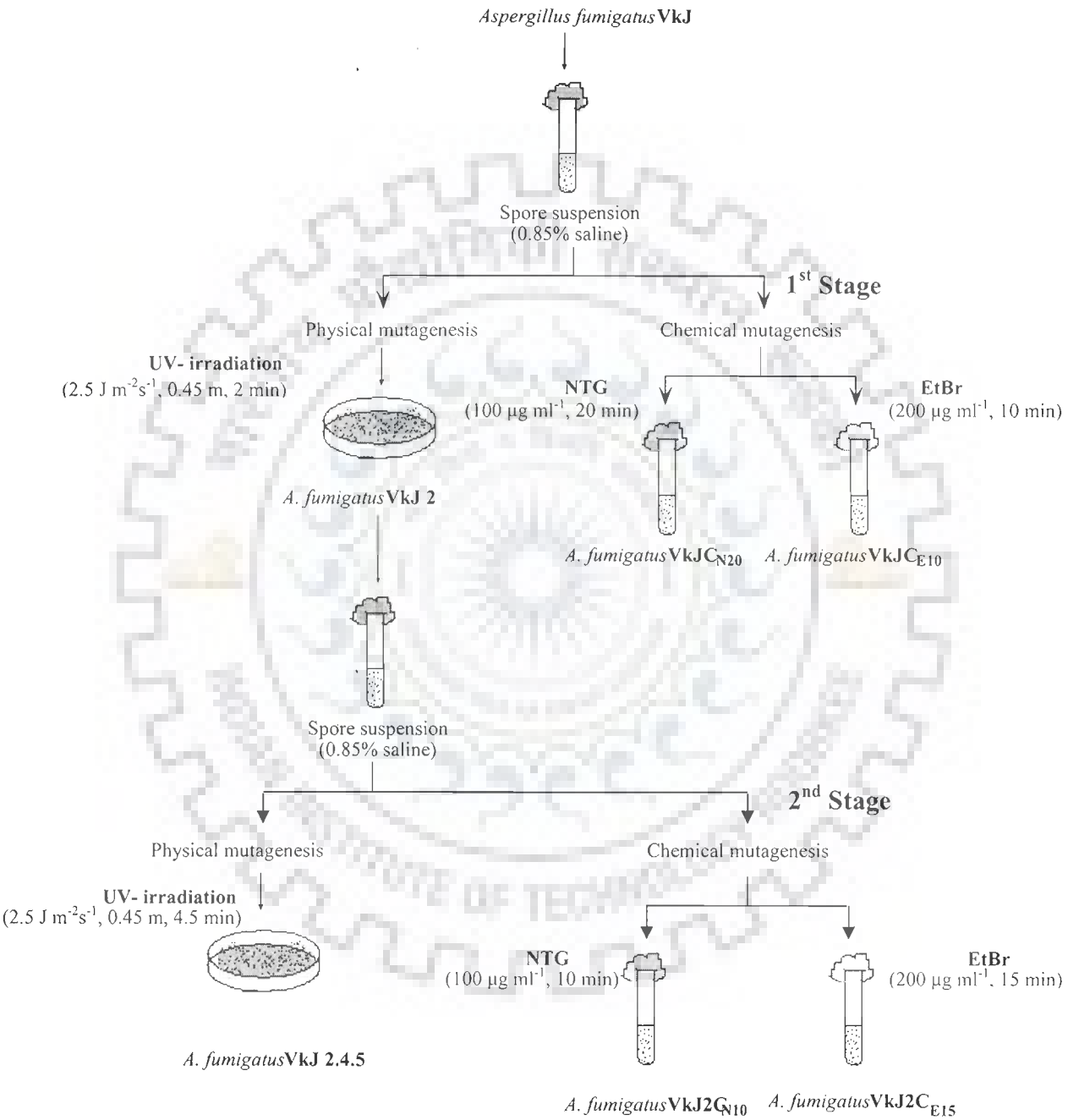


Fig. 7 Mutagenesis of *Aspergillus fumigatus* VkJ for laccase production

3.4 FERMENTATION MEDIUM

The preliminary studies of fermentation for laccase production were carried out as described (Koroleva *et al.*, 2002). The composition of the media used for laccase production was (gl^{-1}): peptone, 3.0; glucose, 10.0; KH_2PO_4, 0.6; $ZnSO_4$, 0.001; K_2HPO_4, 0.4; $FeSO_4$, 0.005; $MnSO_4$ and $MgSO_4$, 0.05. The pH of the medium was adjusted to 6.0 and then autoclaved (121^0C, 15-20 min, 15 p.s.i.).

3.4.1 Surface fermentation

The surface fermentation in defined medium (30 ml) as described (Koroleva *et al.*, 2002) was carried out in the Erlenmeyer flasks (250 ml) at 30°C following inoculation. The fungal mycelia were allowed to grow on the surface of the medium in the form of a mycelial mat with intermittent shaking to maintain the uniform pH of the medium. As and when required, waste gases generated during fermentation were removed by suction pump from the fermentation flask and fresh air (1.5-2.0 l min^{-1}) was circulated over the mycelial mat. The fermentation was carried out for 8 days and samples for the enzyme activity measurement were withdrawn from time to time. Following completion of fermentation, broth was centrifuged (5000 g, 15 min) and used for enzyme assays.

3.4.2 Submerged fermentation process

Submerged fermentation was carried out in 30 ml of defined medium (Koroleva *et al.*, 2002) in Erlenmeyer flasks (250 ml). Medium was inoculated with *A. fumigatus* VkJ2.4.5 and VkJ strains following incubation at 30°C in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (120 rpm). The fermentation was carried out for 8 days and samples for the enzyme activity were taken intermittently. Following completion of the process, broth was centrifuged (5000 g, 15 min) and filtered for enzyme assay.

3.4.3 Solid-state fermentation

Different lignocellulosic (agro and horticultural residues) substrates, i.e. wheat and rice bran, poplar leaves, banana peel, wheat straw and bagasse were washed thoroughly in the double

distilled water to remove the residual dust, air dried, pulverized and utilized as the substrates. Banana peel (10 g) was however initially soaked for an hour in 30 ml of KOH (83 mM) to neutralize organic acids (Stredansky and Conti, 1999). Oven dried solid substrate (4 g) was taken in Erlenmeyer flask (250 ml) and moistened with fermentation medium so as to maintain the appropriate moisture level. Slurry of the fermentation medium containing solid support was autoclaved and inoculated with agar discs (6 discs per flask, 4 mm diameter) slashed from the periphery of petri plates containing freshly grown (72 h) *A. fumigatus* Vk2.4.5. The culture flasks were then incubated at 30°C for 8 days. Waste fermentation gases generated, were removed by connecting the fermentation flasks to a water suction pump and the fresh air (1.5-2.0 l min^{-1}) was circulated over the mycelia.

Following completion of the process, enzyme was extracted from the fermentation system with 100 ml of double distilled water. Each time the whole content was squeezed, extract was centrifuged (5000g, 10 min) to remove the suspended particles and filtered to get rid of the fungal mycelia/spores and clear supernatant was used as enzyme source (Gessesse & Mamo, 1999).

3.5 SCALING UP OF THE PROCESS

Solid-state fermentation process employing banana peel as solid support was scaled up. A column-tray bioreactor was designed for batch production of laccase as shown in fig. 8. Circular aluminium trays (4 nos.) having dimensions of 28 x 2 cm (diameter x height) were vertically arranged and fermentation was carried out in upper three trays. Tray at the bottom was utilized for holding water to maintain humidity in the chamber. Processing of banana peel was performed as described earlier. Appropriate amount (150 g) of solid substrate was mixed with fermentation medium to achieve the derived moisture level, autoclaved and spread evenly in the fermentation trays. Following inoculation, the microbial culture was allowed to grow over the support. The inlet and outlet ports for air circulation were provided. The air was circulated through an air pump unit. Following 6 days of fermentation cycle, broth was recovered aseptically and the same set up of fungal mat and solid support was used for subsequent batches

of fermentation. Initial batch of fermentation was supplied with sufficient amount of medium and incubated for next batch. Following completion of every batch of fermentation, the content was aseptically squeezed to release the accumulated enzyme. The broth, thus recovered was used for estimating the laccase and MnP activity. The trays were then subjected to UV sterilization to inactivate the generated spores.

Table. 5 Chemical composition of the banana peel (% dry matter) (Essien *et al.*, 2005)

Compound (g/100 g)	
Dry matter	14.08
Crude protein	7.87
Crude fat	11.60
Crude fiber	7.68
Total ash	13.44
Carbohydrates	59.51
Moisture	78.4
Mineral and ascorbic acid content (mg/100 g)	
Calcium	7
Sodium	34
Phosphorus	40
Potassium	44
Iron	0.93
Magnesium	26
Sulphur	12
Ascorbic acid	18

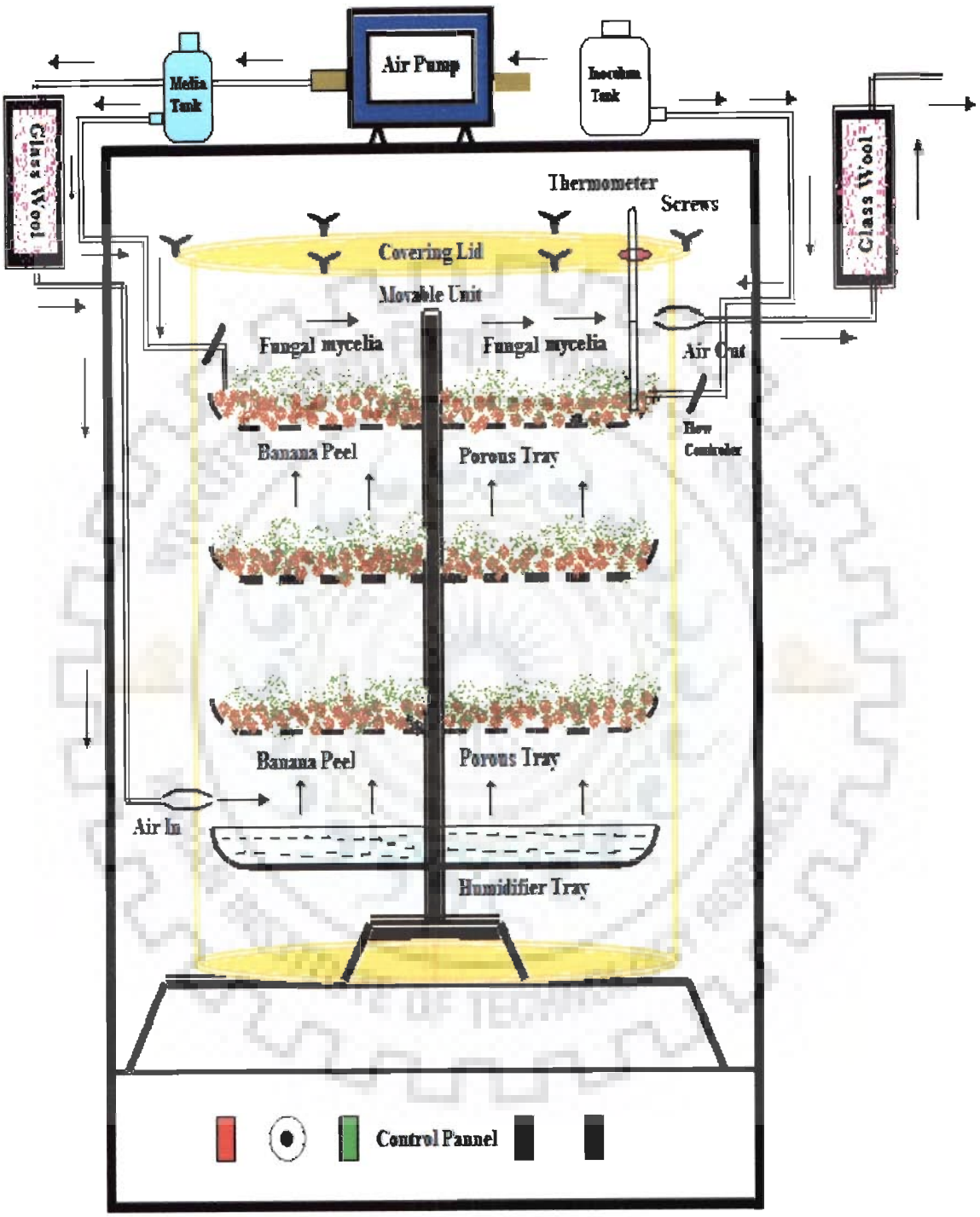


Fig. 8 Schematic representation of the column-tray bioreactor for batch production of laccase under solid-state fermentation

3.6 BIOBLEACHING OF PULP WITH LACCASE FROM *A. FUMIGATUS*

3.6.1 Pulp

The unbleached, mixed wood pulp (*Eucalyptus* sp. and *Populus* sp. 60:40; kappa number 14.0, 35.7% ISO brightness) was procured from Star Paper Mills Limited, Saharanpur, India. Pulp was washed, air dried and stored in air tight polythene bags for further studies.

3.6.2 Production of enzymes

The mutagenized *A. fumigatus* VkJ2.4.5 strain, developed in our laboratory was employed for laccase production under solid-state fermentation using banana peel as solid support as described earlier.

3.6.3 Enzymatic treatment of pulp

3.6.3.1 *Derivation of enzyme dose*

To derive the appropriate enzyme dose for achieving maximum bleaching, pulp (10 g oven dried pulp - odp) was treated in different polythene bags with varying doses of enzyme (0-20 Ug^{-1}) and incubated in a water bath (50°C, 2 h). The pulp was then filtered through muslin cloth and the respective filtrates were collected for further analysis. Untreated pulp was also processed identically except that the enzyme was not added.

3.6.3.2 *Derivation of treatment time*

Derivation of time for achieving maximum enzymatic bleaching (10 Ug^{-1}) was determined by treating the pulp (10g odp) with laccase for varying time periods (0-6 h) in different polythene bags at 50°C. Untreated pulp was also processed identically except that enzyme was not added.

3.6.3.3 *Effect of mediator concentration*

The enzymatic treatment of pulp was performed (10 Ug^{-1}, 2 hrs) in the presence of HBT at different concentrations (0-3%, w/w) to evaluate the effect of mediator on the bleaching efficiency of enzyme.

3.6.3.4 *Colour removal from the pulp*

Following the enzymatic treatment and washing of pulp, effluent thus collected was subjected to scanning (λ 200-400 nm) for determining the lignin removal. The release of phenolic as well as hydrophobic compounds was monitored at A_{237nm} and A_{465nm} respectively (Khandeparkar and Bhosale, 2007).

3.6.3.5 *Application of laccase in multi-step bleaching process*

Fresh pulp was treated with derived dose of laccase washed with distilled water and then treated with different bleaching sequences. Two different bleaching sequences (XCEHH and XODED; where X, laccase; C, chlorination; E, alkali extraction; H, hypochlorite; O, oxygen and D, chlorine dioxide) were used. Conditions applied during the bleaching are given in Table 6. The physical and chemical properties of pulp and paper were determined according to the standard methods (TAPPI 1991). The effluents of treated and untreated samples were also analyzed for AOX and COD as per standard test procedures given in Table 7. The level of laccase mediated delignification was determined by estimating kappa number, brightness and yellowness of pulp. It was then followed by making the handsheets. Various strength properties of the paper sheet like tensile strength, burst strength, tearing resistance, folding endurance etc were also determined as per the standard methods of TAPPI (Table. 7). All the experiments were performed in triplicates.

Table. 6 Conditions applied during different bleaching stages

Sl. No.	Bleaching Sequences	Stage	Dose	Consistency (%)	pH	Temperature (^{o}C)	Time (min)
1a.	CEHH	C	50% of TCD*	3	≥ 2	Ambient	30
		E	$Cl_2/2 + 0.03$	10	10.8	90	90
		H_1	70% of remaining 50% TCD*	10	11.5	45	60
		H_2	30% of remaining 50% TCD*	10	11.5	45	60
1b.	XCEHH	X	10 Ug^{-1}	10	6	50	120
		C	50% of TCD*	3	≥ 2	Ambient	30
		E	$Cl_2/2 + 0.03$	10	10.8	90	90
		H_1	70% of remaining 50% TCD*	10	11.5	45	60
		H_2	30% of remaining 50% TCD*	10	11.5	45	60
2a.	ODED	O	5 $kgcm^{-2}$	10	11.5	90	90
		D_1	1%	10-12	3.5-4.5	70	180
		E	2.5%	10	11.5	60	60
		D_2	0.6%	10-12	3.5-4.5	70	180
2b.	XODED	X	10 Ug^{-1}	10	6	50	120
		O	5 $kgcm^{-2}$	10	11.5	90	90
		D_1	1%	10-12	3.5-4.5	70	180
		E	2.5%	10	11.5	60	60
		D_2	0.6%	10-12	3.5-4.5	70	180

* TCD, Total chlorine demand

Table. 7 Standard test procedures followed for analysis of pulp and paper

Sl No.	Test No.	Description
1.	T 610 om-87	Preparation of indicators and standard solutions
2.	SCAN-C 18:65	Disintegration of pulp for testing
3.	SCAN-C 24:67	Beating of pulp in PFI mill
4.	T227 cm-99	Determination of °SR
5.	SCAN-C 26:76	Forming handsheets for physical testing of pulp
6.	T236 cm-85	Kappa number of pulp
7.	T403	Bursting strength of paper
8.	T404 cm-92	Tensile breaking length and elongation of paper
9.	T414 cm-98	Internal tearing resistance of paper
10.	SCAN-C 11:75	Pulp ISO brightness
11.	SCAN-C 29:72	Chlorine Consumption of pulp
12.	SCAN-C 16:62	Preparation of cupriethylenediamine (CED) solution
13.	T230 cm-99	Viscosity of pulp (capillary viscometer method)
14.	COD	Closed reflux titrimetric method – Thermoreactor CR

3.7 ANALYTICAL METHODS

3.7.1 Estimation of laccase activity

Laccase assay was performed using ABTS as a substrate. Reaction in a total volume of 1 ml contained 200 mM sodium phosphate/100 mM citric acid buffer (pH 5.0), enzyme extract (100 µl) and 1.0 mM of ABTS. Reaction was monitored at 420 nm using UV-Vis spectrophotometer (Varian Cary 100 Bio, Australia) at 25^0C (de Souza-Cruz *et al.*, 2004). One unit of enzyme activity was expressed as 1 µmol of product released per minute.

3.7.2 Estimation of MnP activity

Manganese peroxidase activity was determined by MBTH-DMAB assay. Reaction mixture contained 0.07 mM MBTH (3-methyl-2-benzothiazoline hydrazone), 0.99 mM DMAB (3-dimethylamino benzoic acid), 0.3 mM $MnSO_4$, 0.05% H_2O_2 and 100 mM succinic-lactic acid buffer (pH 4.5). Reaction was monitored at 590 nm at 25^0C (Castillo *et al.*, 1994). One unit of enzyme activity was expressed as 1 µmol of product released per minute.

3.8 CHARACTERIZATION OF THE ENZYME

3.8.1 Thermostability

Thermostability was determined by incubating the culture broth at different temperature (25-70 °C) up to 2 hrs. Enzyme activity was measured as described earlier for which samples were intermittently withdrawn and analyzed for residual laccase activity.

3.8.2 pH stability

pH stability was determined in the pH range of 3.0-11.0 by incubating the enzyme in buffers of different pH; 100 mM citrate-phosphate, pH 3.0-6.0; 100 mM phosphate, pH 6.0-8.0; 50 mM Tris- HCl, pH 8.0-9.0; 50 mM glycine- NaOH, pH 9.0-10.5; 100 mM carbonate-bicarbonate, pH 10.5-11.0. After 2 hrs of incubation, the residual enzyme activities were determined under standard assay conditions as described earlier.

3.9 Sodium-dodecylsulphate polyacrylamide gel electrophoresis

SDS-PAGE of the samples was performed according to Laemmli (1970). Polyacrylamide gels (12%) of 1.5 mm thickness were prepared for electrophoretic analysis.

3.9.1 Reagents

Solution a: Acrylamide solution 29.2% (w/v) containing 0.8% (w/v) bisacrylamide (N, N' methylene-bisacrylamide)

Solution b: Resolving buffer, 1.5 M Tris-HCl, pH 8.8

Solution c: Stacking buffer, 0.125 M Tris-HCl, pH 6.8

Solution d: SDS (10%, w/v)

Solution e: Ammonium persulphate 10% (w/v) (freshly prepared)

Solution f: TEMED (N, N, N', N'-tetramethylethylenediamine)

Electrophoresis buffer, 0.25 M Tris, 0.192 M glycine, pH 8.3

3.9.2 Preparation of resolving and stacking gels

Reagents	**Resolving gel (12%) (ml)**	**Stacking gel (4%) (ml)**
Solution a	12.0	2.66
Solution b	7.50	-
Solution c	-	5.00
Solution d	0.30	0.20
Solution e	0.15	0.10
Solution f	0.015	0.02
Water	10.035	12.2
Total volume (ml)	30.0	20.0

3.9.3 Casting of gel

For casting of gel, glass plates (16 x 18 x 0.3 cm) were assembled and the base of the gel was sealed. Resolving gel mixture was prepared by mixing all the components except ammonium persulphate and TEMED. Mixture was degassed for 15 min, ammonium persulphate and TEMED were added to it. The mixture was gently mixed and poured between the plates leaving the required space at the top for pouring the stacking gel. The water or butanol was added followed by polymerization of the resolving gel, the assembly was tilted to pour off the over layer, washed with sterile distilled water and wiped gently to dry the empty space. Stacking gel mixture was then similarly degassed and layered on the resolving gel; comb was immediately inserted into the mixture to form the wells. Comb was carefully removed after polymerization of stacking gel, wells formed were rinsed with reservoir buffer and the polymerized gel was used for electrophoresis.

3.9.4 Sample preparation

Dialyzed fractions containing protein were concentrated using Centricon (C-10) concentrators (Amicon, USA) and added with SDS-PAGE sample buffer containing 0.125 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue. Samples were heated in a boiling water bath for 3-5 min and loaded onto the gel for electrophoresis.

3.9.5 Electrophoresis

Samples (100 µg of protein) were gently loaded on the gel and electrophoresed at constant voltage (stacking at 100 V, resolving at 120 V). Gel was removed and stained overnight with gentle shaking in coomassie brilliant blue R-250 (0.1%) in methanol: glacial acetic acid: water (5:2:5) at room temperature and destained by washing in destaining solution (Methanol:Acetic acid:Water; 30:10:60).

3.9.6 Zymogram analysis of laccase

Non-denaturing gel (12%) electrophoresis was performed for visualization of enzyme activity under the same conditions as described (Vasdev *et al.,* 2005) except that SDS and β-

mercaptoethanol were not used and samples were not boiled. After electrophoresis, visualization of laccase activity in native-PAGE was determined by soaking the gel in 100 mM citrate phosphate buffer (pH 5.0) containing guaiacol (0.25%). Appearance of dark brown colored bands indicated the presence of laccase.

3.9.7 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-ToF/ToF) Mass Spectrometry

For MALDI-ToF/ToF analysis of the protein, initially the broth was concentrated with Centricon C-10 (Amicon, USA) and then electrophoresed on native gel (12 %). The half of the gel was stained with coomassie brilliant blue R-250 and the other half was subjected to substrate staining (Guaiacol) for zymogram analysis. Protein bands in the coomassie stained gel with the one corresponding to the active band in zymogram were carefully excised and repeatedly washed with 100 mM ammonium bicarbonate-acetonitrile buffer (pH 8.0). Gel slice was dissolved in acetonitrile and vacuum dried followed by washing. Reduction was carried out with 1% dithiothreitol (DTT) followed by 2% iodoacetamide (IAA) modification and tryptic digestion for overnight and acidified by adding 5 µl of 10% tri-fluoroacetic acid (TFA). The digested product was mixed with α-cyano-4-hydroxycinnapic acid matrix in 1:1 ratio. The matrix was made by mixing 0.1% TFA to mixture of acetic acid and water (1:1). Spectra were recorded (Bruker Daltronix Autoflex ToF/ToF, Germany) in a positive mode and the N_2 laser intensity was set at λ 337 nm. The peptide fragments obtained were analyzed with the Flex Analysis Software and database homology search for protein identification was carried out manually using short sequence BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) (Schwend and Gustafsson, 2006).

3.10 SCANNING ELECTRON MICROSCOPY

The detailed morphological analysis of wild and mutant strains and banana peel (uninoculated and inoculated) were carried out using scanning electron microscopy (SEM, LEO 435 VP, England). Fungal mats, mycelia and banana peel were taken and subjected for fixation

using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double distilled water and then treated with the alcohol gradients of 30%, 50%, 70%, 80%, 90% and 100% for dehydration, respectively. The retention time of samples in each alcohol series up to 70% alcohol gradient was 15 minutes, but later, the time period was increased to 30 minutes for alcohol concentrations ranging from 80% to 100%. For scanning electron microscopy of enzyme treated and untreated pulp, fibres were washed thrice in deionized water and treated with 2.0% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 7.2) for 1 h. Further, fibres were separated from glutaraldehyde, washed with the same buffer thrice and dehydrated with ethanol gradient (30-100%) gradually (Kapoor *et al.,* 2007). After complete dehydration, samples were air dried and coated with gold by gold shadowing technique (Gabriel, 1982). The samples were viewed under the SEM and photographs of the samples were taken at the desired magnification.

3.11 High performance liquid chromatography (HPLC)

The action of enzyme on substrate (guaiacol) was analyzed by treatment of the same with enzyme. Substrate was incubated with and without enzyme at 20 °C for different time period (1-3 hrs). HPLC analysis was performed by injecting 20 µl of incubated mixture with a runtime of 15 min (Waters, 1500 series, Ireland) using Nova-pak C_{18} column (3.9 x 150 mm). The elution was done with a mixture of acetonitrile-water-tetramethyl ammonium hydroxide-phosphoric acid (20:80:1:1 v/v) and a UV-detector at 240 nm (Niku-Paavola *et al.*, 1988). Prior to HPLC analysis samples were diluted (100 fold) with the mobile phase and filtered through a 0.2 µm teflon syringe filter for removing any impurities (Barreca *et al.*, 2003).

3.12 Fourier-transformed infrared spectroscopy (FTIR)

Residual lignin from treated and untreated pulp was extracted with methanol/tetrahydrofuran (1:1, v/v) solution in a soxhlet extractor for 3 h and analyzed by FTIR spectroscopy for comparison (Geng and Li, 2002). Lignin samples were embedded in KBr disc and the fourier-transformed infrared spectra were recorded using a Perkin-Elmer 1600 series

spectrometer at room temperature. The assignment of absorbance peaks were based on database for lignin (Buta *et al.*, 1989).

3.13 X-ray diffraction (XRD)

Treated and untreated pulp samples were analysed to evaluate the effect of laccase-mediator system on its crystallinity. The crystallinity of pulp was studied through X-ray diffraction using a diffractometer (Bruker AXS D8 Adv, Germany) operated at 40 KV and 30mA. The measurement range (2θ) was from 5° to 50° with a step size of 1° and step time of measurement was 1 min (Roncero *et al.*, 2003).

3.14 BIOBLEACHING

3.14.1 Analysis of bleach liquor

3.14.1.1 Calcium hypochlorite

In 10 ml of diluted bleach liquor, 10 ml potassium iodide (10%) and 10 ml acetic acid (10%) were added. This solution was titrated with standard sodium thiosulphate solution (0.1N) with starch (0.5%) as indicator. The end point was blue to colorless. The active chlorine (gpl) was calculated as below :

Active chlorine (gpl) = Normality of bleach liquor X 35.5

3.14.1.2 Chlorine dioxide

Sodium chlorite solution (20 gl^{-1}) was titrated by same procedure as used for analysis for calcium hypochlorite solution.

3.14.2 Analysis of residual chlorine

Same procedure as described above was used except that volume of spent bleach liquor was increased to 100 ml and titrated with standard sodium thiosulphate solution (0.1N).

3.14.3 Analysis of H_2O_2

10 ml of H_2O_2 was diluted and made up to 250 ml in a volumetric flask. To 5 ml of this diluted solution, 10 ml of KI (10%), 10 ml H_2SO_4 (4N) and 1 ml of ammonium molybdate (1%) were added. This solution was titrated with standard sodium thiosulphate solution (0.1N) with

starch (0.5%) as an indicator. The end point was blue to colorless. The concentration of H_2O_2 solution was calculated as below:

Concentration of H_2O_2 solution = Normality of H_2O_2 solution X 34

3.15 STATISTICAL ANALYSIS

All fermentation reactions were carried out in triplicate and experimental results represent the mean of three identical fermentations. The statistical analysis of variance (ANOVA) was performed as described (Somasegaran and Hoben, 1994). 1% and 5% levels for ANOVA test was used for comparing the calculated F ratio by using standard tables.

Table 8. Analysis of Variance

Source of Variation	**Sum of square**	**Degree of freedom (df)**	**Mean squares (Ms)**	**F ratio**
Between the table (T)	SST	k-1	SST/ (k-1)	$\frac{SST}{SSE} \times \frac{bkn-k-b+1}{(K-1)}$
With in block (B)	SSB	b-1	SSB/ (b-1)	$\frac{SSB}{SSE} \times \frac{bkn-k-b+1}{(b-1)}$
Error (E)	SSE	Bkn-k-b+1	SSE/ (bkn-k-b+1)	
Total	SS	Bkn-1		

d.f., Degree of freedom; SST, Treatment sum of squares; SSB, Block sum of squares; SSE, Error sum of squares; SS, Total sum of squares; K, Number of treatment; b, Number of blocks (number of replicates per treatment); n, Number of replicates per treatment per block.

Chapter IV

Results

4.1 ISOLATION AND SCREENING OF A POTENT MICROBIAL STRAIN

Overall thirty two fungal strains were isolated from the soil samples of different sites viz. sugarcane and paper industry wastes, forest soil and from dead and decomposing wood materials. Fungal colonies were isolated on the basis of morphological appearance, mycelial and spore colour (Table. 9). These strains were primarily screened qualitatively for laccase production ability on the basis of coloured zones developed on malt agar plates supplemented with gallic acid (Table.10). Out of thirty two strains, eleven isolates had shown laccase production on screening media. Five strains i.e. VkD, VkG2, VkH2, VkJ and VkN2 that had shown clearly demarcated zones on the screening media were selected for further analysis. Among selected strains, largest zone formation and also the maximum laccase activity was detected with VkJ strain (Table. 10). Therefore, strain VkJ was finally selected for further study and it was identified as *Aspergillus fumigatus* (ITCC 6035) from Indian Agriculture Research Institute, New Delhi, India. The strain was maintained by periodical transfers on potato dextrose agar (PDA) slants at 4°C for future studies.

Table. 9 Morphological analysis of isolated fungal strains

S.No.	Isolated Strains	Site of Isolation	Mycelial Colour	Spore Colour	Zone Diameter (cm)
1.	VkA1	Dead tree	Off white	-	-
2.	VkA2	Dead tree	White	-	0.6
3.	VkB	Rotten wood	Off white	-	-
4.	VkC1	Dead tree	Cream	-	0.7
5.	VkC2	Sugarcane wastes	Cream	-	-
6.	VkC3	Paper loading site	White	-	-
7.	VkD	Dead tree	Light yellow	-	0.9
8.	VkE	Sugarcane wastes	Gray	Gray	-
9.	VkF	Sugarcane wastes	Off green	Green	-
10.	VkG1	Dead tree	White	-	-
11.	VkG2	Dead tree	Cream	Green	1.0
12.	VkG3	Dead tree	Cream	-	-
13.	VkH1	Rotten wood	Yellow	Yellow	-
14.	VkH2	Rotten wood	White	Dirty green	0.8
15.	VkH3	Rotten wood	Cream	-	-
16.	VkH4	Rotten wood	Off white	-	-
17.	VkI1	Rotten wood	Dirty gray	Black	-
18.	VkI2	Dead tree	White	-	0.7
19.	VkI3	Dead tree	White	White	-
20.	VkI4	Dead tree	Dirty yellow	-	-
21.	VkI5	Dead tree	Pink	Black	-
22.	VkJ	Sugarcane wastes	White	Gray	2.0
23.	VkK1	Paper loading site	Gray	Black	0.7
24.	VkK2	Paper loading site	Yellow	Green	-
25.	VkL	Dead tree	Off white	Brown	0.6
26.	VkM1	Dead tree	Light Pink	Light Gray	0.7
27.	VkM2	Dead tree	Light yellow	Light yellow	-
28.	VkM3	Dead tree	White	Off white	-
29.	VkM4	Forest soil	Off white	-	-
30.	VkN1	Forest soil	Gray	Gray	-
31.	VkN2	Forest soil	Yellow	Green	0.8
32.	VkN3	Forest soil	Brown	Dark Brown	-

Table. 10 Laccase production from the selected fungal isolates

S. No.	Fungal Strain	Zone diameter (cm)	Laccase (UI^{-1})
1.	VkJ	2.0 ± 0.14	2108.2 ± 49.37
2.	VkG2	1.0 ± 0.11	1623.4 ± 39.78
3.	VkH2	0.8 ± 0.14	1438.1 ± 28.11
4.	VkN2	0.7 ± 0.14	1291.3 ± 49.86
5.	VkD	0.9 ± 0.17	967.4 ± 26.57

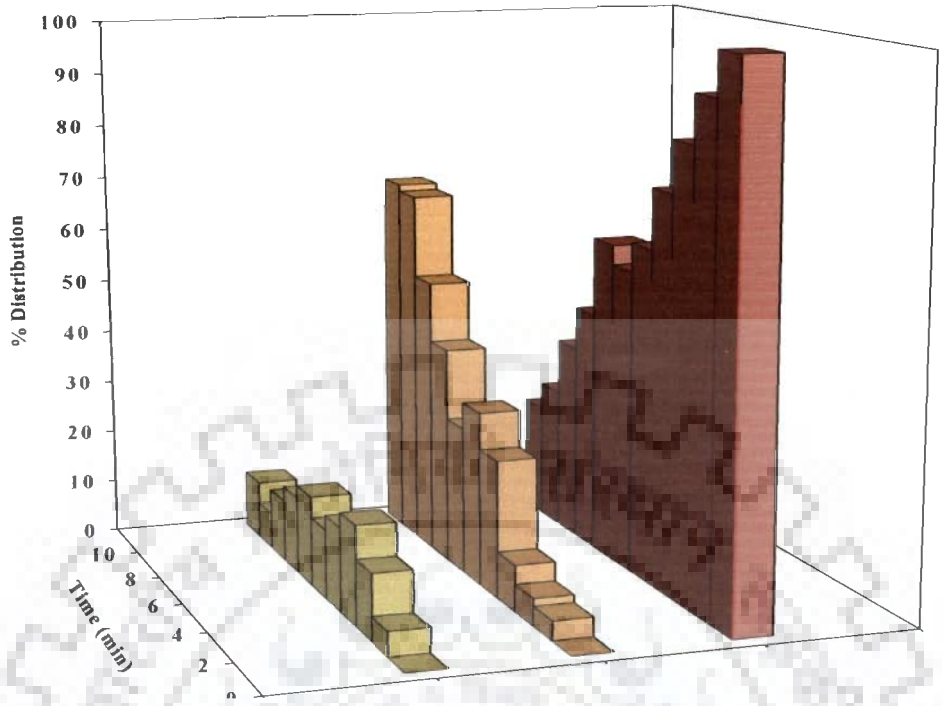
4.2 MUTAGENESIS OF *A. FUMIGATUS* VKJ FOR ENHANCED LACCASE PRODUCTION

Fungal strain, VkJ isolated from site containing sugarcane waste was subjected to physical (UV irradiation), chemical (NTG and EtBr) and mixed mutagenic treatments to find out if an enhancement in the levels of laccase production can be achieved. Spore suspensions of wild type *A. fumigatus* VkJ were irradiated with UV and/or treated with chemical mutagens followed by determining the survivability and percentage distribution of positive, negative and the corresponding colonies.

Following UV irradiation (2 min), a mutant VkJ2 was obtained that had the larger zone diameter (2.8 ± 0.06 cm). Following exposure, a total of 12% colonies had the higher zone diameter compared to the parental strain and were termed as positive mutants, whereas 5% of the colonies had the lesser zone diameter and denoted as negative mutants; rest 82% had the zone diameter (2.0 ± 0.14 cm) equivalent to the parental VkJ strain, hence termed as the corresponding mutants (Fig. 9a). The parental strain VkJ was also subjected to NTG (100 μg ml^{-1}) and EtBr (200 μg ml^{-1}) treatments. $VkJC_{N20}$ mutant obtained after 20 minutes of treatment with NTG had a zone diameter (2.6 ± 0.1 cm) with 3.9%, 3.9% and 94% respectively were negative, positive and unaltered mutants (Fig. 9b) whereas mutant $VkJC_{E10}$ obtained following the EtBr

treatment (200 μg ml^{-1}, 10 min) had a zone diameter i.e. 2.5 ± 0.12 cm (Table. 11). NTG or EtBr treatment for extended period, led to decreased number of corresponding mutants (Fig. 9b, c).

Among the selected positive mutants, VkJ2 that had resulted into maximal zone diameter was subjected to second stage mutagenesis (UV irradiation and chemical mutagens) to find out if a further improvement in laccase production ability of the strain can be achieved. Strain VkJ2 was further subjected to UV treatment (4.5 min). Distribution percentage obtained was found to be 29%, 45% and 24% for positive, negative and corresponding mutants, respectively (Fig. 9d). The mutant strains thus selected on the basis of zone diameter (Fig 10) were evaluated for the laccase and MnP production under submerged fermentation condition. Among positive mutants, mutant VkJ2.4.5 had the maximum levels of laccase and MnP (3852.5 ± 69.13 Ul^{-1} and 644.3 ± 35.20 Ul^{-1} respectively) production (Table 11) which represented about 80% enhancement in laccase production (1.8 times higher) as compared to the wild type VkJ strain.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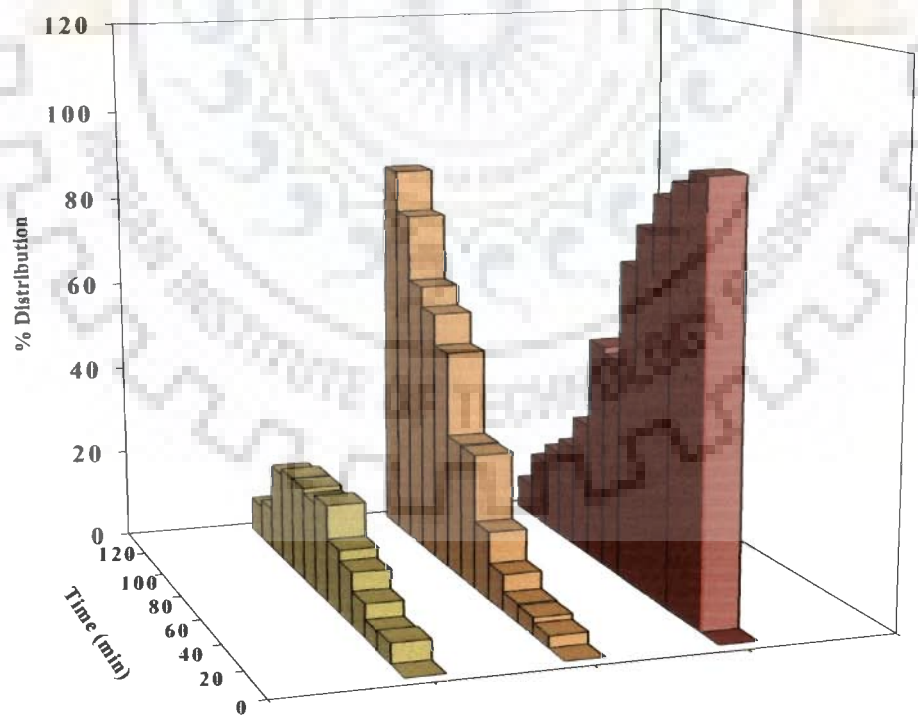
90
80
70
60
50
40
30
20
10
0
Time (min)
10
8
6
4
2

(a)


% Distribution
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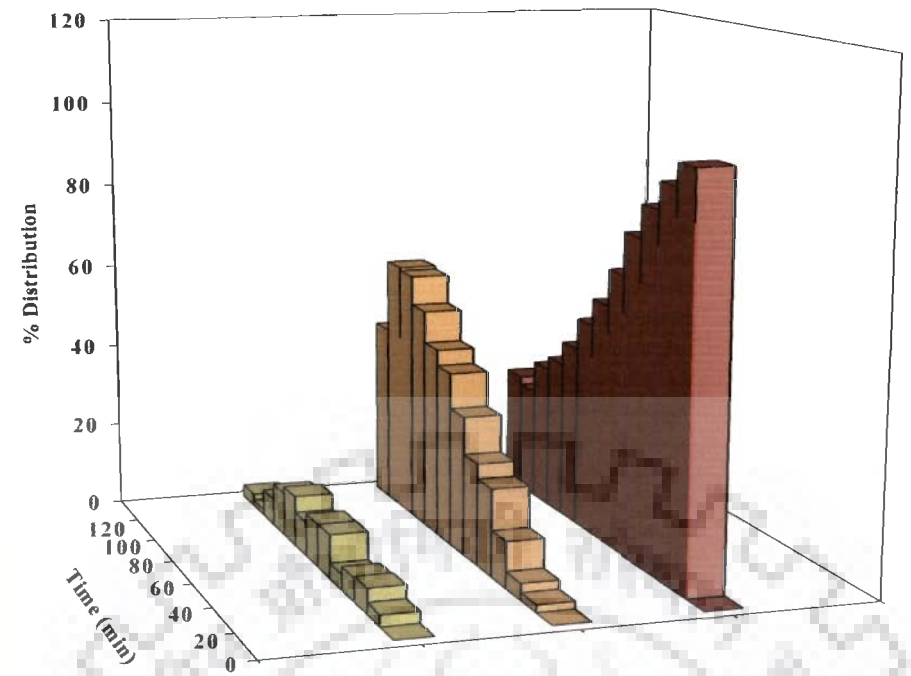
20
0
Time (min)
120
100
80
60
40
20
0

(b)



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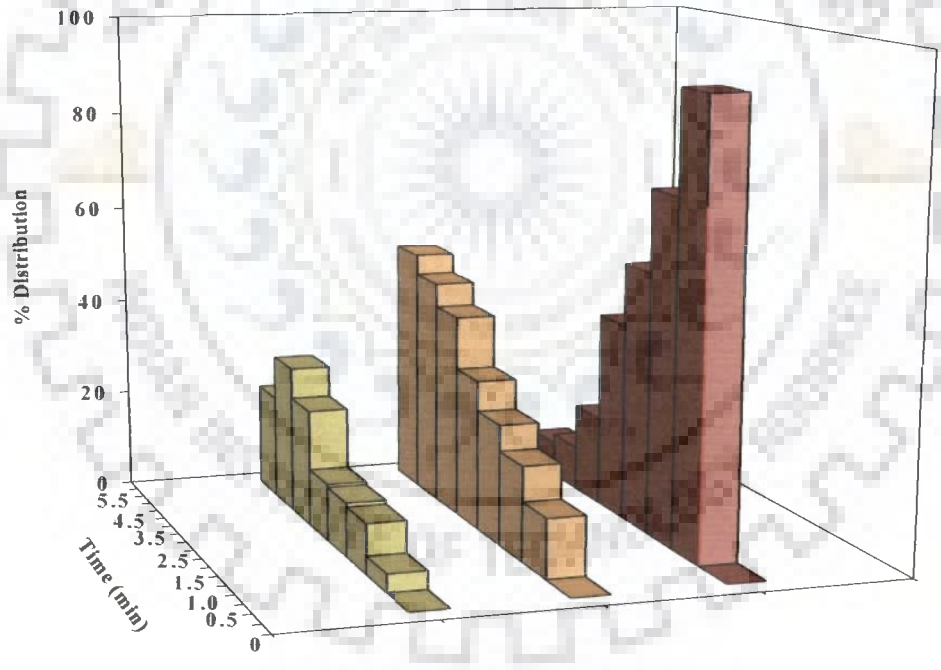
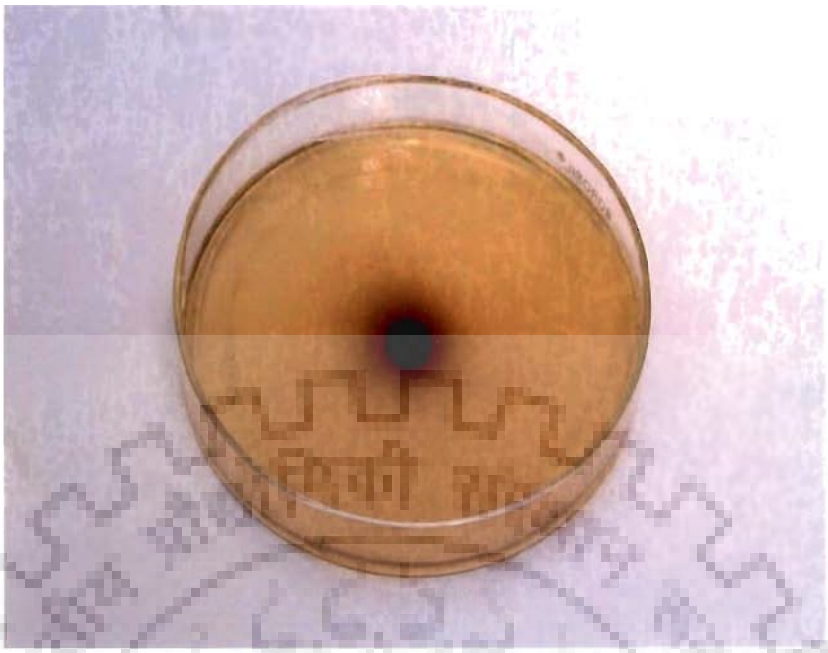


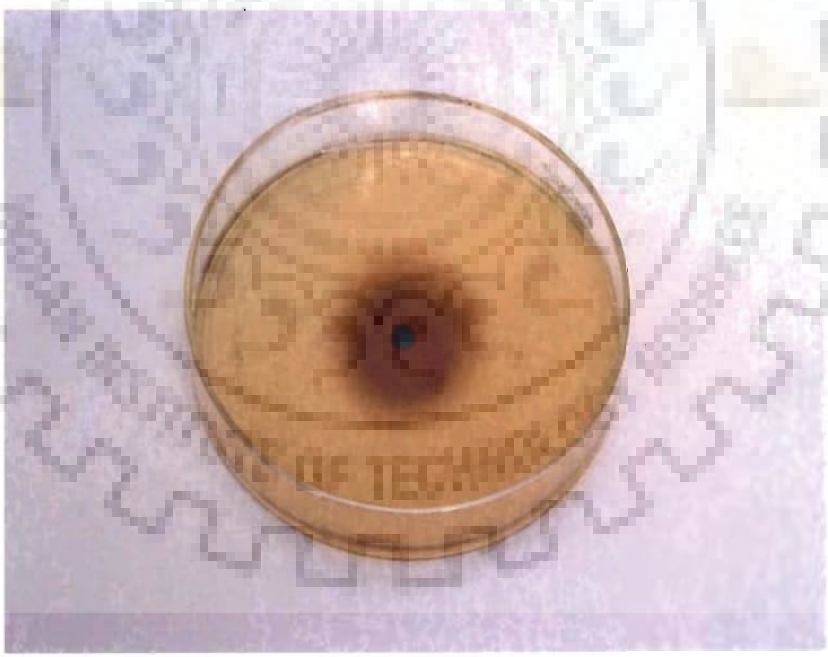
(d)

Fig. 9 Analysis of mutagenesis of *A. fumigatus* VkJ strain (■, ■, ■; represent positive, negative and corresponding mutants)

(a) UV irradiation; (b) N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment; (c) Ethidium Bromide (EtBr) treatment; (d) Double stage (UV+UV) treatment



(a)



(b)

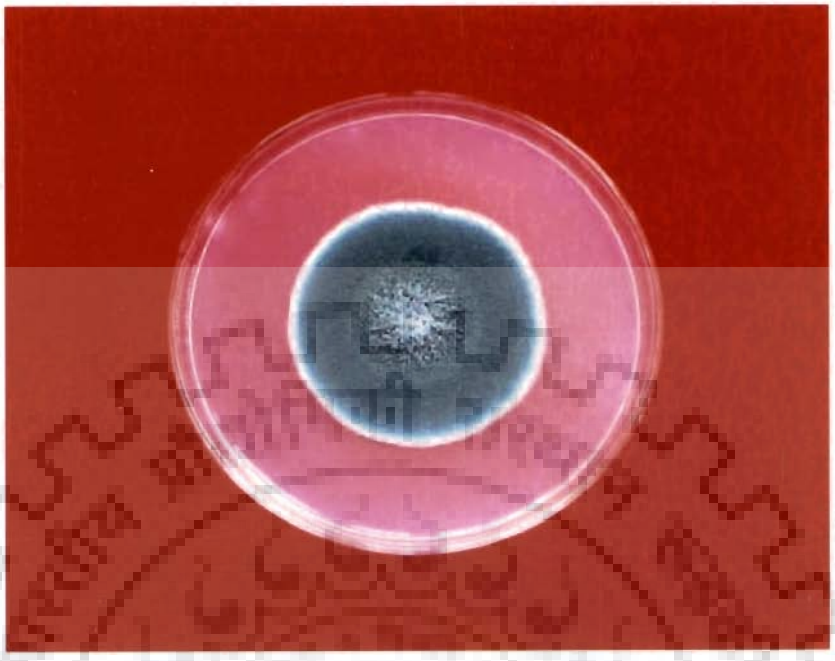
Fig. 10 Zone diameters formed with wild type VkJ (a) and mutant VkJ2.4.5 (b) strains

Table. 11 Selected mutants obtained following mutagenesis of wild type strain *A. fumigatus* VkJ2

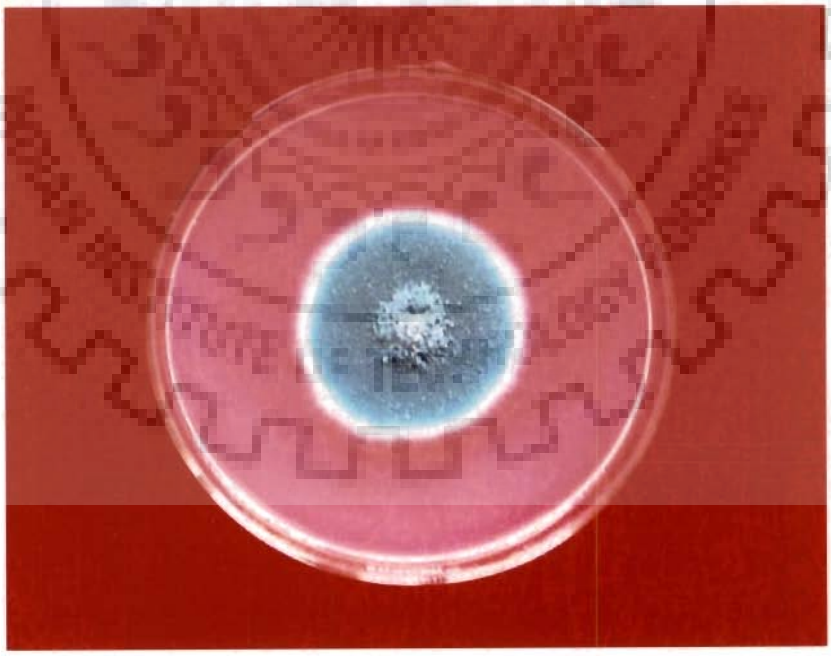
S. No.	Mutagenic agents	Strains of *A. fumigatus*	Zone dia (cm)	Laccase (UI^{-1})	MnP (UI^{-1})
Single Step Mutagenesis					
1.	None	VkJ	2.0 ± 0.14	2108.2 ± 49. 37	446.2 ± 76.29
2.	UV	VkJ2	2.8 ± 0.06	2941.3 ± 42.38	394.4 ± 59.82
3.	NTG	$VkJC_{N20}$	2.6 ± 0.10	2647.6 ± 34.67	375.1 ± 65.66
4.	EtBr	$VkJC_{E10}$	2.5 ± 0.12	2536.5 ± 73.20	524.6 ± 94.74
Mixed Mutagenesis					
1.	UV+UV	VkJ2.4.5	3.5 ± 0.20	3852.5 ± 69.13	644.3 ± 35.20

4.2.1 MORPHOLOGICAL FEATURES

Both wild type and mutant strains could easily be distinguished on the basis of their morphology. Mycelia of wild type strain VkJ were compact and white coloured as compared to the mycelia of the mutant strain VkJ2.4.5 that was loose and creamish white (Fig. 11). However, the spores produced by the parental VkJ strain appeared dark gray unlike that of mutant which was light gray in colour. The finer structural details of the mycelia also showed characteristic features. The hyphae of wild type *A. fumigatus* VkJ were thinner, elongated and ribbon shaped whereas, hyphae of strain VkJ2.4.5 appeared thicker, compact and coiled (Fig 12). In addition, spores of wild type strain *A. fumigatus* VkJ were oval having rougher surface compared to the mutant *A. fumigatus* VkJ2.4.5 that looked flattened with smoother surface (Fig. 13).

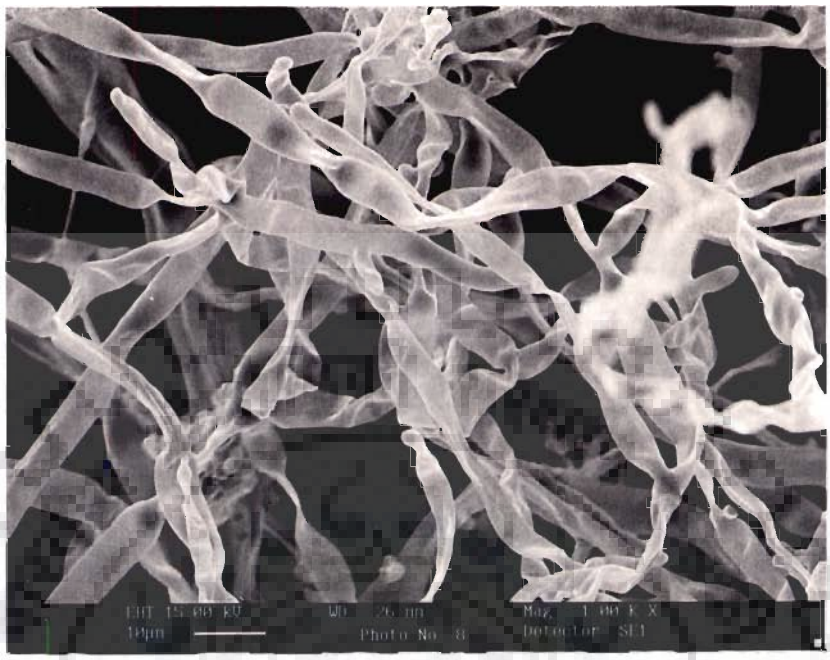


(a)



(b)

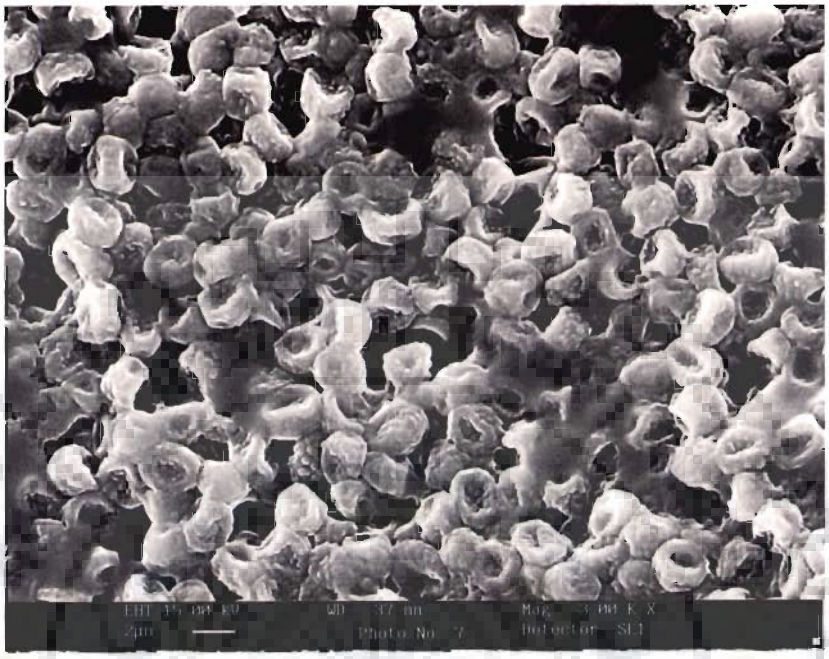
Fig. 11 Morphological features of wild type VkJ (a) and mutant VkJ2.4.5 (b) Strains



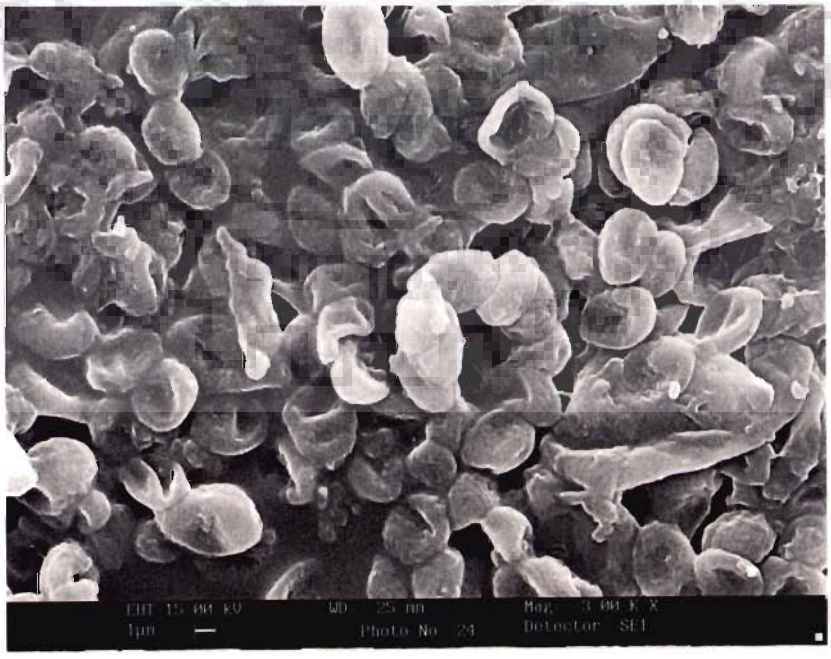
(a)

(b)

Fig. 12 Scanning electron microscopy of mycelia of wild type VkJ (a) and mutant VkJ2.4.5 (b) strains



(a)



(b)

Fig. 13 Scanning electron microscopy of spores of wild type VkJ (a) and mutant VkJ2.4.5 (b) strains

4.3 EVALUATION OF *ASPERGILLUS FUMIGATUS* VkJ2.4.5 FOR ENZYME PRODUCTION IN VARIOUS FERMENTATION SYSTEMS

Enzyme production ability of *A. fumigatus* VkJ2.4.5 strain was analyzed in different fermentation systems i. e. surface, submerged and solid-state fermentation systems (Fig 14a-c). Solid-state fermentation (SSF) using sugarcane bagasse as easily available solid support appeared promising for achieving higher levels of enzymes (4561.6 ± 69.11 Ul^{-1}, 1133.7 ± 57.18 Ul^{-1}). During SSF, maximum levels of laccase and manganese peroxidase were obtained following six days of fermentation (Fig. 14c). Since solid-state fermentation had resulted into maximum levels of production, hence, further studies were carried out using SSF system to derive critical parameters for achieving further, enhanced levels of enzyme production.

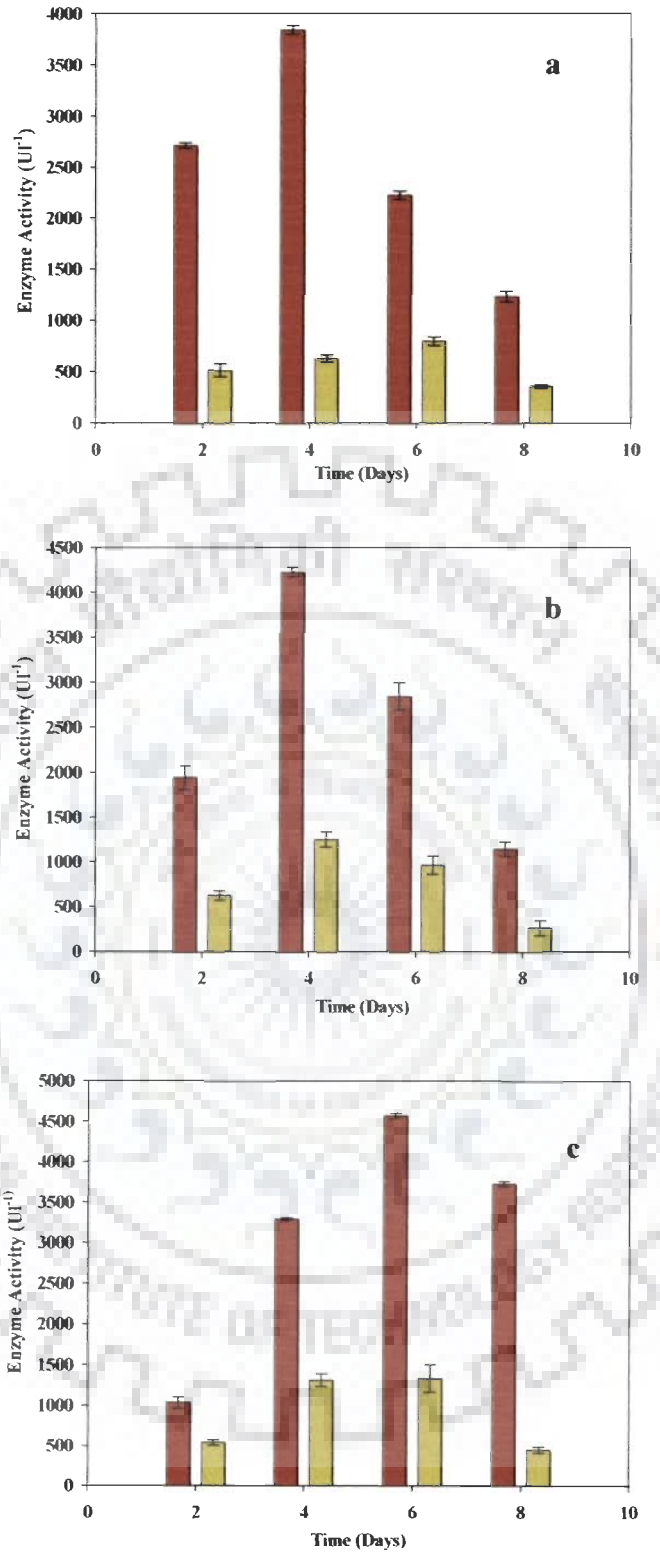


Fig. 14 Enzyme production by *A. fumigatus* VkJ2.4.5 strain under surface (a), submerged (b) and solid-state fermentation systems (c) ■, ■; represent laccase and manganese peroxidase respectively

4.4 ANALYSIS OF SOLID-STATE FERMENTATION CONDITIONS FOR ENZYME PRODUCTION

Solid-state fermentation had resulted into comparatively higher levels of the laccase and manganese peroxidase production as compared to other two fermentation systems. Therefore, in order to economize the process and attain further higher levels of production various low cost solid supports or the ones that are available freely were evaluated.

4.4.1 Analysis of the substrates for enzyme production under solid-state fermentation

A variety of agro-horticultural residues that are easily and abundantly available either at considerably low-price or with no cost, were chosen as substrate for enzyme production (Fig. 16). All solid supports significantly encouraged the growth and enzyme production by mutant *A. fumigatus* VkJ2.4.5 strain but among all, banana peel led to maximum levels of laccase (5792.0 ± 40.95 Ul^{-1}) and considerable levels of manganese peroxidase (1334.6 ± 167.32 Ul^{-1}) production (Fig. 15). The order of the substrate suitability for enzyme production was; Banana peel > Bagasse > Wheat Bran > Poplar leaves > Wheat straw > Rice bran. This showed that all the substrates could serve as source of carbon and energy for growth of the fungus but luxuriant growth of the VkJ2.4.5 strain was observed on banana peel which indicated this to be most suitable matrix for colonization and fermentation and also a cost effective matrix for fermentation. Hence, banana peel was selected for further studies. A significant variation ($P < 0.01$) in enzyme production was observed with various solid supports (Table 12).

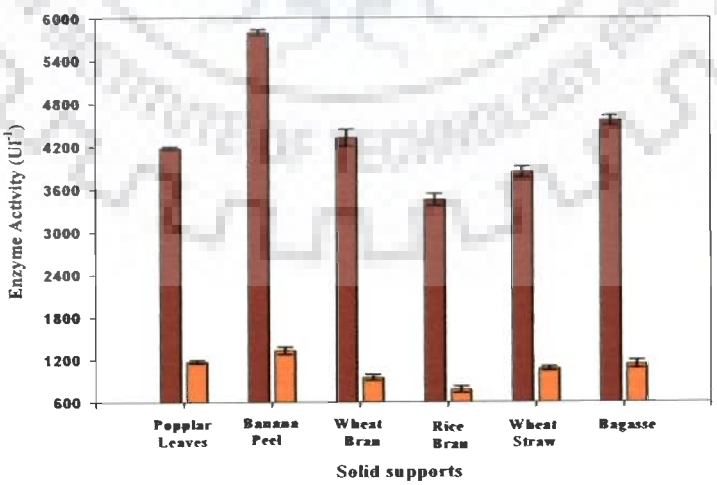


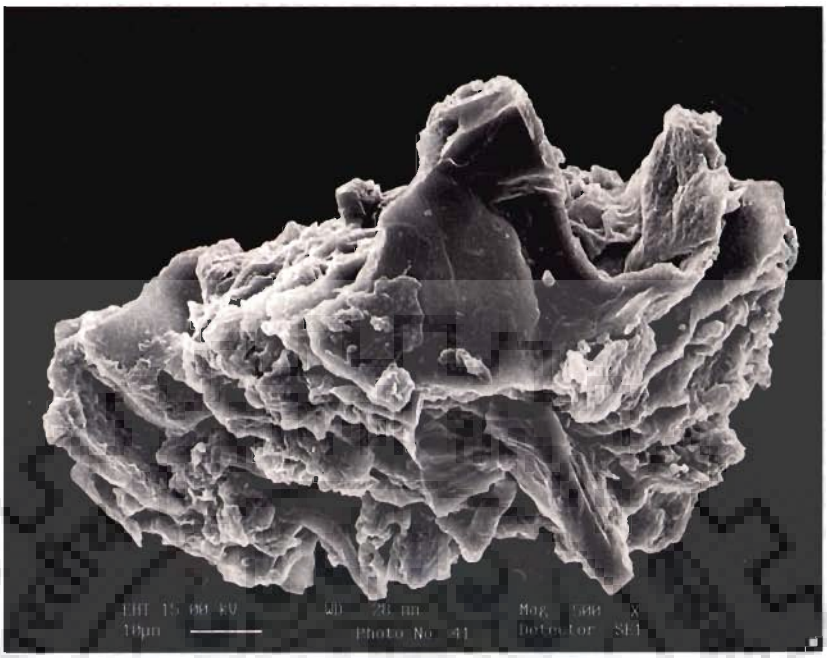
Fig. 15 Evaluation of different solid supports for enzyme production by mutant *A. fumigatus* VkJ2.4.5 strain (■ ■; represent laccase and manganese peroxidase levels respectively)

Fig. 16 Different solid supports used during solid-state fermentation

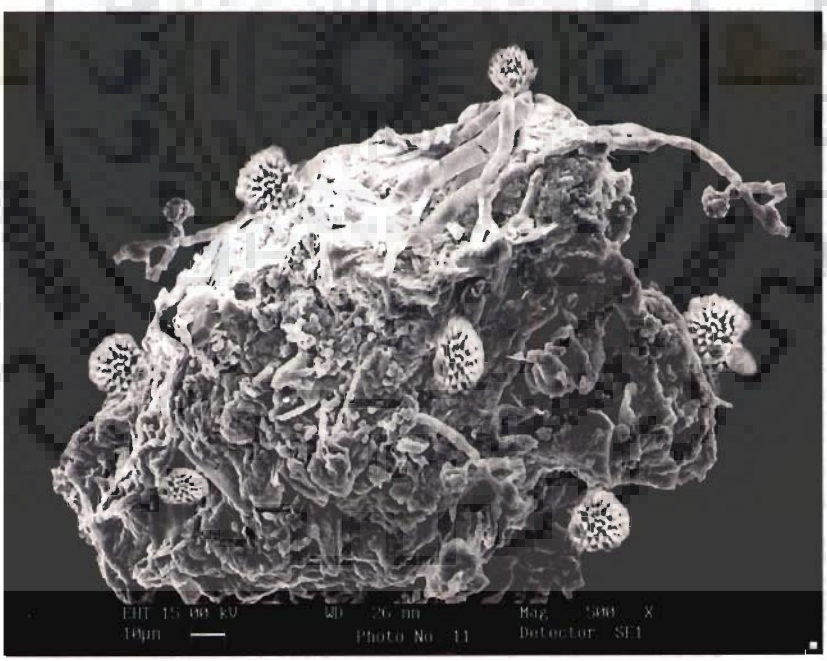
(a) Banana peel; (b) Bagasse; (c) Wheat Bran; (d) Poplar leaves; (e) Wheat straw; (f) Rice bran

4.4.2 Banana peel in solid state fermentation

As observed through scanning electron microscopy, morphological features of banana peel seemed to be supportive for fungal entrapment and anchorage. Uninoculated banana peel appeared porous and hence emerged as a better substrate for fungus to adhere and penetrate. Banana peel following inoculation had profound colonization by *A. fumigatus* VkJ2.4.5. Thus, it seems to be a suitable matrix supporting the metabolic activity of fungus (Fig. 17).



(a)



(b)

Fig. 17 Scanning electron micrograph of uninoculated particle of banana peel

(a) and (b), banana peel entrapped with *A. fumigatus* VkJ2.4.5 under solid state fermentation (4 days old)

4.4.3 Analysis of the amount of substrate for enzyme production under solid-state fermentation

Banana peel in different amounts (2-10 g) was evaluated to find out its effect on enzyme production. It was observed that 4g of substrate was most suitable for production of laccase (5797.3 ± 101.78 UI^{-1}), where as MnP secretion (1348.3 ± 92.27 UI^{-1}) was higher at 6g of substrate concentration (Fig. 18). At various amounts of substrate significant variation ($P < 0.01$) was observed in enzyme production (Table 12).

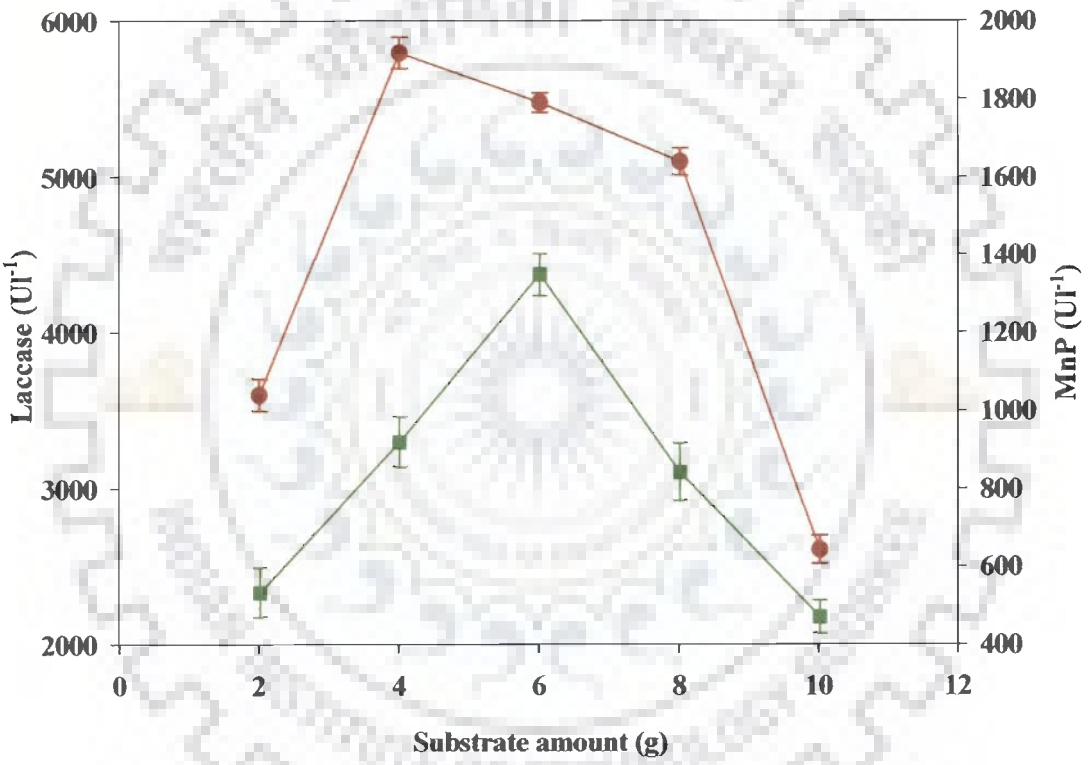


Fig. 18 Effect of amount of the substrate on enzyme production under solid-state fermentation by *A. fumigatus* VkJ2.4.5 (●, ■; represent laccase and manganese peroxidase levels respectively)

4.4.4 Analysis of particle size of banana peel for enzyme production

Particle size of particular substrate is a major parameter in SSF for microbial anchorage, growth and activity. Agro-horticultural residues of different particle size are employed as the solid matrix for SSF process. The fine particles (0.1-0.5cm) of banana peel were observed to be more supportive for laccase and MnP production (5841.0 ± 77.44 UI^{-1}, 1149.6 ± 146.58 UI^{-1} respectively). The fine particles appeared to be suitable for fungal entrapment, growth and led to maximal enzyme production (Fig. 19). A significant variation ($P < 0.01$) in enzyme production was observed with various particle size of solid support (Table 12).

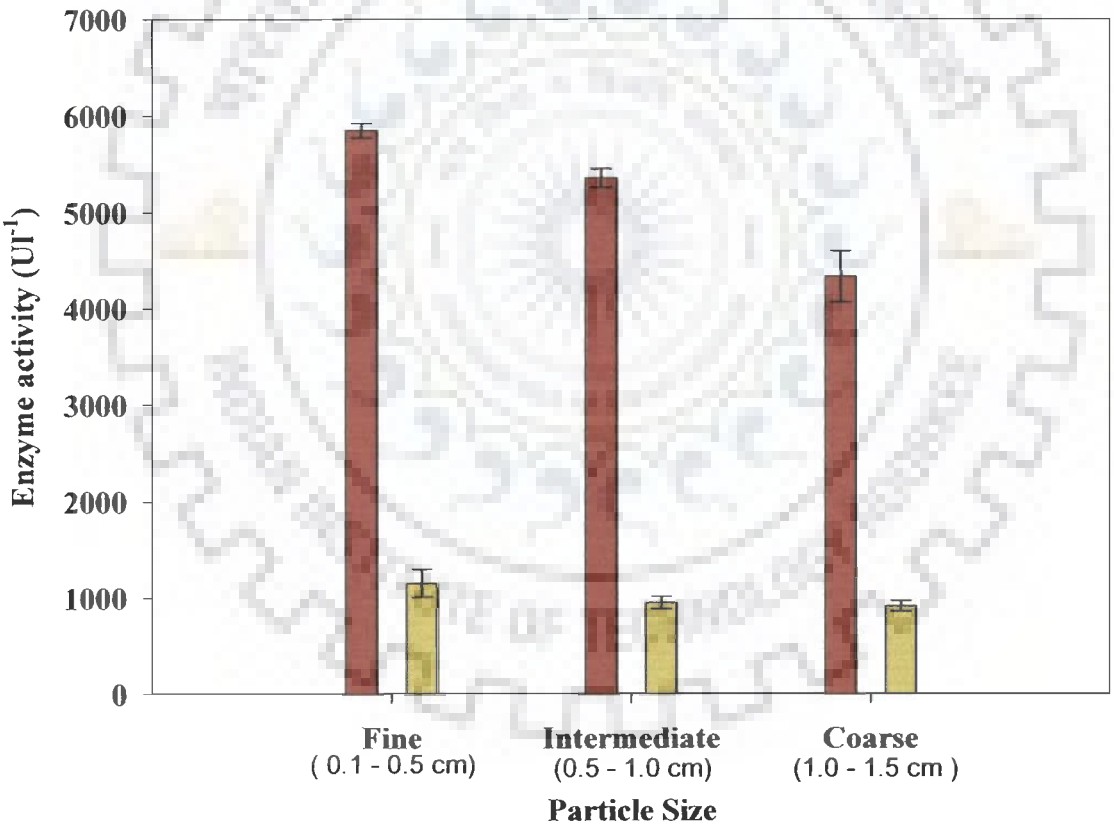


Fig. 19 Effect of banana peel particle size on enzyme production under solid-state fermentation by *A. fumigatus* VkJ2.4.5 (■, ■; represent laccase and manganese peroxidase levels respectively)

4.4.5 Analysis of the moisture level

SSF is distinct from the submerged fermentation by the fact that microbial growth and product formation occurs at low moisture content. The effect of moisture level on enzyme production is depicted in fig. 20. Moisture level at 80% was detected to be appropriate for maximal levels of laccase production (5873.2 ± 34.07 Ul^{-1}), notable level of MnP was also detected (1359.3 ± 141.01 Ul^{-1}). Too low or higher levels of moisture didn't support the enzyme production. At various levels of moisture a significant variation ($P < 0.01$) was observed in enzyme production (Table 12).

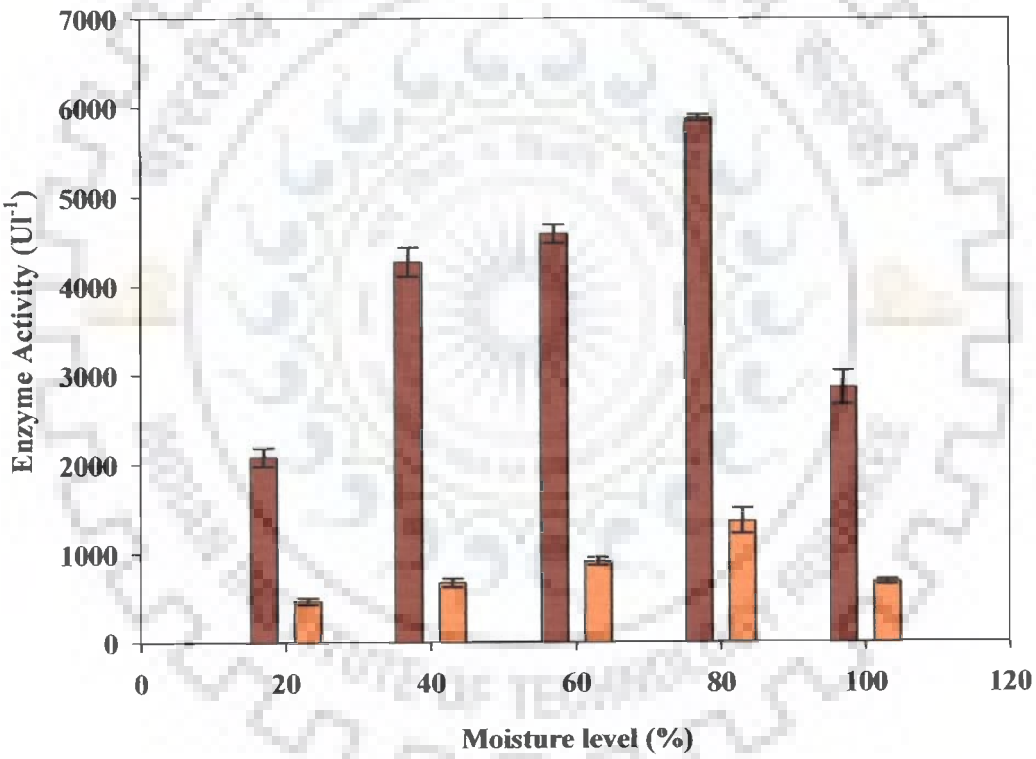


Fig. 20 Effect of the moisture level (%) on enzyme production during solid-state fermentation (■, ■; represent laccase and manganese peroxidase levels respectively)

4.4.6 Analysis of additives on laccase production

The stimulating effect of various additives was also evaluated for enhancing the production of enzymes. Among the various additives used, incorporation of yeast extract (1%)

was found to be promotive for laccase (6205.3 ± 123.13 Ul^{-1}) and equally supportive for MnP production (1339.0 ± 131.23 Ul^{-1}). However, a slight increment was also observed by employing cheese whey and molasses (Fig. 21).). A significant variation ($P < 0.01$) in enzyme production was observed with various additives (Table 12).

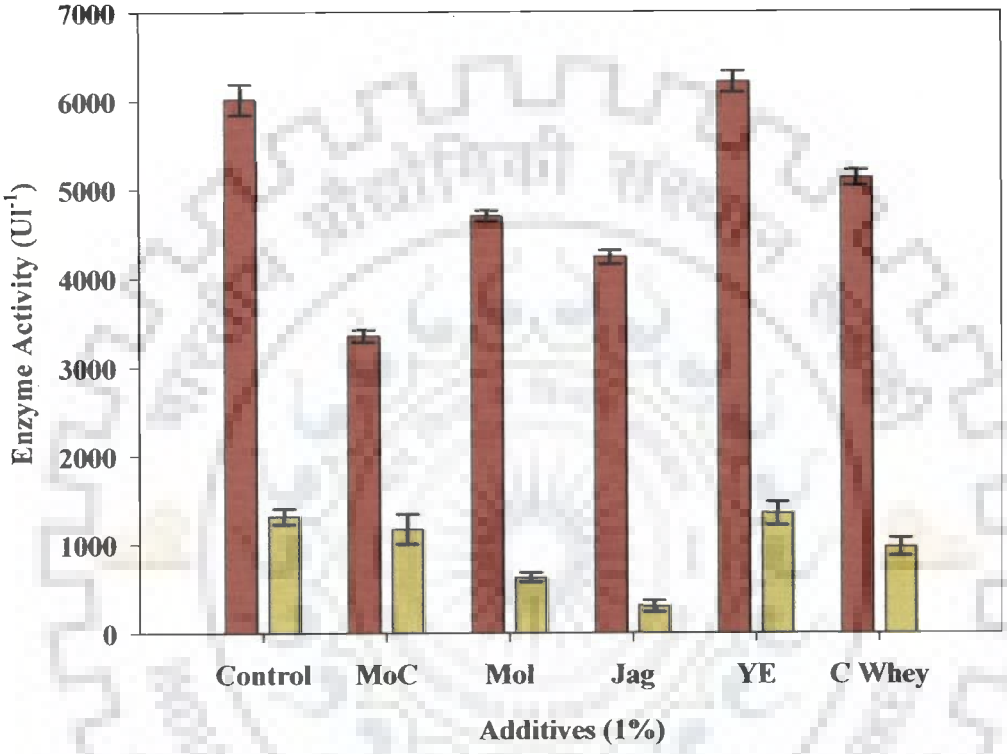


Fig. 21 Effect of different additives on enzyme production during solid-state fermentation

(■, ■; represent laccase and manganese peroxidase levels respectively)

4.4.7 Analysis of aeration volume on laccase production

Aeration plays crucial role in SSF system for laccase production. Different volumes of sterile air were supplemented into the fermentation media to evaluate the influence of the same. An aeration level of 2.5 l min^{-1} led into maximum levels of production (6281.4 ± 63.60 Ul^{-1}). A

further increase in aeration level was not supportive for production (Fig. 22).). A significant variation ($P < 0.01$) in enzyme production was observed at various aeration volume (Table 12).

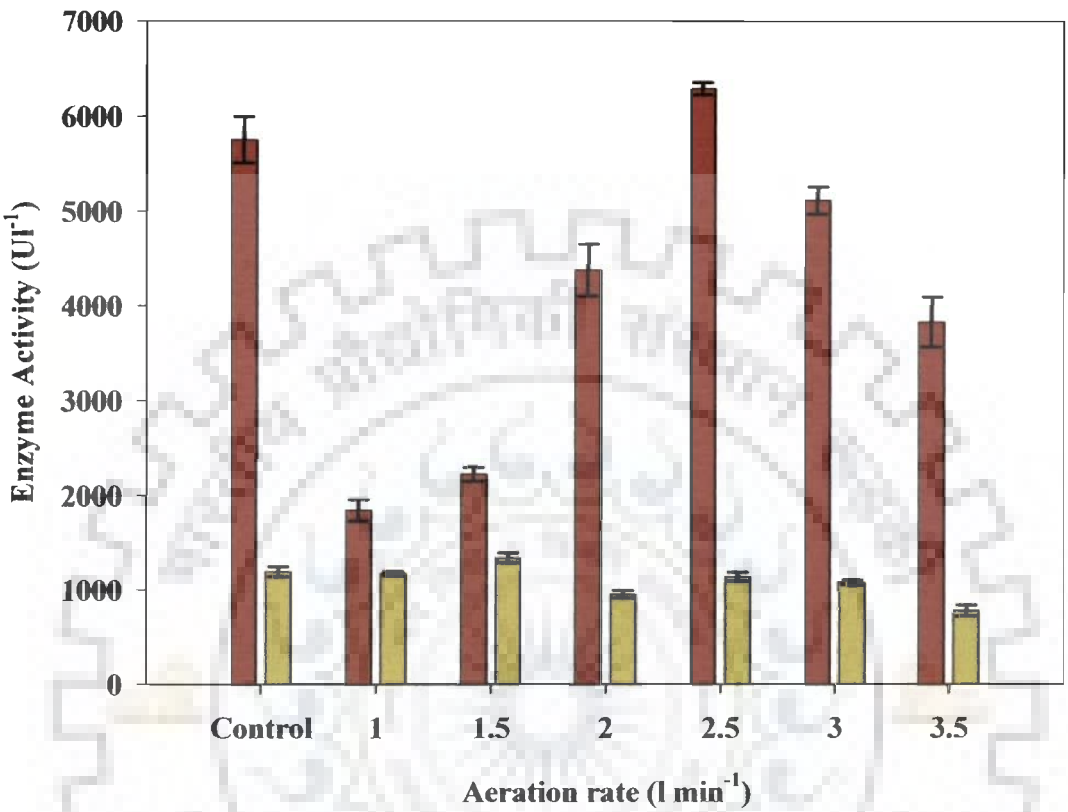


Fig. 22 Effect of aeration volume on enzyme production during solid-state fermentation

(■ , ■; represent laccase and manganese peroxidase levels respectively)

ANOVA was performed to assess the statistical significance of differences of means between more than two groups. Variations in the enzyme production under solid-state fermentation process were studied by the statistical analysis and results of ANOVA are given (Table. 12).

Table. 12 Analysis of variance for different parameters for laccase production by mutant VkJ-2.4.5 under solid-state fermentation

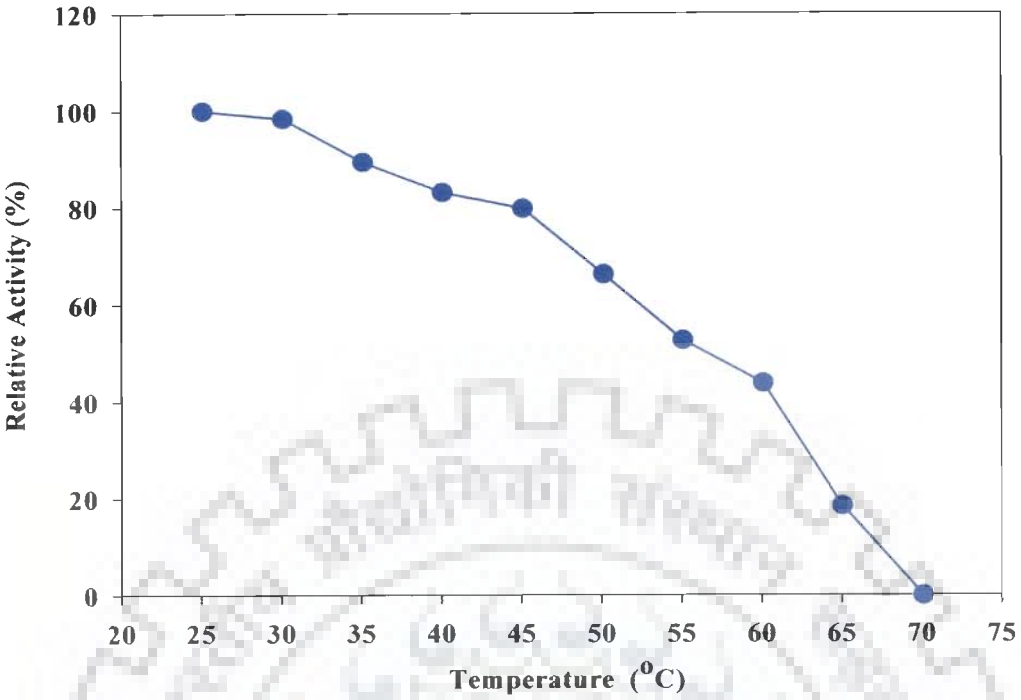
Parameters	Variation in laccase production	Degree of freedom	Sum of square	Mean square	F ratio observed
Solid support	Among samples	5	9296061	1859212	110.2934
	Within samples	2	36110.33	18055.17	1.07108
	Error	10	168569.7	16856.97	
Particle size	Among samples	1	366548.2	366548.2	16.4455
	Within samples	2	28617.33	14308.67	0.641971
	Error	2	44577.33	22288.67	
Substrate amount	Among samples	4	26804226	6701056	335.3474
	Within samples	2	13322.53	6661.267	0.333356
	Error	8	159859.5	19982.43	
Moisture level	Among samples	4	26804226	6701056	335.3474
	Within samples	2	13322.53	6661.267	0.333356
	Error	8	159859.5	19982.43	
Additives	Among samples	4	13513362	3378341	129.3723
	Within samples	2	25043.2	12521.6	1.322504
	Error	8	75744.8	9468.1	
	Among samples	8	75744.8	9468.1	

* Significant at 1% level

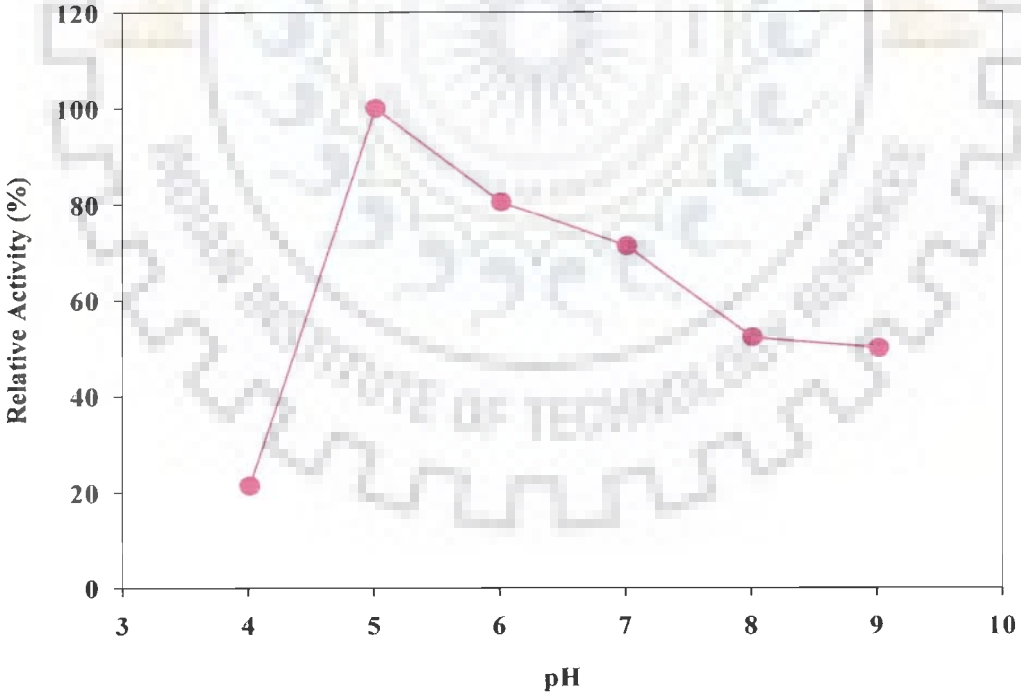
4.5 BIOCHEMICAL CHARACTERIZATION OF LACCASE PRODUCED BY *A. FUMIGATUS* VkJ2.4.5 UNDER SOLID-STATE FERMENTATION

4.5.1 Temperature and pH stability of the laccase

To evaluate the thermal and pH stability of enzyme, broth was kept for 2 hours at various temperatures and enzyme activity thereafter, was determined. Laccase appeared to be quite stable upto a temperature of 50°C for 2 h. Increase in temperature up to 60°C led to a 50 % loss in activity (Fig. 23a). The enzyme activity of fungus is greatly influenced by H^+ concentration. Laccase was found to be stable between the pH of 5.0-9.0 and retained upto 50% of the relative activity at pH 8.0. Higher and lower pH decreased the enzyme activity (Fig. 23b). In general, laccase activity has a bell shaped profile with an optimal pH that varies considerably.



(a)



(b)

Fig. 23 Temperature (a) and pH (b) stability of laccase obtained from *A. fumigatus* VkJ2.4.5

4.5.2 Zymogram Analysis

The protein profile of mutant VkJ2.4.5 and wild type VkJ was compared by analyzing the fermentation broth. SDS-polyacrylamide gel analysis had shown increased levels of 34±1 kDa protein from mutant VkJ2.4.5 as compared to that from wild type VkJ. A protein (22 kDa) was also detected in the protein profile of mutant VkJ2.4.5 (Fig. 24a). The zymogram analysis of the partially purified broth indicated an enhanced level of a 34 kDa protein in the mutant VkJ2.4.5 (Fig. 24b) than the wild type strain. This corresponded to the higher laccase activity as observed in the mutant strain than the wild type strain.

4.5.3 Determination of internal peptide sequence

The internal amino acid sequencing of laccase was performed by MALDI-ToF/ToF analysis that provides significant new insights into the nature of laccase and also the sequence homology search observations. Enzyme from *A. fumigatus* VkJ2.4.5 possesses resemblance to those of reported laccase in ascomycetes, actinomycetes, plants and basidiomycetes. All seven peptides generated during MALDI ToF/ToF analysis of the *Aspergillus fumigatus* VKJ2.4.5 laccase were subjected to the homology search with other laccase from multi copper oxidase family. The amino acid sequence of the generated peptides showed high levels of identity with laccase of ascomycetes mainly from *Fusarium oxysporum* strains (50%), *Phaeosphaeria* sp. (54%); actinomycetes like *Streptomyces cyaneus* (50%) and *Strongylocentrotus* sp. (50%) and plants like *Arabidopsis thaliana* (67%) and *Rhus vernicifera* (50%). Further, low identity level was observed with laccase from basidiomycetes like *Pleurotus* sp. (35%), *Trametes* sp. (33%) and *Pycnoporus sanguineus* (27%) (Table 13).

4.5.4 Analysis of catabolic activity of laccase

As the reaction time of enzyme-substrate (guaiacol) is prolonged, rate of degradation of substrate also increases. It has been observed that monophenols having hydroxy or methoxy groups were most affected by the enzyme treatment. During HPLC analysis, it was observed that original substrate tend to disappear and new, unidentified components appeared following enzymatic treatment. Most of the compounds affected by laccase contain free phenolic hydroxy group that included substituted *o* and *p*-dihydroxybenzene, trihydroxybenzene and *p*-phenylene-diamine (Fig. 25).

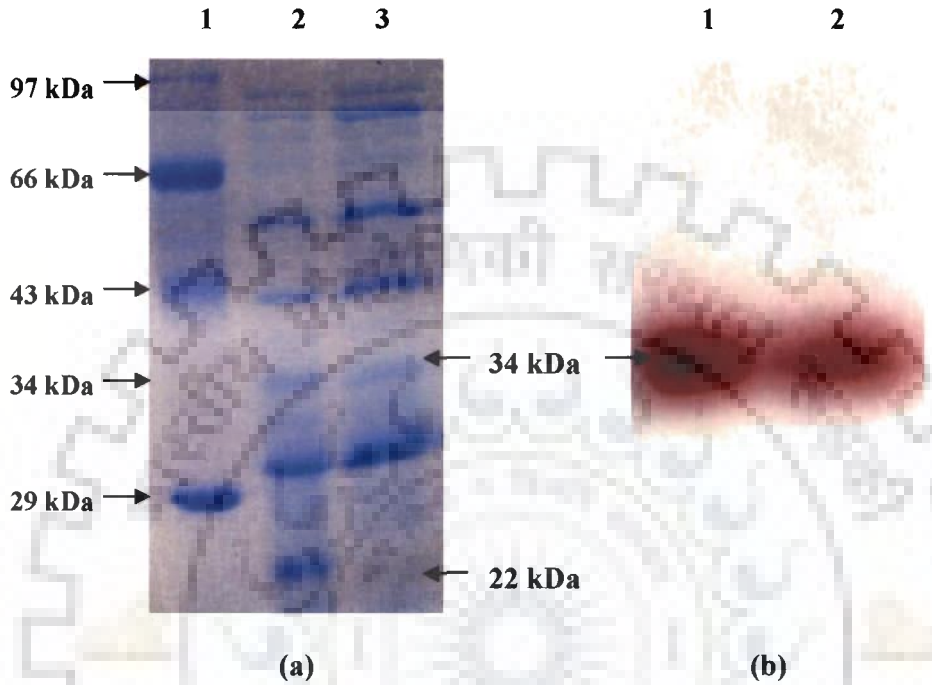


Fig. 24 SDS-PAGE (a) and Zymogram (b) analysis of proteins produced by mutant *A. fumigatus* VkJ2.4.5 and wild type *A. fumigatus* VkJ after 4 days of incubation

a; Lane 1, marker protein; Lane 2, protein profile of mutant *A. fumigatus* VkJ2.4.5; Lane 3, protein profile of *A. fumigatus* VkJ, b; Zymogram analysis Lane 1, laccase activity band of mutant *A. fumigatus* VkJ2.4.5; Lane 2, laccase activity band of wild type *A. fumigatus* VkJ

Table. 13 Sequence homology for internal peptides of laccase by NCBI BLAST short sequence search

Accession no	Organism		Sequence		Identity %
Peptide 1	*Aspergillus fumigatus*	39	-MMMIER-	44	-
ABS19941	*Fusarium oxysporum*	582	GMMMVIQD	589	50
NP195724	*Arabidopsis thaliana*	433	KMMFPERK	440	67
BAB63411	*Rhus vernicifera*	410	LMIEYGEA	417	50
Peptide 2	*Aspergillus fumigatus*	45	-VRGEEEER-	52	-
ABP49580	*Streptomyces cyaneus*	103	LVRDDEEDAL	112	50
NP199621	*Arabidopsis thaliana*	166	DVRVEVEEFV	175	63
XP00120228	*Strongylocentrotus sp*	142	IVREPEEDNP	148	50
Peptide 3	*Aspergillus fumigatus*	15	-KGGEEEGEAK-	24	-
ABS19943	*Fusarium oxysporum*	239	DGIYTEEAEAEM	250	40
AAN17283	*Phaeosphaeria sp*	77	HVSGEGVGEYAK	85	40
XP001177603	*Strongylocentrotus sp*	436	NRMQNEEGEAIH	447	50
Peptide 4	*Aspergillus fumigatus*	16	-GGEEEGEAKGR-	26	-
ABS19941	*Fusarium oxysporum*	264	GLGNEEALLPGTF	276	36
ABK58288	*Streptomyces sp*	249	PAGEHPAELDGRL	261	45
AAN17283	*Phaeosphaeria sp*	75	VSGEGVGEAKMNV	87	54
XP001177603	*Strongylocentrotus sp*	437	RMQNEEGEAIHWH	449	45
Peptide 5	*Aspergillus fumigatus*	1	-MPLALDSDGGESAR-	14	-
ABS19942	*Fusarium oxysporum*	484	VEDPLALQASLKLPQNH	498	29
ABP49580	*Streptomyces cyaneus*	214	PGIVHQIGSDGGLLPRP	228	29
AAN17292	*Phaeosphaeria halima*	216	QDMPKALEVGFDDDLGS	232	29
Peptide 6	*Aspergillus fumigatus*	27	-QMTREWLSTLGR-	38	-
ABS19938	*Fusarium oxysporum*	430	YNSPTLLLSKLGNH	443	33
ABK58288	*Streptomyces sp*	74	VIVLDDWLDGIGRT	87	33
XP789245	*Strongylocentrotus sp*	215	VIMMSDWTDTLSMQ	228	33
Peptide 7	*Aspergillus fumigatus*	1	-MPLALDSDGGESARK-	15	-
ABS19942	*Fusarium oxysporum*	483	VEDPLALQASLKLPQNH	499	27
ABP49580	*Streptomyces cyaneus*	212	PGIVHQIGSDGGLLPRP	228	27
AAN17292	*Phaeosphaeria halima*	215	QDMPKALEVGFDDDLGS	231	27

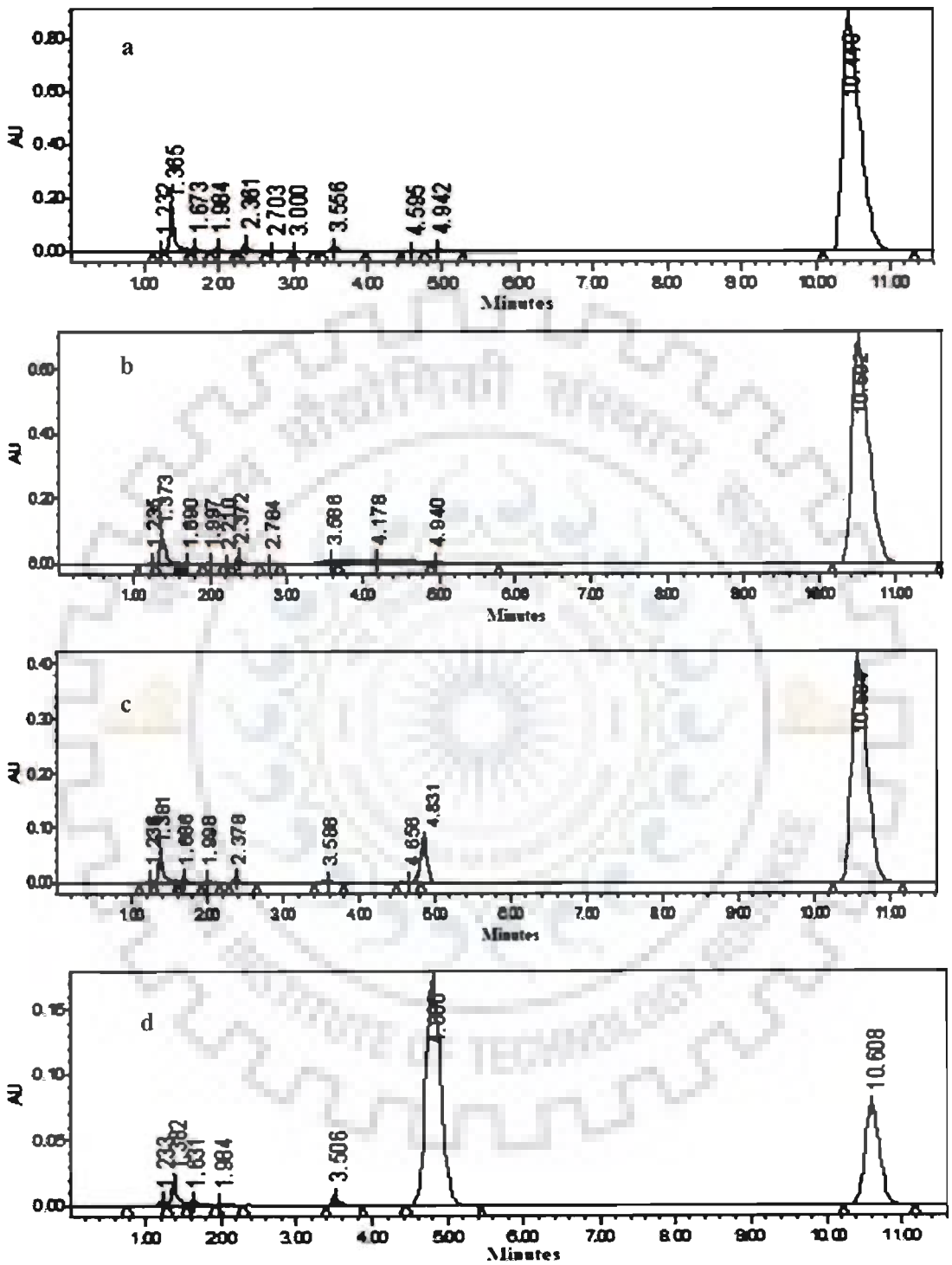


Fig. 25 HPLC analysis of untreated (a) and laccase treated guaiacol (b; 1hr, c; 2hr, d; 3 hr)

4.6 EVALUATION OF LACCASE FROM MUTANT *A. FUMIGATUS* VKJ 2.4.5 FOR BLEACHING OF PAPER PULP

4.6.1 Derivation of reaction conditions for enzymatic bleaching

4.6.1.1 Enzyme dose and reaction time

Mixed wood pulp was treated with different dosage of enzyme (0-25 U g^{-1} of odp). An enzyme dose of 10 U g^{-1} of odp was found to be suitable to achieve higher bleaching effects (Fig. 26a). Treatment time of 2 h was found to be effective when 10 U g^{-1} of laccase was used for pretreatment of mixed wood pulp. Further, prolonged treatment had no notable impact on pulp bleaching (Fig. 26b).

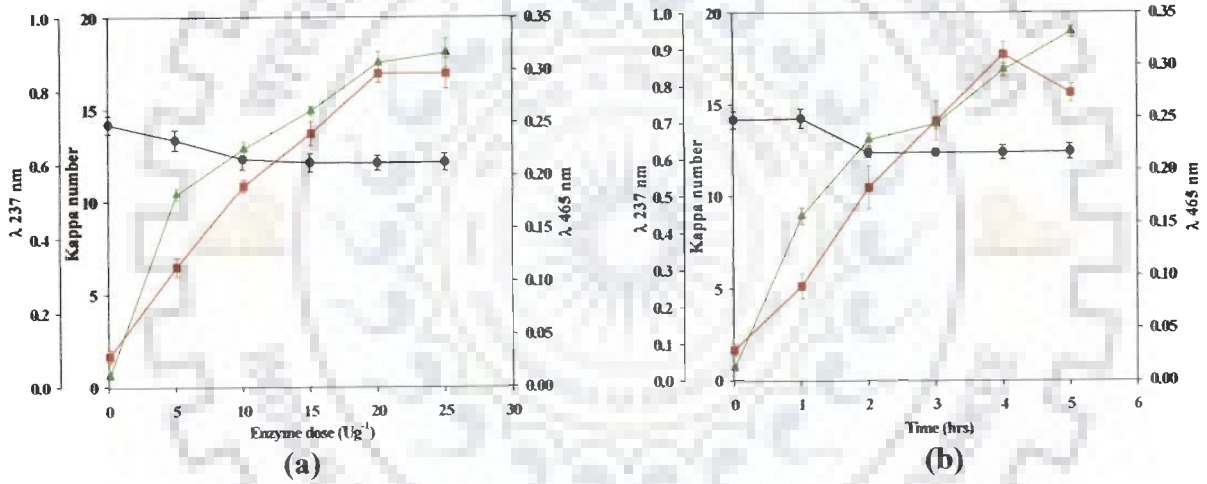
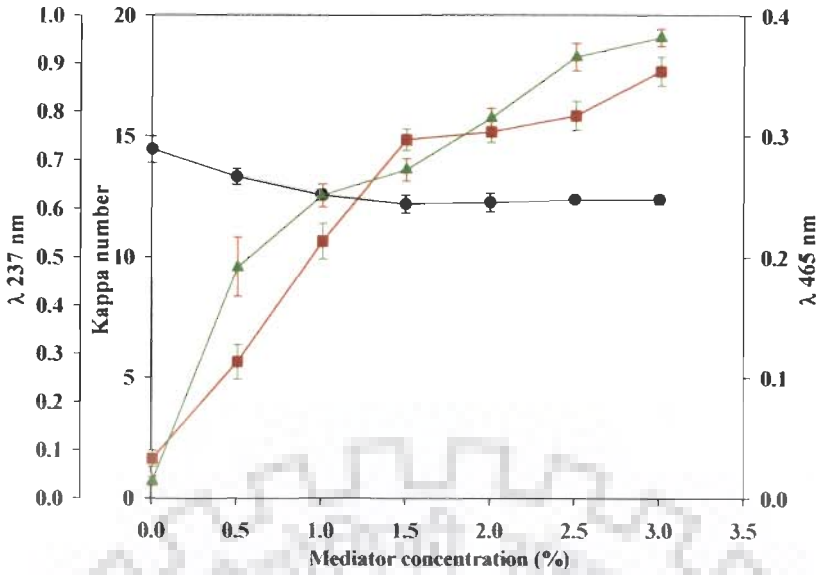


Fig. 26 Derivation of enzyme dose (a) and reaction time (b) for enzymatic treatment of pulp (●, ■ ▲; represent kappa number, release of hydrophobic and phenolic compounds at $\lambda 465_{nm}$, $\lambda 237_{nm}$ respectively)

4.6.1.2 Mediator concentration

Addition of a mediator, HBT (1.5%, w/w) with enzyme in prebleaching was found promising. Further, increased concentration of HBT had no significant impact on pulp bleaching (Fig. 26c).



(c)

Fig. 26 Derivation of mediator concentration for enzymatic treatment of pulp

(●, ■, ▲; represent kappa number, release of hydrophobic and phenolic compounds at $\lambda 465_{nm}$, $\lambda 237_{nm}$ respectively)

Treatment of mixed wood pulp with laccase mediator system under derived conditions (10 U g^{-1}, 2 h, 1.5% HBT,) had resulted into increment in brightness (7%), decrease in kappa number (14%) and yellowness (4%) (Table. 14).

Table. 14 Effect of laccase-mediator system on mixed wood pulp

S. No.	Parameter	Initial	After treatment	% Improvement
1	Kappa number	14.0 ± 0.55	12.0± 0.36	14.28
2	Brightness	35.7± 0.62	38.3 ± 0.86	7.3
3	Yellowness	30.4 ± 0.42	29.0 ± 0.63	4.6

Release of phenolic and hydrophobic compounds in effluent during enzymatic bleaching were also analyzed spectrophotometrically by measuring the absorbance at 237 nm and 465 nm

respectively. Enzymatic treatment of pulp under derived conditions was notably effective and associated with maximum release of chromophores (Fig. 27a-c). Further increase in enzyme dose did not affect the release of chromophores.

4.6.2 Colour removal from the pulp

Following bleaching of pulp under derived conditions, effluent was subjected to scanning (200-400 nm) for evaluating the lignin removal. The peak observed at 280 nm in the UV spectra indicated the presence of lignin in the effluent that became prominent with increase in the enzyme dose (Fig. 27).

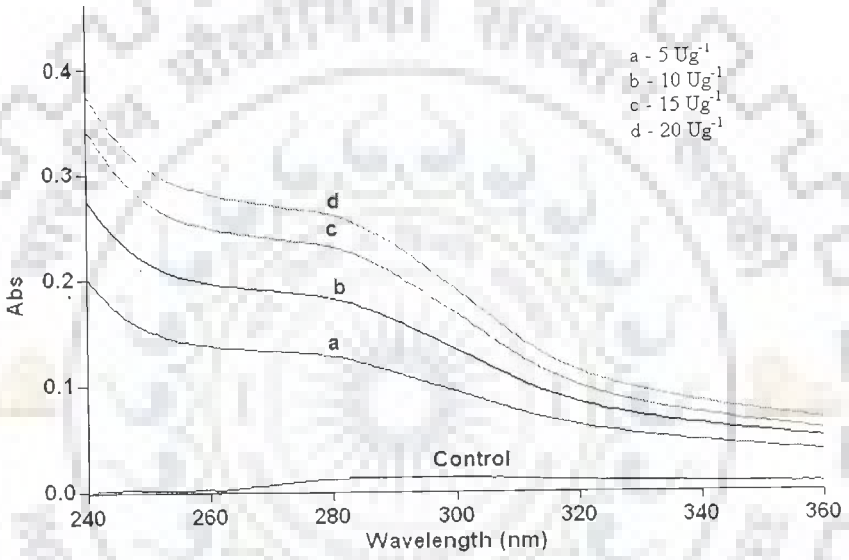
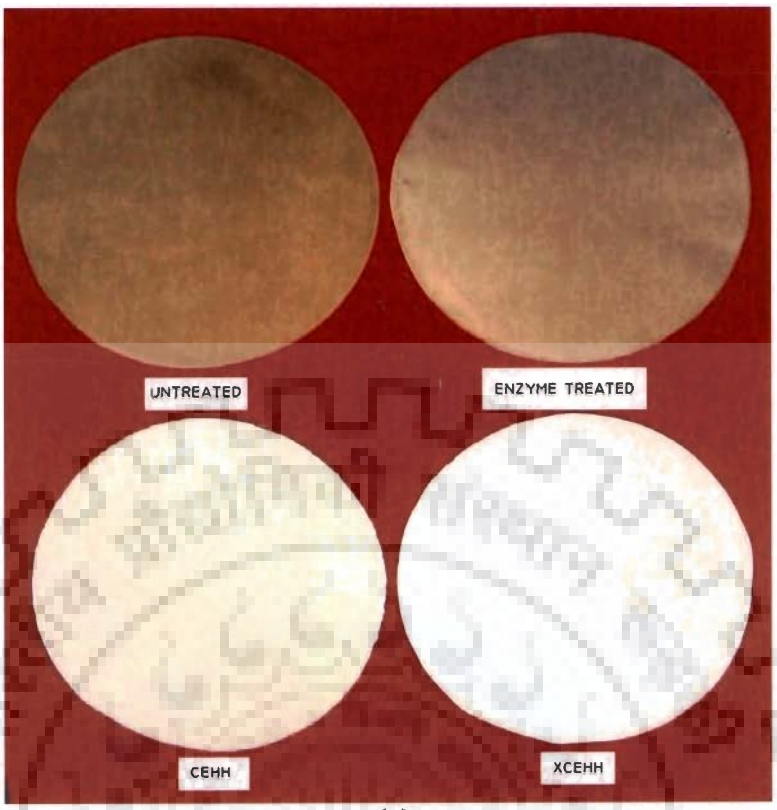


Fig. 27 UV absorption spectrum of effluent released during enzymatic treatment (0-20 U g^{-1}) of pulp

4.6.3 Analysis of pulp and paper properties

Following enzymatic pre-treatment of pulp handsheets were made. Strength properties of pulp and papers in both the sequences (CEHH, XCEHH, ODED and XODED) were evaluated. XODED works better than XCEHH sequence. Observations had denoted improved pulp and paper properties following biobleaching stages where kappa number and yellowness has decreased (14% and 4% respectively), brightness has increased (7%). Similarly paper properties also had improved (Fig. 28, Table. 15).



(a)

(b)

Fig. 28 Colour of the handsheets made from mixed wood pulp (a) CEHH; **(b)** ODED

Table. 15 Effect of enzymatic treatment on pulp and paper properties

		Pulp properties				Paper properties		
Sl. No.	Bleaching sequences	Kappa No	Viscosity (cp)	Brightness (% ISO)	Yellowness (%)	Tensile Strength ($N\ mg^{-1}$)	Tear Index (mNm^2g^{-1})	Burst Factor ($KPam^2g^{-1}$)
1.	Control	14.0 ± .55	10.7 ± 0.08	35.7 ± 0.62	30.4 ± 0.42	-	-	-
2.	Treated	12.0 ± .36	11.2 ± 0.12	38.3 ± 0.86	29.0 ± 0.63	-	-	-
3a.	CEHH	-	9.0 ± 0.12	71.53 ± 0.65	21.43 ± 0.6	42.44 ± 0.90	8.34 ± 0.36	3.90 ± 0.16
3b.	XCEHH	-	9.6 ± 0.10	77.46 ± 0.44	16.1 ± 0.17	38.32 ± 0.53	7.69 ± 0.44	2.30 ± 0.07
4a.	ODED	-	9.8 ± 0.09	79.0 ± 0. 37	19.3 ± 0.34	48.61 ± 0.63	7.63 ± 0.36	3.23 ± 0.16
4b.	XODED	-	10.0 ± 0.07	84.33 ± 0.57	15.2 ± 0.32	46.03 ± 0.38	7.42 ± 0.65	2.53 ± 0.15

4.6.4 Analysis of chemical requirement by pulp and effluent properties

Decrease in the kappa number as a consequence of enzymatic treatment of pulp had led into reduction in overall consumption of bleaching chemicals (Cl_2 and chlorine dioxide). The conventional bleaching sequences (CEHH and ODED) required more chemical (Cl_2) consumption as compared to the enzyme (X) pretreated sequences viz. (XCEHH and XODED) as shown in Table. 16. Hence, enzymatic pre-treatment would result into reduced chemical load as well as improved paper properties in pulp and paper manufacturing.

The environmental parameters to a large extent depend on composition and concentration of bleach chemicals. Higher the concentration more will be the value of AOX and COD. As a result of decrease in requirement of chlorine compounds due to enzymatic bleaching discharge of chlorinated compounds had also reduced (Table. 16).

Table. 16 Effect of enzymatic treatment on bleach chemical requirement and effluent properties

Sl. No.	Bleaching Sequence	Chlorine consumption (%)	Yield (%)	AOX (Kg t^{-1})	COD (mg l^{-1})
1a.	CEHH	93.5	92.5	5.1	949 ± 11.25
1b.	XCEHH	72	91.9	4.3	889 ± 27.63
2a.	ODED	94	91.1	3.8	766 ± 20.51
2b.	XODED	69.4	90.7	3.2	746 ± 19.54

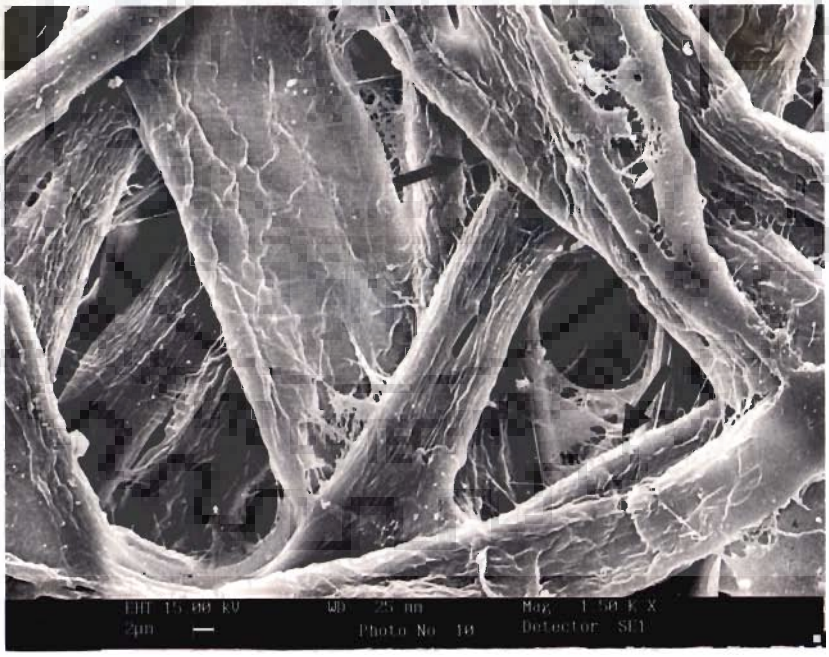
(X, enzyme; C, chlorination; E, alkali extraction; H, hypochlorite; O, oxygen; D, chlorine dioxide)

4.7 EFFECT OF ENZYME TREATMENT ON LIGNOCELLULOSIC COMPONENTS

4.7.1 Fiber morphology

Fiber morphology following enzymatic treatment was analyzed by scanning electron microscopy (SEM) which had shown characteristic variations on the fiber surface due to lignin removal. The fibers in the untreated pulp were uniform and straight having a smoother and sleek surface with compact texture. In addition they appeared softer with no sign of external fibrillation. On the other hand the fibers of the laccase treated pulp were less straight with rougher surface, were heterogeneous and striated, indicating these having the process of peeling with more external fibrillation resulting into remarkable changes in the morphology. Thus the surface appears to be flayed or peeled. The microfibrils were also separated from the initial connected structures and fully exposed, thus increasing the external surface area and porosity. The treated fibers appeared to have grooves and were more conspicuous than the untreated ones (Fig. 29a, b).

(a)



(b)

Fig. 29 Scanning electron microscopy of pulp (a) untreated and (b) treated with laccase mediator system

4.7.2 Pulp crystallinity

In order to assess the pulp delignification during biobleaching the crystallinity of pulp was analyzed. The X-ray diffraction analysis of enzyme treated and untreated pulp had indicated that these had distinct degree of crystallinity. As a result of enzymatic action hemicellulose and lignin are removed, leaving thereafter the higher proportions of amorphous cellulose that led to decreased crystallinity of the pulp. The X-ray diffraction analysis of untreated and treated pulp is shown in fig. 30. As detected higher band width was observed for laccase treated pulp than for the untreated one, hence pulp after treatment appeared to have the decreased crystallinity. Thus bleaching efficacy of laccase mediator system was remarkably higher compared to the untreated one, leading to the removal of lignin and making the pulp less crystalline.

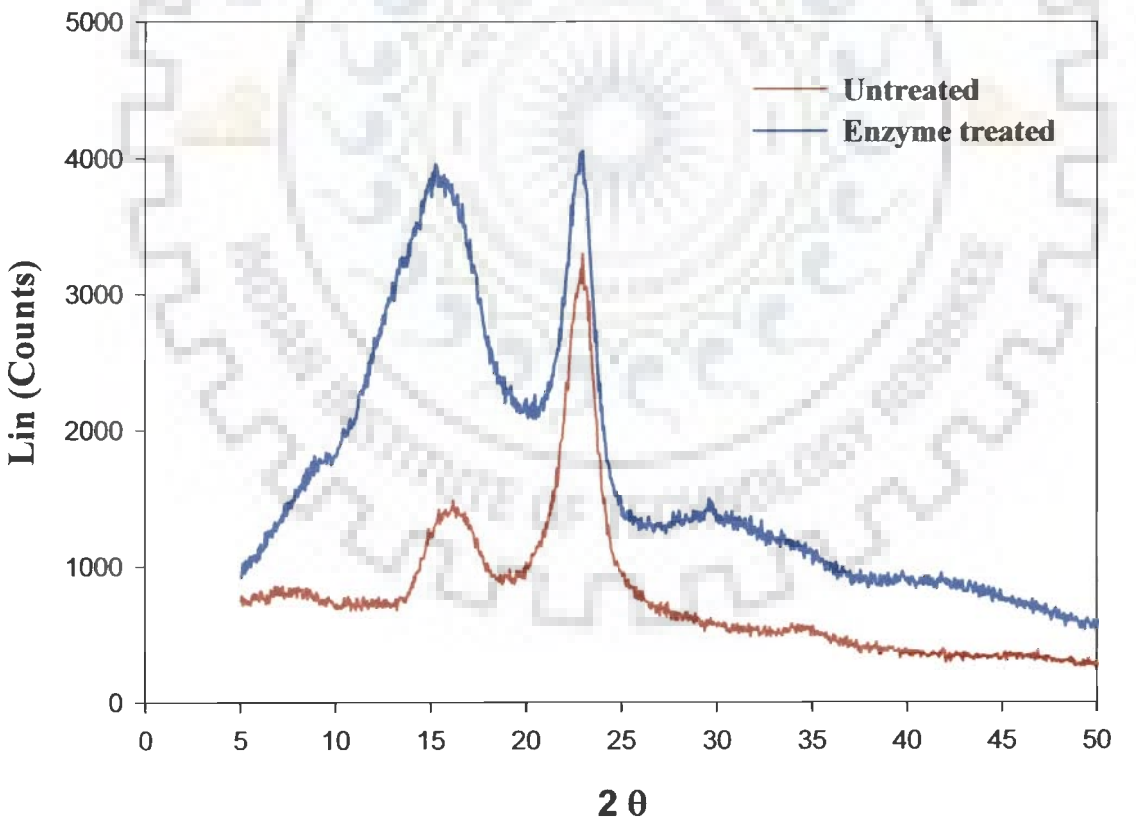
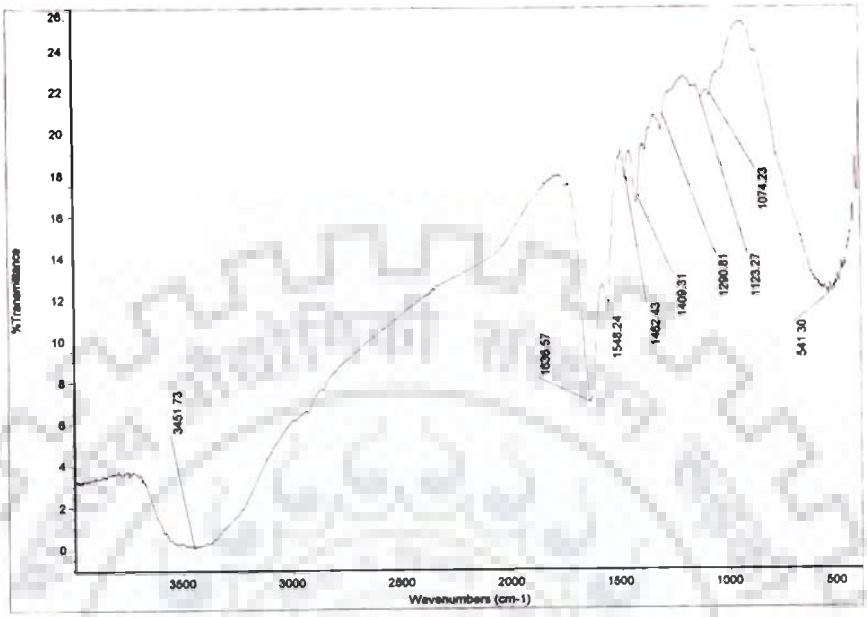


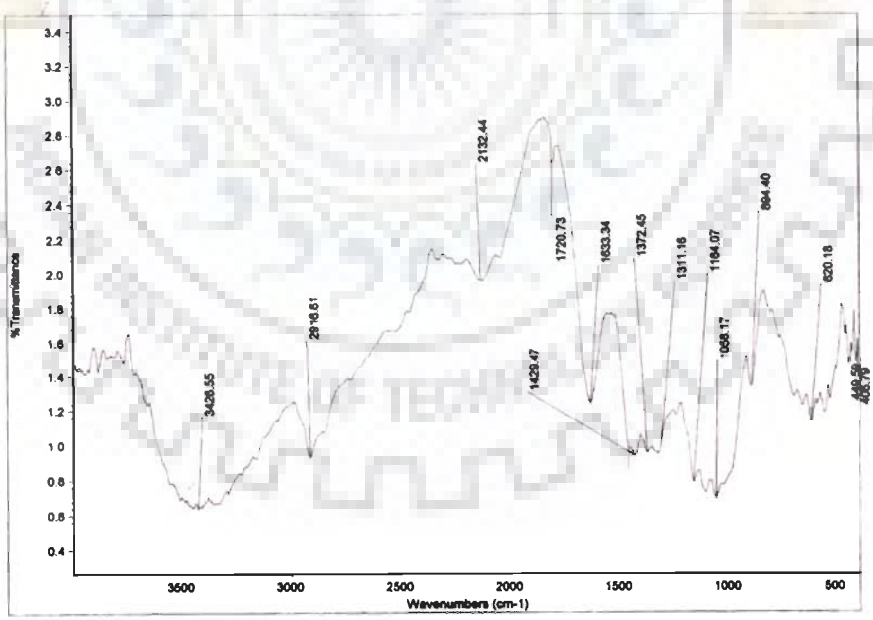
Fig. 30 X-ray diffraction analysis of pulp (a) untreated and (b) treated with laccase mediator system

4.7.3 Spectroscopic analysis of residual lignin

The FTIR spectra of the lignin from treated and untreated pulp was analyzed. FTIR spectra of laccase treated pulp had denoted clear structural differences in lignin. Enzymatic treatment led to removal and degradation of lignin, as indicated by the characteristic changes in the spectra. This may be attributed to the pronounced oxidation and degradation ability of the laccase mediator system. The peak at 3400 cm^{-1} appeared due to presence of -OH groups in lignin of the treated pulp. The peak intensity at 2900 cm^{-1} of treated pulp was higher as compared to untreated one which is attributed to presence of methyl/methylene (-CH) groups. In addition, new strong peak at 1720 cm^{-1} appeared in enzyme treated pulp. This peak is attributed to release of free carbonyl groups (>C=O) as a result of action of enzyme on aromatic ring of lignin. In untreated samples these carbonyl groups remain associated with aromatic rings, hence resulting into no absorption. Besides this, peaks also had appeared in the range 1600-1400 cm^{-1} and 1300-1200 cm^{-1} due to change in the ratio of syringyl and guaiacol groups (Fig. 31a, b).



(a)



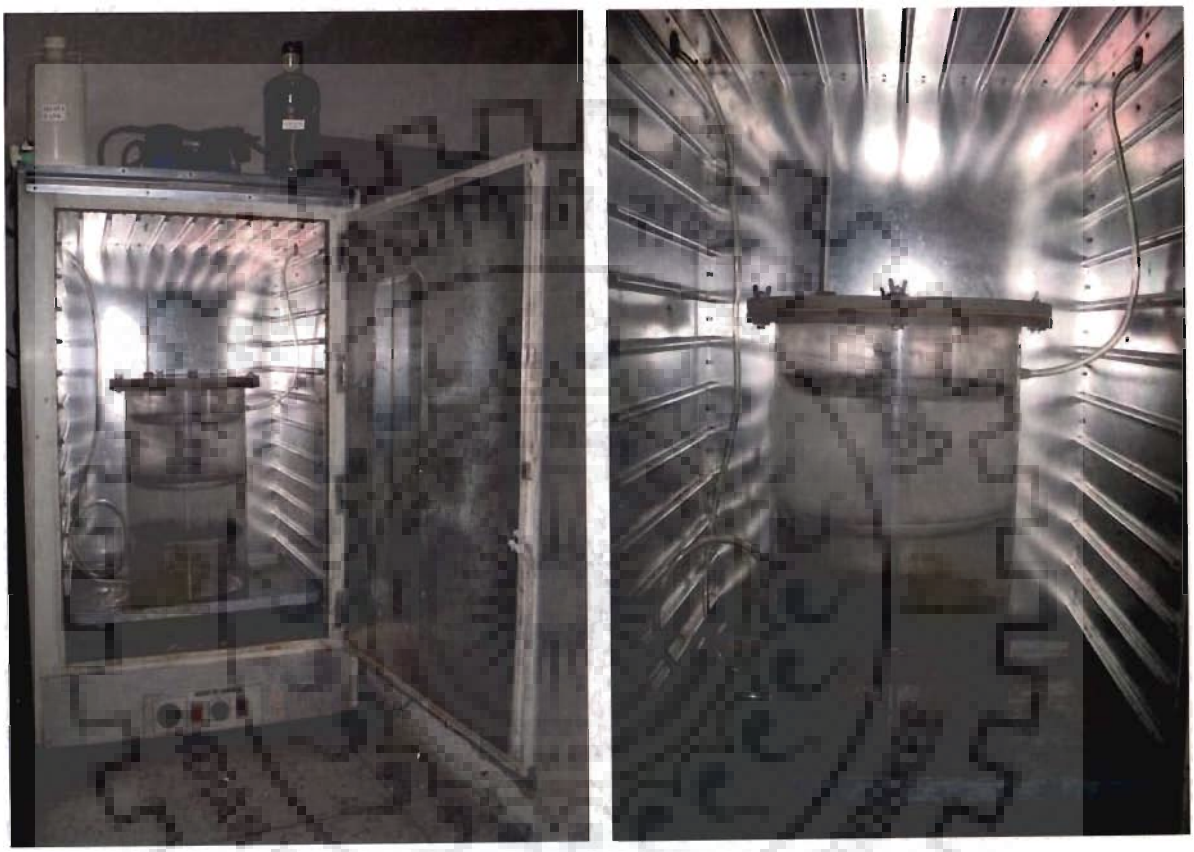
(b)

Fig. 31 FTIR spectra of lignin (a) untreated and (b) treated with laccase mediator system

4.8 COLUMN-TRAY BIOREACTOR FOR LACCASE PRODUCTION

SSF had led to higher levels of laccase and MnP production as compared to the submerged fermentation system. Thus, a column-tray bioreactor was designed for scaled up production of the enzymes in batch fermentation using earlier derived conditions (Fig. 32). In this bioreactor, banana peel impregnated with fermentation medium was added and inoculated with mutant VkJ2.4.5 strain. Humid and sterile air was circulated through air pump for proper aeration from the bottom of tray having autoclaved water for maintaining humidity. The scrupulous growth of fungus occurred in the bioreactor and enzyme was harvested on 6^{th} day of incubation period.

Enzyme was extracted from the biomass and estimated. A significant level of laccase was produced during batch fermentation. Laccase activity was detected on the 2^{nd} day and then increased thereafter reaching to its maximum levels on the 6^{th} day (6267.4 ± 71.72 Ul^{-1}), the activity decreased on further incubation. Similar trend was also observed for MnP activity. These values are in close association with the production levels as observed for the laboratory scale SSF process. Following I cycle of fermentation, fungal biomass was utilized for enzyme production in the subsequent cycles (Fig. 33).



(a) (b)

Fig. 32 Column-tray bioreactor for batch production of laccase and manganese peroxidase using banana peel as solid support (a) Exterior view; (b) Interior view

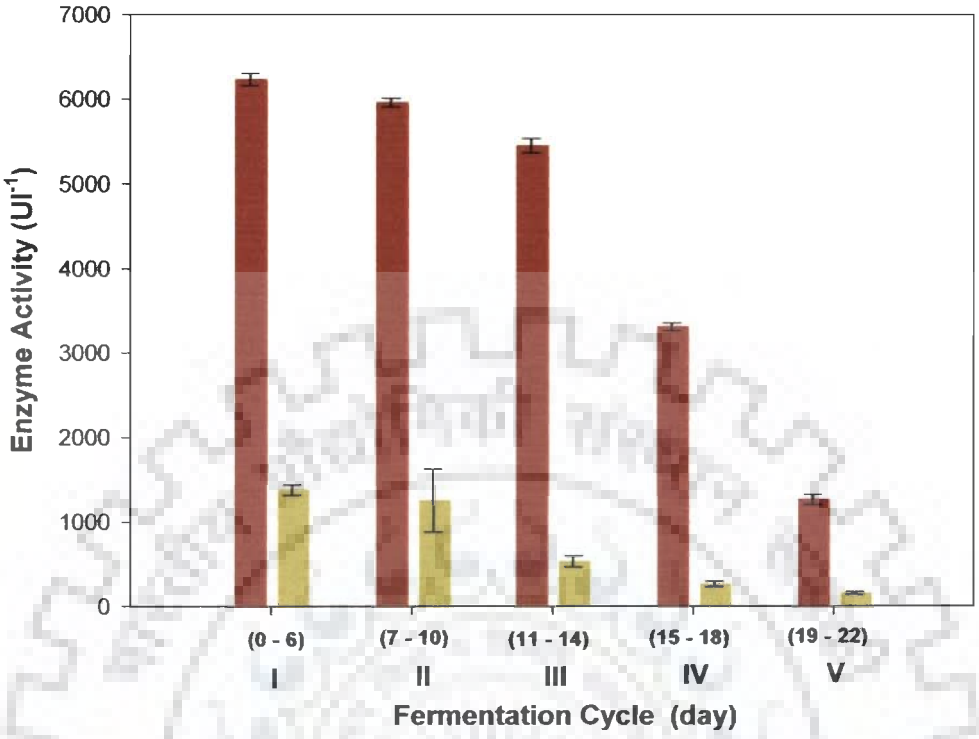


Fig. 33 Batch production of enzymes in column-tray bioreactor under SSF

(, ; represent laccase and manganese peroxidase respectively)

Significant levels of enzyme were produced during I-III fermentation cycles. Enzyme production in the IV and subsequent cycles decreased due to depletion of nutrients and sporulation. Thus, economical levels of enzyme production can be accomplished for bleaching application using the column-tray bioreactor.

Chapter V

Discussion

DISCUSSION

Maximal enzyme production is one of the goals in any biotechnological process and to achieve the same several strategies such as, strain development, derivation of medium, bioprocess optimization and other crucial parameters have widely been under scrutiny. For developing an industrial fermentation, derivation of medium is of utmost importance as metabolite concentrations and growth of fungi are strongly influenced by media components. Process optimization may involve derivation of biochemical and physical parameters including media formulation/design and culture parameters.

5.1 IDENTIFICATION AND SCREENING OF LACCASE PRODUCING MICROORGANISM

The laccase has wide range of applications including in pulp and paper industries, treatment of various industrial effluents, enzymatic decolourization and in bioremediation. One of the limitations to the large scale application of the enzyme is due to the gap relating to competent and economically viable technologies for the scaled-up production of industrially compatible enzyme. These limitations can be addressed by screening for naturally occurring potential strains or employing the genetic manipulation approaches for developing hyper secretory microorganisms. Profuse diversity of microorganisms with various crucial functions is not concealed in nature, environmental factors greatly influence the ability of fungi to produce the metabolites of specific types. Microorganisms consisting bacteria, actinomycetes and fungi produce detectable levels of laccase. Fungi look more promising as these represent extracellular multiple enzyme system with less specificity and hence a broad substrate range, which eventually leads to enhanced delignification of the lignocellulosic biomass. (Alexander and Zhulin, 2000; Record *et al.*, 2002; Saparrat *et al.*, 2002).

Among the thirty two fungal strains isolated from different natural sites, fifteen were from dead tree, six from rotting wood, four each from forest soil and sugarcane wastes and three from paper loading site. Among these, eleven isolates had shown laccase production following screening and five were further selected for quantitative estimation of laccase production. Among the selected strains i.e. VkD, VkG2, VkH2, VkJ and VkN2, maximal level of laccase production (2108.2 ± 49.37 Ul^{-1}) was observed by strain VkJ under submerged fermentation. The strain VkJ was identified as *Aspergillus fumigatus* ITCC 6035 from Indian Agricultural Research Institute, New Delhi, India.

The development of an economical and industrially feasible process mainly depends on the production levels obtained from the strain selected for the process. An attempt was made for improvement in the level of enzyme production by subjecting the wild type strain to physical and chemical mutagenesis and various low cost or freely available agro-horticultural residues were evaluated as substrate for laccase production.

5.2 IMPROVEMENT OF STRAIN BY MUTAGENESIS

Strains isolated from natural sources have generally the limited ability for the production of metabolites and can be genetically manipulated for improvement. The wild type *A. fumigatus* VkJ strain was mutagenized by physical and chemical mutagens for obtaining a strain with increased levels of production. The strain was subjected to various dosages of UV, NTG and EtBr and positive and negative mutants were identified on the basis of zone diameter. The viable colonies obtained in first stage of UV irradiation (2 min) were further subjected to UV irradiation. Among different mutants obtained, *A. fumigatus* VkJ2.4.5 was found to secrete maximum laccase and MnP (3852.5 ± 69.13 Ul^{-1} and 644.3 ± 35.20 Ul^{-1} respectively). Strain VkJ2.4.5 was subcultured for several generations and found to be stable with minor variations in levels of production. The induced mutagenesis and strain selection is widely practiced for strain improvement. Mutant *Penicillium variabile* M-80.10 developed by physical mutation (UV

irradiation) was observed to be an overproducer of glucose oxidase (127% higher) than wild type *Penicillium variabile* P16 (Petruccili *et al.*, 1995). *Candida* sp 99-125 developed following the series of mutagenic treatments increased production of lipase by ten folds as compared to wild type strain (Tan *et al.*, 2003). Chand *et al.*, (2005) has reported increased cellulase production in mutant *Aspergillus* CMV5-A10 than wild type *Aspergillus* CMV5 following the treatment with EtBr and MNNG. Dhawan *et al.*, (2003) had reported that fungus *Cyathus bulleri* when grown with sublethal concentration of mutagen (EtBr), had about four fold increase in the laccase production. Group of researchers had already attempted strain improvement for enzyme production in different microorganisms (Suryanarayan, 2003).

5.3 COMPARATIVE ANALYSIS OF DIFFERENT FERMENTATION SYSTEMS

Modifications in culturing process of the microbes affect the metabolite production. Dong *et al.*, (2005) had observed that change in culturing process of *Trametes gallica* significantly influenced production of laccase. Laccase production by *Pycnoporus sanguineus* under submerged fermentation system was evaluated utilizing lignocelllose residues and it was observed that addition of wood fibres increased the production level by 12 folds (Pointing *et al.*, 2000). Increased levels of laccase production have been reported by *Phaerochaete chrysosporium* NCIM 1197 under SSF than the static culture system (Gnanamani *et al.*, 2006). Maximum levels of enzyme production may be achieved in different fermentation systems viz. surface, submerged and SSF. Several groups have made earlier attempts to identify and regulate the critical parameters for laccase production using an assortment of microorganisms; *Coriolus hirsutus, Botryosphaeria* and *Pleurotus* sp. (Koroleva *et al.*, 2002, Da Cunha *et al.*, 2003, Stajic *et al.*, 2006). Initial trial for attaining maximum levels of laccase and MnP production from the improved mutant VkJ2.4.5 strain, developed in our laboratory was performed by evaluating different fermentation systems.

Mutant VkJ2.4.5 was analyzed for laccase and MnP production in different fermentation systems using glucose (surface and submerged fermentation) and agro-horticultural residues (solid-state system) as carbon source. Solid-state fermentation using sugarcane bagasse as solid support was found to be most productive (laccase, 4561.6 ± 69.11 UI^{-1}; MnP, 1133.7 ± 57.18 UI^{-1}) than the submerged and surface fermentation. Laccase production had been largely carried out under submerged fermentation by various groups (Lorenzo *et al.*, 2002; Da Cunha *et al.*, 2003; Palonen *et al.*, 2003; Stajic *et al.*, 2006). Comparatively lower levels of production were observed in submerged and surface fermentations. However, few groups had also evaluated SSF using *Trametes versicolor* (Couto *et al.*, 2002, 2006), *Pleurotus* sp. (Reddy *et al.*, 2003), *Coriolopsis rigida* (Gomez *et al.*, 2005), *Pycnoporus sanguineus* (Vikineswary *et al.*, 2006) for laccase production. SSF is advantageous over submerged fermentation (SmF) due to its simplicity and compatibility to the natural surroundings that are suitable for growth and survival of many microorganisms (Virupakshi *et al.*, 2005). Moreover, higher enzyme productivity as well as increased stability of secreted enzymes has been the other notable features of SSF (Pandey *et al.*, 1999; Viniegra-Gonzalez *et al.*, 2003; Holker *et al.*, 2004). Since SSF had resulted into maximum levels of enzyme production, hence, further studies were carried out for laccase and MnP production in solid-state fermentation.

5.4 ANALYSIS OF MAJOR FACTORS REGULATING SOLID-STATE FERMENTATION

SSF is carried out with microbes growing on nutrient impregnated solid substrate with little or no free water. The process may be directly carried out with abundantly available low-cost biomaterials (agro-horticulture residues, lignocellulosic materials) with minimal or no pretreatment, and thus is relatively simple, uses less energy than SmF and can provide unique microenvironments conducive to microbial growth and metabolic activities. Currently, SSF is undergoing a renewed surge of activities, primarily because of the opportunities it offers for

increased productivity, low energy consumption and yielding into concentrated product as compared to submerged system. The process can utilize a wide range of agro-horticultural byproducts, generates lesser waste-water which could be advantageous for product recovery and could also reduce the cost of downstream processing.

For SSF, six different solid supports viz. banana peel, bagasse, wheat bran, poplar leaves, wheat straw and rice bran impregnated with the fermentation medium as derived in this study, were utilized. Among the solid substrates employed, maximum levels of laccase and MnP were achieved with banana peel (5792.0 ± 40.95 Ul^{-1}, 1334.6 ± 167.32 Ul^{-1}) respectively. It is possibly due to the presence of various suitable nutrients in banana peel, its particle size and consistency required for anchorage. It contains significant amount of carbohydrate, proteins and organic acids ensuring abundant growth of fungi. In addition, it also contains water soluble aromatic compounds capable of inducing or stimulating biosynthesis of ligninolytic enzymes therefore, prompting better fungus growth and enzyme production. The porosity of banana peel also helps in efficient absorption of nutrient and air inside the peel, leading to proper growth and production of enzyme. Laccase production by *Trametes pubescens* CBS 696.94 has been observed (1570 Ul^{-1}) using solid-state fermentation with banana skin as soild support (Osma *et al.*, 2007).

The critical role of moisture on enzyme production is attributed to interference of moisture with physical properties of substrate. Moisture level during SSF ranges from 30% to almost 80% depending on the solid support employed. Moisture level of 80% was found suitable for mutant VkJ2.4.5 strain for maximal levels of laccase and MnP production. Moisture less than 60% or more than 80% didn't effectively endorse enzyme production. High moisture decreases porosity, declines air volume and exchange of oxygen to the microorganism and hence reduces the ability of laccase secretion in fermentation process. Lower moisture level may lead to reduced solubility of nutrients, decreased degree of swelling of substrates and elevated water tension resulting into lower yield of enzyme (Lonsane *et al.*, 1985; Gawande & Kamat, 1999).

Supplementation of banana peel with various additives stimulated the laccase production by mutant VkJ2.4.5. Among the different additives used, yeast extract led to maximum enhancement in the laccase production followed by cheese whey. Molasses and jaggary had also induced the enzyme production. The increase may be due to the micronutrients and the growth stimulating factors present in the yeast extract (Chong *et al.*, 2005; Djekrif-Dakhmouche *et al.*, 2006). Oxygen is another factor affecting growth profile. A proper saturation of oxygen is required in solid-support that interacts with the active mesh of fungal mycelia at the surface as well as to its interior regions (Lenz *et al.*, 2004). Medium was aerated intermittently.

5.5 CHARACTERISTIC FEATURES OF LACCASE FROM MUTANT *A. FUMIGATUS* VkJ2.4.5

A. fumigatus VkJ2.4.5 laccase was detected as a single band of 34±1 kDa protein in crude culture filtrate as visualized by CBB and activity staining (guaiacol). Many of the fungal laccases mainly from *Phlebia radiata*, *Schizophylium commune*, *Neurospora crassa*, *Monocillium indicum* have the molecular weight in the range of 62-100 kDa (Thurston, 1994). Nyanhongo *et al.*, (2006) have reported two distinct laccase isozymes from *Trametes modesta* with the molecular weights of 77.6 and 52.5 kDa. Murugesan *et al.*, (2007) observed laccase of 43 kDa from *Ganoderma lucidum* KMK2 involved in dye decolorization. A laccase of low molecular mass (43 kDa) was reported from *Albatrella dispansus* by Wang and Ng (2004). However, lately laccase of lower molecular weight similar to the one as detected in the present study has also been reported from *Pleurotus eryngii*, (34 kDa) (Wang and Ng., 2006). Many other groups have also reported laccase with diverse molecular weight (Palonen *et al.*, 2003; Sulistyaningdyah *et al.*, 2004).

Laccase from *A. fumgiatus* VkJ2.4.5 revealed significant identity with the laccase of ascomycetes mainly from *Fusarium oxysporum* and *Phaeosphaeria* sp., actinomycetes - *Streptomyces cyaneus, Strongylocentrotus* sp. and plants that are *Arabidopsis thaliana* and *Rhus*

vernicifera. Further, it has lower degree of identity with basidiomycetes mainly from *Pleurotus* sp., *Trametes* sp. and *Pycnoporus sanguineus* when compared to the sequences of other reported laccases and multi-copper oxidases in database search. It is noticeable that the amino acid sequences of laccases are sufficiently diverse in composition and the degree of identity.

Laccase was also analyzed for its activity with substrate (Guaiacol). As the reaction time of enzyme-substrate increases, the rate of degradation of substrate also increases. It has been observed that monophenols having hydroxy or methoxy groups were most affected by the enzyme treatment. During HPLC analysis, it was observed that original substrate gradually decreased and new, unidentified components appeared following enzymatic treatment. Most of the compounds affected by laccase contain free phenolic hydroxy groups that included substituted *o* and *p*-dihydroxybenzene, trihydroxybenzene and *p*-phenylene-diamine. Niku-Paavola *et al.*, (1988) have also reported similar findings.

Enzyme preparation from *A. fumigatus* VkJ2.4.5 was found to be stable at temperature ranging from 25^0 to 65^0C, whereas at 70^0C, laccase was completely inactivated within 2 h. Laccase retained more than 50% of the initial activity up to 55°C for 2 h. It has been observed that when enzyme is subjected to high temperature copper ions may be released. This depletion of copper ions not only inactivated the enzyme but also uncouple the domains of copper-depleted protein (Koroleva et al., 2001). Wang and Ng (2006) had reported maximal laccase activity from *Pleurotus eryngii* at 70°C and at pH range of 3-5. *Peniophora* sp produced thermostable laccase having half life of 5 h at 60°C (Niku-Paavola *et al.*, 2004). Other group of researcher had also observed the similar findings (de Carvalho *et al.*, 1999; Jordaan *et al.*, 2003, Sulistyaningdyah *et al.*, 2004,). The stability of the enzyme preparation was also evaluated at different pH. Enzyme showed acceptable activity within a pH range of 5.0-9.0. At pH 7.0, 70% of the enzyme activity was noted. The higher enzyme activity at pH 5 may be due to unfolding of molecule at acidic pH in beginning and helping the substrate to reach the active site

(Martinez-Alvarez *et al.*, 2008). The enzyme preparation had considerable levels of activity at alkaline pH and hence appears appropriate for its application to the pulp and paper industry. Thus, the laccase from *A. fumigatus* VkJ2.4.5 was stable at a wide range of temperature and pH and its stability profile was comparable with laccases from *Cythus stercoreus* (Sethuraman *et al.*, 1999) and from *Phaenerochaete chrysosporium* (Coll *et al.*, 1993), *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996) reported earlier.

5.6 BIOBLEACHING OF MIXED WOOD PULP BY LACCASE MEDIATOR SYSTEM

Environmental concerns are of prime significance to substitute chlorine derivatives by enzymes for pulp bleaching. Paper production requires separation and degradation of lignin in wood pulp but ecological concerns urge to replace conventional and ecotoxic chlorine based delignification reaction. Hence, in the present study the potential of laccase-mediator system (LMS) from *A. fumigatus* VkJ2.4.5 for lignin removal from mixed wood pulp was monitored during bleaching. Maximum delignification was achieved with laccase (10 Ug^{-1} odp) following 2 hrs of treatment in presence of HBT (1.5%, w/w) which acts as a mediator. Different findings had denoted the role of LMS as prebleaching agent so as to improve the quality of pulp. Sigoillot *et al.*, (2005) had observed significant levels of delignification of non-wood pulp when LMS treatment was performed in presence of HBT (15-40 mg g^{-1} of odp). Ibarra *et al.*, (2006) had reported that laccase from *Pycnoporus cinnabarinus* with mediator (HBT) delignified the eucalyptus pulp significantly leading to decrease in kappa number by four points as well as 6% increase in ISO brightness. Improvement in ISO brightness as well as decrease in kappa number (15% in both) had been observed by Camarero *et al.*, (2007) following the laccase mediator treatment of eucalyptus pulp. Further it has been observed that a combination of laccase and MnP may degrade lignin effectively (Nakamura *et al.*, 1999). The laccase and MnP less mutants from *Trametes versicolor* were found to be virtually ineffective in degrading the lignin as

compared to the strain possessing laccase and MnP enzymes (Addleman *et al.*, 1995). Laccase and MnP from *Rigidoporus lignosus* were studied for lignin degradation. When isolated, neither laccase nor the MnP alone was able to solubilize the radioactive lignin. In contrast, two enzymes when added to the reaction medium at the same time, led to the effective solubilization of the lignin. Thus laccase and MnP acted synergistically for degrading the lignin (Galliano *et al.*, 1991). Thus, *A. fumigatus* VkJ2.4.5 which produces laccase along with MnP in considerable amounts may be a potential strain for biobleaching.

Additionally, phenolic (A_{237nm}) and hydrophobic compounds (A_{465nm}) were released under derived conditions. Comparison of resulting final pulp properties showed that following enzymatic treatment of pulp, 14 and 4% reduction respectively in kappa number and yellowness and 7% increment in ISO brightness was attained respectively. Lignin degradation was observed by LMS that had resulted into enhanced discharge of chromophores, phenolic and hydrophobic compounds. Release of chromophores, phenolic as well as hydrophobic compounds was higher under derived conditions and no further increase in the release of these compounds was observed following increasing dose of the enzyme and reaction time. Thus, release of hydrophobic and phenolic compounds is an appropriate indicator of the kinetics of enzymatic attack on the pulp. Similar findings have also been reported by other investigators (Garg *et al.*, 1998; Khandeparkar and Bhosle, 2007).

Chemical modifications in lignin followed by laccase mediator system treatment of pulp were analyzed. FTIR spectra of untreated and laccase treated pulp indicated the structural changes. Enzymatic treatment led to removal and degradation of lignin; as indicated by the characteristic changes in FTIR spectra following the treatment. This may be attributed to the high oxidation and degradation power of laccase-mediator system. The peak at 3400 cm^{-1} appeared due to presence of OH groups (Faix, 1992) in lignin and the peak intensity was higher in treated pulp as compared to untreated one. The peak intensity at 2900 cm^{-1} of treated pulp was higher as compared to untreated which is attributed to presence of methyl/methylene (-CH)

group. In addition, new strong peak at 1720 cm^{-1} appeared in enzyme treated pulp. This peak is attributed to release of free carbonyl groups (>C=O) as a result of action of enzyme on aromatic ring of lignin (Buta *et al.*, 1992). In untreated samples these carbonyl groups remain associated with aromatic rings, hence resulting into no absorption.

Lignin is a highly branched polymer with a wide variety of functional groups providing many active centers for chemical and biological interactions. The main functional groups in lignin are hydroxyl, methoxy, carbonyl and carboxylic groups. Understanding the biodegradation of lignin would have significant implications in developing an ecofriendly technique for selective removal of lignin in getting high-value paper products. Much interest has been focused on functional group analysis by various research groups (Sealey and Ragaukas, 1998; El Mansouri and Salvado, 2007).

The possible mechanism of delignification by LMS lies in the redox cycles in which oxygen oxidizes the reduced form of laccase to the native laccase, which in turn oxidizes a mediator to produce oxidized mediator species and the original laccase. Lignin moieties in the residual lignin in pulp then undergo oxidation by the mediator oxidized species, resulting in degradation and dehydrogenative polymerization of lignin (lignin oxidized) and reduction of mediator (Call and Mucke, 1997). The release of chromophoric materials from pulp was monitored by analyzing the components dissolved in effluent. Effluent obtained followed by bleaching of pulp under derived conditions, was subjected to scanning (λ 200-400 nm) for evaluating the lignin removal. The peak observed at 280 nm in the UV spectra indicated the presence of lignin in the effluent that had increased with an increasing enzyme dose. Khandeparkar and Bhosle, (2007) have also noticed the similar pattern. The release of chromophoric materials seemed to be promoted by the action of enzyme as untreated pulp samples didn't cause any significant release.

The enzymatic prebleaching reduced the total Cl_2 demand of the pulp. A decrease to the extent of 22 and 26% respectively for chlorine consumption was observed for CEHH and ODED

bleaching sequences, due to reduction of kappa number of pulp followed by the action of laccase from *A. fumigatus* VkJ2.4.5. Levels of adsorbable organic halide, biological and chemical oxygen demands had also declined significantly due to enzymatic treatment of pulp. Adsorbable organic halide got reduced by 15% in both bleaching sequences, COD by 6 and 2% and in BOD by 15 and 14% in CEHH and ODED bleaching sequences respectively. Increment in the pulp brightness and decrease in yellowness was observed in the pulp. It indicated that the LMS applied may have direct brightening effect on the fibers. Also, the enzyme pretreated bleaching sequences showed comparatively higher brightness than the untreated ones indicating that treated pulp fibers may be more accessible to chemical sequences in the following steps of bleaching. The analysis of physical properties viz. brightness, tear index, burst factor etc of handsheets prepared from treated pulp exhibited considerable improvements because of fiber modifications which had led to easy availability of bleaching chemicals during full bleaching in subsequent stages, when compared with respective untreated handsheets. Camarero *et al.*, (2004) had reported significant improvements in paper properties following laccase treatment. Thus, enzymatic prebleaching leads to higher pulp yield and had retained the desired mechanical strength properties along with higher brightness and reduced pollution load.

Fiber morphology following enzymatic treatment was analyzed by scanning electron microscopy (SEM). The fibers in the untreated pulp were uniform and straight having a smoother and sleek surface. In addition these appeared softer with no sign of external fibrillation. On the other hand the fibers of the laccase treated pulp were less straight with a rougher surface, heterogeneous and striated. These morphological changes therefore indicate that fibers undergo in process of peeling with more external fibrillation. The treated fibres appeared to have grooves and were more conspicuous than the untreated ones that may be because of erosion of surface material. Abrasion and splitting in fibers have also been observed apparently which substantiates that treatment may remove some components from the pulp and led to modification in fiber morphology. Similar morphological variations have also been

observed with eucalyptus pulp bleached with xylanase (Roncero *et al.*, 2000, 2005; Torres *et al.*, 2000; Zheng *et al.*, 2000). The enzymatic removal of lignin which is conjugated on the surface of fibers had resulted into detachment of fibres to form grooves or cracks that may lead to enhanced bleaching in subsequent bleaching stages.

In order to further assess the pulp delignification during biobleaching, crystallinity of pulp was analyzed. As a result of enzymatic action hemicellulose and lignin are removed, leaving high proportions of amorphous cellulose which leads to decreased crystallinity of the pulp (Xu *et al.*, 2007). As shown in diffractograms higher band width was detected for laccase treated pulp than for the untreated one, hence pulp following treatment appeared to have a decreased crystallinity. Thus bleaching efficacy of laccase mediator system was remarkably higher compared to the untreated one, leading to the removal of lignin and making the pulp less crystalline.

5.7 EVALUATION OF COLUMN-TRAY BIOREACTOR FOR BATCH PRODUCTION OF LACCASE

A column-tray bioreactor was developed and evaluated for scaled up production of laccase and MnP from *A. fumigatus* VkJ2.4.5. Banana peel as the solid support and the various parameters that led to maximal levels of production at solid-state process were utilized for production. However, at larger scale in SSF temperature rise is observed in substrate beds resulting into inconsistent regulation of temperature (Mitchell *et al.*, 2000). Higher metabolic activity of the microorganisms as well as lower thermal conductivity of the solid matrices used may be responsible for rise in temperature; hence the heat removal may be tedious and time consuming process. The increase in temperature may thus affect fungal growth and secretion of enzyme during fermentation. Temperature and oxygen levels remain uniform at substrate bed in SSF at earlier stages of fermentation. As process continues, aeration has a significant role to play for maintaining the optimum temperature and oxygen levels by heat transfer (Pandey, 2003).

Hence, sterile humid air was circulated throughout the chamber. Similar strategy was employed by the other groups for regulation of temperature in bioreactor (Suryanarayan, 2003; Lenz *et al.*, 2004). The mutant strain *A. fumigatus* VkJ2.4.5 had the significantly higher levels of laccase (6267.4 ± 71.72 Ul^{-1}) and MnP (1358.0 ± 32.41 Ul^{-1}) production in batch fermentations. Subsequent batch fermentations with the biomass generated following initial cycle of fermentation had yielded significant levels of enzyme production for up to further two cycles of fermentation. Therefore, the batch reactor set up and the utilization of banana peel as an appropriate solid matrix, that is available in plenty and with no cost promoted the higher levels of enzyme production and contributed to diminish the production cost.

Chapter VI

Summary and Conclusions

SUMMARY AND CONCLUSIONS

An attempt was made to derive and define an enzymatic system for biobleaching of paper pulp. Various critical and major parameters were evaluated so as to obtain an active and economical system for biobleaching applications. Thirty two fungal strains were isolated from samples collected from different sites mainly from sugarcane industry waste, decomposing wood, manure and from paper industry waste. Eleven isolates had shown laccase secretion following screening. These strains had distinct morphological features, colour and growth pattern. The strain VkJ had resulted into maximum zone diameter and was identified to be *A. fumigatus* (ITCC 6035) from Indian Agricultural Research Institute, New Delhi, India and was selected for further study. Higher levels of laccase along with notable levels of manganese peroxidase was detected in *A. fumigatus* VkJ strain.

To achieve an improvement in the enzyme production ability of the selected strain, physical and chemical mutagenesis of the strain was attempted. Mutagenesis at every stage was followed by determining the survival rate and distribution profile of the mutants. Initial mutagenic treatment (UV irradiation, 2 min) led to development of the VkJ2 strain with increased level of laccase production. VkJ2 was successively exposed to UV irradiation (4.5 min) resulting into 29% of positive mutants of the total surviving colonies. Among these, mutant *A. fumigatus* VkJ2.4.5 strain, had 80% higher laccase production ability than the wild type *A. fumigatus* VkJ strain and was selected for further study. The mycelia of the mutant VkJ2.4.5 strain was loose and dirty white as compared to mycelia of wild type VkJ strain that was compact and white coloured. Scanning electron microscopy revealed demarcated changes in the morphological features of the fungal strain following mutagenic treatments. Hyphae of themutant VkJ2.4.5 strain were thick, compact and coiled as compared to hyphae of wild type *A. fumigatus* VkJ that were thinner, elongated, ribbon shaped and straight.

To derive a system, production of enzyme was evaluated in surface, submerged and solid-state fermentation (SSF) systems. The SSF system led to maximum production of enzyme and the process was further economized by evaluating the low cost or freely available agro-horticultural residues as solid supports for fermentation. Among six different solid supports employed, banana peel appeared to be the most suitable substrate for laccase as well as MnP production. Maximum laccase production (6281.4 ± 63.60 Ul^{-1}) was achieved on 6^{th} day of incubation requiring a moisture level of 80% and aeration rate of 2.5 l min^{-1}. Similarly higher levels of MnP was also detected under these conditions. Yeast extract (1%, w/w) as an additive, improved the enzyme production ability of mutant VkJ2.4.5 strain. Characterization of the enzyme revealed this be of 34±1 kDa. The amino acid sequence of the laccase of *Aspergillus fumigatus* VKJ2.4.5 shares significant identity with the laccase of *Fusarium oxysporum, Streptomyces cyaneus, Strongylocentrotus* sp, *Phaeosphaeria* sp and *Arbidopsis thaliana*. It is noticeable that the amino acid sequences of laccases are sufficiently diverse in composition and the degree of identity is present in various groups of organisms. Substantial level of enzyme activity (50%) remained after incubating the same at 55°C for 2 hours, in addition, enzyme was detected to be stable at a wide range of pH (5.0-9.0).

An attempt to scale up the production was made by designing a column-tray bioreactor using the conditions as derived earlier. Following the cultivation of fungus in the bioreactor the stationary phase of growth was observed after more than 4 days of cultivation. However, increasing level of laccase activity was observed after 48 hrs indicating thereby that the laccase production started in the exponential phase of growth and continued up to the stationary phase. The cultivation of mutant VkJ2.4.5 in bioreactor had resulted into comparable levels of laccase and MnP production (6267.4 ± 71.72 Ul^{-1}, 1358.0 ± 32.41 Ul^{-1}) respectively. In the bioreactor, biomass was recycled for different batches of the fermentation and significant level of enzyme was detected for up to further 2 cycles of fermentation. The level of production declined thereafter which may be due to the depletion of nutrients and sporulation.

Laccase thus obtained was evaluated for biobleaching applications of the mixed wood pulp. Enzymatic treatment of pulp (10 Ug^{-1} odp) was performed for 2 hrs along with a laccase mediator system (1.5% HBT). A significant reduction in the kappa number (14%), yellowness (4%) and increase in brightness (7%) of the mixed wood pulp was obtained. HPLC analysis had revealed that treatment of substrate with laccase had resulted into new unidentified peaks which indicated the degradation of the substrate. Similar observations were made following FTIR analysis of the treated mixed wood pulp by applying laccase mediator system under derived conditions.

The enzymatic pretreatment led to a decline in the chlorine requirement of mixed wood pulp in successive stages of bleaching. In addition, decreased level of AOX and COD of the effluent was also detected. The paper generated from treated pulp had shown superior mechanical and optical properties in comparison to paper made from untreated pulp.

Hence, treatment of pulp using laccase-mediator system appears to be safer, effective and an eco-friendly process. The following are the major conclusions derived from the present study,

a. Among the thirty two fungal isolates, *A. fumigatus* VkJ that was isolated from the sugarcane residual materials was found to be a potential strain producing laccase and also the detectable levels of manganese peroxidase.
b. Strain VkJ was subjected to UV exposure in two successive stages of mutagenesis that had led to the mutant *A. fumigatus* VkJ2.4.5 having 82 % higher levels of laccase and 44 % higher levels of the MnP than the wild type strain.
c. Solid-state fermentation had resulted into higher levels of laccase and MnP production than the other fermentation systems evaluated. Banana peel was found to be the most persuasive solid substrate for laccase production.
d. Laccase produced by *A. fumigatus* VkJ2.4.5 strain was detected as protein of 34±1

kDa which had a higher stability profile when treated at 55°C for 2 hr and also at a wide range of pH variations (5.0-9.0). The amino acid sequence of the laccase of *Aspergillus fumigatus* VKJ2.4.5 shares significant identity with the laccase of *Fusarium oxysporum, Streptomyces cyaneus, Strongylocentrotus* sp, *Phaeosphaeria* sp and *Arbidopsis thaliana.*

e. Solid-state process was scaled up by designing a column-tray bioreactor using the earlier derived parameters. A comparable level of enzyme production (laccase, 6267.4 ± 71.72 Ul^{-1}; MnP, 1358.0 ± 32.41 Ul^{-1}) was obtained under these conditions. Biomass following recycling can yield higher levels of enzyme production for up to a further two cycles of fermentation.

f. Pre-treatment of mixed wood pulp with laccase-MnP preparation as obtained by *A. fumigatus* VkJ2.4.5 had resulted into significant increase in ISO brightness and better quality of the paper. Enzymatic pre-bleaching had reduced the chlorine requirement by 22 %, thus the process can be considered as environment and ecofriendly and a safer process.

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2. Dwivedi P, **Vivekanand,** Sabharwal N and Singh R P (2007). "Fungal co-cultivation: An approach for simultaneous production of xylanase and laccase under submerged fermentation using *Parthenium* sp. as a novel plant biomass". 29th Symposium on Biotechnology for Fuels and Chemicals, Denver Adams Mark Hotel, Denver, Colorado, USA, 29th April – 2nd May, 2007.
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   Note: *This poster got First Prize in technical poster session 1 at Natl. Symp. Biohorizon-2006, IIT Delhi, New Delhi, India.*

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