BIOPROCESS OPTIMIZATION FOR ENZYMATIC DELIGNIFICATION AND BIOCONVERSION OF *Bambusa balcooa* BIOMASS INTO FERMENTABLE SUGARS FOR BIOETHANOL PRODUCTION

Thesis

Submitted to the

G. B. Pant University of Agriculture & Technology

Pantnagar-263145, Uttarakhand, INDIA

By

Raj Kumar Pandey

M.Sc. (Microbiology)

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

(Microbiology)

December, 2016

Acknowledgements

To the two most important people who are behind all what I am today, Maa-Pitaji...

First and foremost, I bow my head in front of almighty God, for showers of blessings, mercy and for providing me this opportunity and granting me the capability to proceed it successfully.

With profound sense of graduate, I wish to pay my heartiest thanks to my esteemed advisor and chairperson of my advisory committee Dr. Lakshmi Tewari, Professor, Department of Microbiology, for her benevolent guidance, supervision, constructive counsel, and inspirations before and during the preparation of this manuscript which had provided me a stimulating atmosphere and kept my interest alive in the research. I have found my work incomplete without conveying my special thanks to her for untiring efforts, valuable ideas, and moral support during my research work and preparation of this script.

With sincerity and immense pleasure, I acknowledge my inexplicable and deep sense to gratitude to the members of my advisory committee Dr. Virendra Kasana, Professor, Deptt. of Chemistry; Dr. Gohar Taj, Assoc. Professor, Deptt. of MBGE, and Dr. Manvika Sahgal, JRO, Deptt. of Microbilogy, for their valuable suggestions and kind cooperation throughout the course of my investigation.

I express my sincere thanks to Hon'ble Vice Chancellor; Director, Experiment Station; Dr. N S Murty, Dean PGS; Dr. S P Singh, DSW; Dr. Uma Melkania, Dean CBSH; and LSRB, (DRDO) for providing me the necessary facilities and financial support for the research work.

I feel extremely proud to express my sincere thanks to Dr. Reeta Goel, Professor & Head, Deptt. of Microbiology for allowing me to carry out the research in the department and for her caring, scientific and loving attitude throughout my research work.

I owe earnest thanks to Dr. Salil K. Tewari, Er. Khan Chand, Dr. Anita Sharma, Dr. Ajay Veer Singh, Dr. VD Sharma, Dr. Sanjeev Agrawal, Dr. Atul Kumar, Dr. Ashutosh Dubey, Dr. Alolk Shukla, Dr. AK Shukla, Dr. AK Verma, Dr. JPN Rai, Dr. Vir Singh, Dr. RK Shrivastava, Dr. SP Singh, Dr. DK Shukla, Dr. Raman Rao, Dr. Anil Kumar (Head MBGE), Dr. AK Gaur, Dr. Sundeep Malik, Dr. Pushpa Lohani, Dr. Sandeep Arora, Dr. DS Murthy (former DSW), Dr. GK Singh (Dean CVASc), Mr MP Singh and other deans and directors, and all other teachers of the University for their constant support, guidance, and encouragement, during the pursuit of present study.

I would also like to acknowledge Pathak Sir, Padalia Sir, Pandey Sir, Chitra Mam, Mr. Pankaj, Mrs. Kanak, Kaloo bhaiya, Joshi ji, Nabban and non teaching staff of the Dean office, Department of Biochemisty, Department of Environmental Sciences and Department of MBGE.

Worldly thanks cannot express my respect towards my seniors especially Deep Sir and Disha mam, Tarannum mam, Govind sir, Rekha mam, Geeta mam, Shubhi mam, Komal mam, Harshita mam, Narendra sir, Hemu mam, Ishwar sir, Krishna sir, Mathpal sir, Ashish sir,

Gaurav sir, Avikal sir, Anil sir, Puneet sir, Pawan sir, Rakesh sir, Nitin sir, Kunal sir, Himanshu sir, Katiyar sir, Santosh sir, Ankur sir, Bharti sir, Uma Mageshwar sir, Snigdha mam, Anita Yadav mam, Asmita mam, Neha mam, Tripti mam, Shubhpriya mam, Bhawna mam, Kavita mam, Priyanka Giri mam, Pallavi mam. I also thankful to my lovely juniors Anamika, Hina(2), Priyanka, Shailendra, Rachna, Pragati, Hemant, Hema, Shikha, Nikita, Shalini, Puneet and group, Smriti, Sameekshya, Shivani, Sateesh, Yash, Saurabh(2), Kaapu, Rahul, Ruchi, Jyoti(2), Deepti, Shivam, Divya, Shivangi, Raj, Aamir, Swati, Damini, Ankita, Yogita, Pooja, Vinita, Roshni, Nidhi, Neha, Vandana, Vaibhav for their cooperation, valuable help and regards.

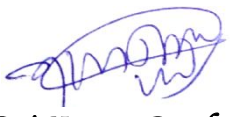
The thesis would be incomplete without a mention of the support and love given me by my cherished group of friends (The Gairi Gang) namely Dr. Saurabh, Dr. Sateesh, Dr. Anupam, Dr. Pankaj, Dr. Dinesh, Dr. Chandan & 'the sadad 'Gairi Sir' (All are soul out of my soul); and my dear and always supportive best friends namely Mantoo, Padam, Mutthu, Rajdev, Chaudhriji, Naru daa, Anjul, Parul, Anwesa, Meenakshi, Dinesh Rai sir, Prateeksha, Karishma, Rajesh Pathak Sir, Deepika, Veena, Bhawna, Manu, Naveen, Priyanka, and others; and my best juniors Babita, Seema and Saloni who kept my sprits alive.

I would like to express my special thanks to PG Society, Pantnagar especially Dr. Upadhyay, Dr Gurdeep, Dr. Ghosh, Dr. Danish, Dr. Raj, Dr. Nitin, Priyanka, Divyanshi, Chandola, Geetanshi, Beenu, Iqra, Hina, Sonia, Ishika, Shweta, Priyanka, Sandeep, Manpreet, Ravi, Abhay, Ashwani, Shauquat and group and all others for their cooperation and valuable support during the stay in Pantnagar. I am highly grateful and thankful for their whole hearted cooperation and timely help. Also I cannot forget those very special moments of PG nights and programmes.

Words in my lexicon fail to express the depth of my feelings for my respected Pitaji and Maaji who displayed love, care, affection, encouragement, always constant help, and moral support in each and every step of my life. Words fail to express my love and gratitude towards my family members Lt. Mr. DN Pandey (Dadaji), Aamma, Mr. Hemant Pandey (Kaka) and family, Mr. HC Pandey (Taauji) and family, my beloved sister Neha Pandey (banna), Renu bua and family, Kunnu bua and family, Rani bua and family, Pinku Bua and family Babli di and family, , and all others members for their help and love.

I would like to express my special thanks to my all batchmates, Shastri Bhawan Inmates and staff, GBPUAT staff, The Fartiyal and staff, and others for their support at the different stages of my work. As a step out of blissful environment of this great University, I express my tribute to G.B.P.U. A. & T. Pantnagar in few words:

"God shall give me strength to behold your fame, may you always shower your earnest blessing on my name".



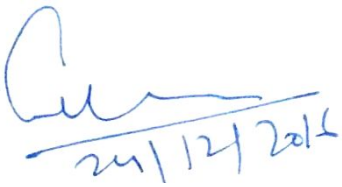
Pantnagar
December, 2016

(Raj Kumar Pandey)
Author

CERTIFICATE-I

This is to certify that the thesis entitled **"BIOPROCESS OPTIMIZATION FOR ENZYMATIC DELIGNIFICATION AND BIOCONVERSION OF *Bambusa balcooa* BIOMASS INTO FERMENTABLE SUGARS FOR BIOETHANOL PRODUCTION",** submitted in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** with major in **MICROBIOLOGY**, of the College of Post-Graduate Studies, G. B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by **MR. RAJ KUMAR PANDEY, Id. No. 44125** under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged.



Pantnagar
December, 2016

(Lakshmi Tewari)
Chairperson
Advisory Committee

CERTIFICATE-II

We, the undersigned, members of the Advisory Committee of **MR. RAJ KUMAR PANDEY, Id. No. 44125,** a candidate for the degree of **DOCTOR OF PHILOSOPHY** with major in **MICROBIOLOGY**, agree that the thesis entitled **"BIOPROCESS OPTIMIZATION FOR ENZYMATIC DELIGNIFICATION AND BIOCONVERSION OF *Bambusa balcooa* BIOMASS INTO FERMENTABLE SUGARS FOR BIOETHANOL PRODUCTION"** may be submitted in partial fulfillment of the requirements for the degree.

(Lakshmi Tewari)
Chairperson
Advisory Committee

(Virendra Kasana)
Member

(Gohar Taj)
Member

(Manvika Sahgal)
Member

Head of the Department
(Ex-Officio Member)

CONTENTS

No.	PARTICULARS	PAGE
	List of Tables	
	List of Figures	
	List of Plates	
1.	INTRODUCTION	
2.	REVIEW OF LITERATURE	
3.	MATERIALS AND METHODS	
4.	RESULTS AND DISCUSSION	
5.	SUMMARY AND CONCLUSIONS	
	LITERATURE CITED	
	VITA	
	ABSTRACT	

LIST OF TABLES

S. No.	Title	Page
2.1	Fuel properties of some liquid transportation fuels	
2.2	Potential lignocellulosic biomasses and their composition (% dry weight)	
2.3	Data representing the area of bamboo forest	
2.4	The fuel characteristics of some common crop based lignocellulosic biomass as feedstocks for fuel production	
2.5	Physical pretreatment conditions for potential lignocellulosic feedstocks used for bioethanol production	
2.6	Biological pretreatment methods used to modify lignocellulosic biomass	
2.7	Summary of various processes used for pretreatment of lignocellulosic biomass	
3.1	Detailed description of the fungal and bacterial isolates/cultures used during present investigation	
3.2	Manual optimization of alkali concentration for delignification	
3.3	Coded and actual (uncoded) levels of the independent variables for design of experiments for enzymatic saccharification	
3.4	Box-Behanken design for three variables at three levels each for enzymatic saccharification	
3.5	Box-Behnken design for enzymatic saccharification	
3.6	Instrument used in the present study	
4.1	Selection and diversification of indigenous wood rotting fungal isolates for lignolytic potential	
4.2	Relative extracellular overall lignin modifying enzyme activity (I_{lig}); laccase enzyme activity (I_{lac}); and lignin peroxidase enzyme activity (I_{per}) indices of selected wood rot fungal cultures as a qualitative measure of lignin degrading enzyme production	
4.3	Time period required for maximum percent removal of different synthetic dyes from liquid media by the selected lignolytic fungal cultures	

4.4	Initial extracellular lignin peroxidase enzyme (a), Mn dependent peroxidase enzyme (b), and laccase enzyme (c) activities in the filtrates of the selected fungal cultures	
4.5	Initial (crude) extracellular crude cellulase enzyme activities (Uml-1) in the filtrates of selected fungal cultures	
4.6	(a) Influence of exogenous carbon source on production and yield of extracellular laccase enzyme from the white rot fungal isolate WDP2 (b) Influence of wheat bran concentration on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2	
4.7	(a) Influence of exogenous nitrogen source on production and yield of extracellular laccase enzyme from the white rot fungal isolate WDP2 (b) Influence of concentration of nitrogen source (ammonium sulphate) on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2	
4.8	Influence of pH on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2	
4.9	Influence of temperature on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2	
4.10	Influence of exogenous surfactants on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2	
4.11	Influence of the exogenous inducer (veratryl alcohol) on laccase production by the fungal isolate WDP2 under static/shake culture conditions	
4.12	Comparative evaluation of lignocellulolytic fungal cultures for laccase enzyme activity under optimised culture conditions	
4.13	(a) Effect of pH and temperature on activity of extracellular laccase enzyme produced by fungal isolate WDP2 (b) Effect of pH, temperature and storage on stability extracellular laccase enzyme produced by fungal isolate WDP2	
4.14	Moisture contents of wood biomass from selected bamboo genotypes	
4.15	Chemical composition of wood biomass from selected bamboo genotypes used during present study	
4.16	Cellulose and lignin content after pretreatments using titenium dioxide and its combinations.	

4.17	Influence of oxidative/alkaline oxidative pretreatment using H_2O_2 with or without metallic induction	
4.18	Selection of medium for whole cell biological (fungal) pretreatment of *Bambusa balcooa* biomass	
4.19	Experimental response of dependent variable (% delignification) after alkaline hydrogen peroxide pretreatment of *Bambusa balcooa* powder using Box-Behnken design (BBD)	
4.20	Optimization of oxidative pretreatment for delignification of bamboo (*B. balcooa*) biomass	
4.21	Experimental response of dependent variable (saccharification efficiency) after enzymatic saccharification of alkaline hydrogen peroxide pretreatment of *B. balcooa* powder using Box-Behnken design (BBD).	
4.22	ANOVA for response surface quadratic model (analysis of variance table)	
4.23	Effect of individual parameters on saccharification efficiency	
4.24	ANOVA for saccharification efficiency	
4.25	Results of regression model for percent saccharification	
4.26	Constraints for optimization of variables for saccharification efficiency	
4.27	Optimum values of parameters for percent saccharification	
4.28	Compositional changes in *B. balcooa* wood after various pretreatments	
4.29	The reported chemical changes in the functional groups of lignocellulosic biomass as observed through FTIR analysis based on absorbance bands	
4.30	Phenotypic (fruiting body/colony/mycelial) characteristics of selected wood rot laccase producing fungal cultures	
4.31	Biochemical characteristics of selected fungal cultures	
4.32	5.8S ITS r-RNA gene sequences retrieved from NCBI database showing identity (%) with that of selected laccase producing fungal isolates	

LIST OF FIGURES

S. No.	Title	Page
2.1	Ultra-structure of lignocellulosic biomass	
2.2	Basic structural units of lignin and structural model of a section of cork lignin	
2.3	Countries with prominent bamboo resource	
2.4	Major steps in lignocellulosic bioethanol production pathway	
2.5	Schematic representation of influence of pretreatment on lignocellulosic biomass	
2.6	General scheme of the catalytic cycle of peroxidases during oxidation of any compound	
2.7	The catalytic cycle of laccases	
2.8	Basic mechanism of action of cellulase enzyme complex on cellulose	
2.9	Schematic view of degradation of arabinoxylan	
4.1	Relative enzyme activity indices of the selected lignolytic fungal cultures for the three lignin degrading enzymes: overall lignin modifying enzyme (I_{lig}), laccase enzyme (I_{lac}) and lignin peroxidase enzyme (I_{per}).	
4.2	Relative cellulase enzyme activity indices (I_{cel}) of the selected lignolytic and non-lignolytic fungal cultures	
4.3	Laccase enzyme activity in dye containing decolourized broth by potential fungal isolate AAFP5	
4.4	Differential extracellular cellulase enzyme activities (Uml-1) in the filtrates of selected fungal cultures	
4.5	Effect of carbon source on production of extracellular laccase enzyme by wood rot fungal isolate WDP2	
4.6	Effect of wheat bran concentration on extracellular laccase production by wood rot fungal isolate WDP2	
4.7	Effect of nitrogen source on extracellular laccase production by wood rot fungal isolate WDP2	

4.8	Effect of different concentrations of nitrogen source on extracellular laccase production by wood rot fungal isolate WDP2	
4.9	Effect of pH on extracellular laccase production by wood rot fungal isolate WDP2	
4.10	Effect of incubation temperature on extracellular laccase production by wood rot fungal isolate	
4.11	Percent increase in laccase yield upon addition of soap-nut extract in comparison to Tween 80 and Triton X 100, from the fungal isolate WDP2	
4.12	Effect of agitation and inducer (veratryl alcohol) on laccase production using wood rot fungal isolate WDP2	
4.13	Percent increase in laccase activity by the fungal isolate WDP2 in optimised medium as compared to that in initial synthetic medium	
4.14	Impact of pH on activity and relative activity of extracellular laccase enzyme produced by the fungal isolate WDP2	
4.15	Impact of temperature on activity and relative activity of extracellular laccase enzyme produced by fungal isolate WDP2	
4.16	Effect of pH on the stability of the extracellular laccase enzyme from fungal isolate WDP2	
4.17	Effect of temperature on stability of the extracellular laccase enzyme from the fungal isolate WDP2	
4.18	Storage stability of extracellular laccase enzyme from fungal isolate WDP2 stored at 4^0C in a refrigerator	
4.19	Comparative evaluation of wood powder from different bamboo sp for biomass yield & chemical composition	
4.20	Influence of various physical and physicochemical pretreatments on cellulose and lignin content of woody biomass of the selected bamboo sp. (*B. balcooa*)	
4.21	Influence of various chemical pretreatments on exposed cellulose and lignin content of the selected bamboo (*B. balcooa*) biomass	
4.22	Influence of biological pretreatment using fruit juice on cellulose and lignin content of selected bamboo sp. (*B. balcooa*)	
4.23	Influence of biological pretreatment on lignin content of the bamboo wood biomass along with the extracellular laccase production by the fungal isolate WDP2 with fermentation medium containing only water, synthetic medium, and optimised medium	

4.24	Linear surface contour plots (a, b) showing the interaction of various parameters during saccharification of *B. balcooa* powder	
4.25	3D surface contour plots showing the interaction of various parameters during saccharification of B. balcooa powder	
4.26	UV-Vis spectra of the hydrolysate of the wood samples subjected to physical, chemical, and biological pretreated showing chemical bonding shifts as compared to untreated (control) wood filtrates	
4.27	FTIR spectra of *B. balcooa* samples before and after physical, chemical and fungal pretreatment	
4.28	Boot strapped consensus phylogenetic tree showing evolutionary relatedness of selected wood rot fungal isolate WDP2 with other wood rot fungi based on 5.8S ITS r-RNA gene sequences	
4.29	Phylogenetic tree showing evolutionary relatedness of selected wood rot fungal isolate WFPn1 with other wood rot fungi based on 5.8S ITS r-RNA gene sequences	

LIST OF PLATES

S. No.	Title	Page
4.1	Some selected mushroom fruiting bodies used for isolation of wood rotting fungi	
4.2	Colonial growth pattern (*In vitro*) on PDA, and photomicrographs depicting mycellial structure of selected wood rotting fungal isolates	
4.3	Qualitative detection for overall lignin modifying enzyme activity; laccase enzyme activity; and lignin peroxidase enzyme activity using ABTS+H_2O_2 (e, f) and using azure B assay of two wood rotting fungal isolates WFPn1 & WDP2 respectively	
4.4	Qualitative detection of cellulase enzyme production by some fungal isolates using CMC as sole carbon source	
4.5	*In vitro* decolourization of synthetic industrial dyes by potential lignolytic fungal isolates	
4.6	SDS PAGE analysis and Native PAGE analysis of extracellular proteins of various lignolytic fungal cultures	
4.7	Zymogram analysis of extracellular laccase enzyme produced by the selected fungal isolates	
4.8	Processing of Bamboo biomass from forest to laboratory for bioprocessing studies	
4.9	Biological pretreatment of (*B. balcooa*) biomass using lignolytic wood rot fungal isolate WDP2, and fungal growth on the bamboo biomass during pretreatment in a flask	
4.10	Scanning electron photomicrographs of *B. balcooa* wood powder untreated and pretreated at 500X and at 1000X	
4.11	Scanning electron photomicrographs of biologically (fungal) pretreated *B. balcooa* wood sample after enzymatic saccharification at 500X and at 1000X.	
4.12	Fruiting body morphology of the selected wood rot fungal cultures at their natural habitat.	
4.13	Phase contrast photomicrographs showing mycelial structures of the unstained live fungal preparations at 40X and 100X	
4.14	Sugar/ organic compound assimilation efficiency of selected fungal cultures	
4.15	Electrophoretic separation of genomic DNA on agarose gel, PCR amplicon of 5.8S ITS rRNA gene of two selected lignolytic fungal cultures	

Introduction

Chapter 1 INTRODUCTION

Global warming and energy crisis are the great concern to governments and people around the world. The demand of energy is globally increasing in twenty first century and one of the major challenges in front of the society is the generation of the polluting gases into the atmosphere due to combustion of a large amount of liquid fossil fuel. In addition, the rising costs of fossil fuel due to its depletion within the earth crust and the predicted world energy consumption within next 10 years, which is expected to increase by 54%, considerable attention is being directed towards the development and the use of eco-friendly, sustainable, and cost effective domestic energy source that can be able to replace completely or displace a significant amount of the petroleum. Bio-ethanol from bio-renewable sources including sugars, starches and lignocellulosic materials could be one such alternative that can be used as liquid transportation fuel (**Wyman, 2007**). The agreement implemented by Policy Energy Act and the Energy Independence and Security Act aims to reach 36 billion gallons of bioethanol by the year 2022.

The biofuels are classified as primary and secondary biofuels. The primary biofuels are the energy sources taken from renewable biomass and used in an unprocessed form, basically for heating, cooking or electricity production such as dry wood and wood chips etc. The secondary biofuels are produced by processing of the renewable biomass such as ethanol, biobutanol and biodiesel etc. that can be used in vehicles and various industrial processes. The secondary biofuels are further divided in to first generation, second generation and third generation biofuels on the basis of substrate or the raw material used for their production **(Nigam and Singh, 2010)**.

The first generation liquid biofuels that are produced from sugars, starch or other edible materials (grains and seeds) are not preferable due to the feed or food competition, use of arable land, high water and fertilizer requirements for their production, and lack of well mannered agricultural practices. This has caused greater attention for second generation biofuels that are produced from the agricultural lignocellulosic plant residues (**Nigam and Singh, 2010**). . Furthermore, the ethical concerns about the use of food crops such as corn, and sugarcane etc. as fuel raw materials have encouraged the research efforts to be more concentrated on the potential of inedible feedstock alternatives (**Limayem and**

Ricke, 2012). Other potential benefits of the use of second generation biofuels include utilization of agro-residues, no use of additional fertile land for substrate production, promotion of rural development and improving economic conditions in emerging and developing regions (**Singh *et al*., 2011**).

Ethanol is excellently used as a transportation fuel and can be blended with gasoline at about 10% levels as gasohol (E10) as is used in U. S., or it can be used as 20% blends or E20 as is used in Brazil. Bio-ethanol alone accounts for 85% of total global biofuel production, with biodiesel as secondary fuel. Alternatively, ethanol can be mixed in a proper proportion with isobutylene to form ethyl tertiary butyl ether (ETBE), which in addition is used to increase octane rating, oxygenate benefits of direct ethanol blends, reduce the vapour pressure of the gasoline to which it is added, and further improves the suitability of gasoline for meeting clean air requirements (**Wyman, 2007**). Among potential alternative resources for bioethanol production, lignocellulosic plant biomass has been identified as the primary source. Lignocellulosics from agricultural residues, industrial extracts and forest sources account for the majority of total plant biomass present in the world and thus provide vast renewable resource. Importantly, the lignocellulosic raw materials not competing with food production, thus, can provide environmental, economic, and strategic benefits for the production of biofuels (**Vikari *et al*., 2012**). The goals and ambitions of the U.S. Department of Energy is to supply 30% of the gasoline demand by the year 2030, and the European Union with a vision of 10% of the EU's transportation fuels deriving from biofuels by 2020. Today, the main aim is not only to replace the petroleum products by any type of biofuel but also to review the greenhouse gas emission saving thresholds. The fuel bio ethanol can be obtained from energy crops and lignocellulosic plant biomass. The complexity of generation of proper process depends on the availability of feedstock. In this way, the spectrum of designed technologies goes from the simple conversion of sugars by fermentation, to the multi-stage conversion of recalcitrant lignocellulosic plant biomass into ethanol.

Lignocellulosic biomass comprise about 85-90% of cellulose, hemicelluloses and lignin and content of these constituents vary from 20-50% cellulose, 20-35% hemicelluloses, 10-35% lignin **(Kumar *et al*., 2009)**. Cellulose is the most abundant component and is a linear and un-branched homo-polysaccharide, consisting of 10,000 to 15,000 D-glucose units linked by β- 1, 4 glycosidic bonds; and is most amenable for

production of bio-ethanol **(Sannigrahi *et al.*, 2010)**. Hemicelluloses are second most abundant polysaccharides in lignocellulose, composed of complex carbohydrates which are non-cellulosic and having low molecular weight than cellulose. Hemicelluloses are heterogenous in nature, mainly consists of different polymer like pentoses (xylose and arabinoise), hexoses (glucose, mannose and galactose), and sugar acids **(Sun *et al.*, 2002; Hendriks and Zeeman, 2009)**. Lignin, a non-fermentable phenyl-propane unit; comprises the second most abundant class of polymeric organic substance on the Earth after cellulose **(Saritha *et al.*, 2012)**. This amorphous, hetero-polyphenol polymer lacks a defined primary structure and consisting of three phenyl propeonic alcohols or monolignols *viz p*-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (quaiacyl propanol), and sinapyl alcohol (syringyl propanol); that held together by different kinds of linkages occurs in vascular tissues and specialized for liquid transport and mechanical strength, thus very tough to degrade **(Kotake *et al.*, 2013)**. Different feed-stocks contain different amount of lignin that must be removed via pretreatment to enhance accessibility of cellulose for enzymatic digestibility.

About $2.9x10^3$ million tons of the lignocellulosic residues are available from the cereal crops worldwide, $3x10^3$ million tons from oil seed crops and $5.4x10^2$ million tons have been produced from plantation crops annually worldwide. A plenty amount of the lignocellulosic plant biomass is available in India (**Sukumaran *et al.*, 2010**). The reported main feedstock for ethanol production is sugarcane which is either in the form of cane juice or molasses. About 79% of ethanol in Brazil is produced from fresh sugar cane juice and the remaining is from cane molasses (**Wilkie *et al.*, 2000**). In India, Sugarcane molasses are the main feedstock for bio-ethanol production but the cane juice is not presently used for this purpose. Beet molasses are other sources of fermentable sugars for ethanologenic fermentation. Agriculture residues such as wheat straw, corn stover, rice husks, and the woody crops are other lignocellulosic biomasses that are renewable and inexpensive. Although the resources are vast, but almost 90% of this material goes unused, and, the burning of such waste materials can cause the environmental pollutions (**Richardson *et al.*, 2002**). The estimated global use of wood is approximately four billion cubic meters and of which 55% is used as fuel wood. Part of agri-residue (about 16 million tons/yr) is used for power generation. The wood waste has the great potential as raw material resource for the production of the second generation biofuels *i.e.* bioethanol.

Bamboo is perennial woody grass belonging to the family Gramineae and distributed widely in Asia. China contributes as a major bamboo-producing country and has approximately 300 species in 44 genera that occupy 3% of the total forest area (**Scurlock *et al.*, 2000**). Bamboos are group of perennial ever-green grasses and enjoying wide distribution in India, especially in the north eastern regions where it is an important resource for the multiple applications in daily life. *Dendrocalamus* sp. is the most common species that occupies more than 50% of the total area under bamboo growth in India. India is the second largest producer of bamboo biomass in the world with the production rate of about 32 million tons per year. About 5.4 million tons of bamboo residues are generated in the country every year by the bamboo processing industries of which about 3.3 million tons remains as surplus .Compared to other available feedstocks, bamboo biomass has a relatively high cellulose and low lignin content which make it suitable for bioethanol production (**He *et al.*, 2013**). Furthermore the growth rate of the grass is very high, then high biomass yield can be obtained and thus high specific ethanol production can occur if compared with other biomasses. Due to the high growth rates, the oxygen release and the absorption of the environmental carbondioxide is also high, that lead to the environmental cleanup. These properties make the bamboo biomass a desirable feedstock for the production of ethanol and other biomass based materials. Thus Bamboo is an interesting feed stock for bioethanol production due to the relatively higher growth rate and their abundant and sustainable availability. *Bambusa* sp. and *Dendrocalamus* sp. are important bamboo varieties with high amount of cellulose that are very common in India. Of them large amounts of biomass is annually generated, that can be efficiently utilised for bio-ethanol production. **Kuttiraja *et al*. (2013)** reported the ethanol production potential is about 473 million litters from the 3.3 million tons of total bamboo process waste available in the country. Thus, with improved strategies for solid liquid separation and improvements in enzymatic delignification, saccharification and fermentation, the huge amount of ethanol can be generated with significantly improved economics.

In processing of any woody lignocellulosic plant substrate, there are four stages *viz* pretreatment, hydrolysis or saccharification, fermentation and distillation. Feedstock pretreatment is required to alter the biomass recalcitrant structures so that hydrolysis of the carbohydrate fraction of the biomass to monosaccharides can be achieved more fastly with the greater fermentable sugar yield.

Effective pretreatment is fundamental for optimal successful hydrolysis of the lignocellulosic substrates and product recovery. Pretreatment upstream processesses include mainly physical, (i.e., biomass size reduction) and thermochemical or chemical processes that involve disruption of the recalcitrant material of the plant biomass. This upstream operation increases substrate porosity with lignin redistribution or lignin degradation. Therefore, it enables maximal exposure of cellulases to the explored surface area of fibres to reach an effective hydrolysis with minimal energy consumption and maximum sugar recovery. Several pretreatment methods, namely, physical/mechanical, chemical or microbiological have been used to remove the recalcitrance of lignocellulosic plant biomass depending on the raw material being extracted. More recently, there has been considerable advancement in development of biological or microbial pretreatment processes. Although, most of these treatments can liberate hemicellulose and cellulose from the cell wall, some of them remain economically unfeasible due to key technical issues (**Limayem and Ricke, 2012**).

The physical and chemical pretreatment methods are sometimes harsh and cost/energy intensive. On the contrary, biological pretreatment processes are considered to be the mild and environment friendly methods. They employ micro-organisms, mainly white rot fungi, sometimes actinomycetes and bacteria which can degrade lignin through the action of lignin-degrading enzymes such as peroxidases (lignin peroxidase, LiP and manganese peroxidase, MnP) and laccases (Lac). *Phanerochaete chrysosporium* has been the model organism for studies of lignin degradation by white rot fungi. But the laccase production less been reported using the fungi *P. chrysosporium* (**Dittmer *et al.*, 1997**). Moreover, families of lignolytic enzymes such as phenol oxidase (laccase) and peroxidases (lignin peroxidase, LiP and manganese peroxidase, MnP) are widely considered to play a key role in the enzymatic degradation of lignin. Besides these, other enzymes, such as glyoxal oxidase, glucose oxidase, oxido-reductase and methanol oxidase might also be involved in lignin degradation (**Saritha and Arora, 2012**). Laccases generally belong to the blue copper oxidoreductive enzyme family & are similar to other phenol-oxidizing enzymes which preferably polymerize lignin. Recently, some bacterial cultures such as *Azospirillum lipoferum, Bacillus subtilis* etc. have also been reported to produce laccase enzyme. The more effective combination of laccase with either LiP and/or MnP in several other white rot fungi seems to be a more common combination of enzymes than the LiP/

MnP pattern found in standard culture of *Phanerochaete chrysosporium.* Thus fungal peroxidases, and laccases have been implicated in the biodegradation of lignin.

The other common wood rot fungi producing lignolytic enzymes include *Ceriporiopsis subvermispora*, *Phlebia* sp., *Physisporinus rivulosus*, *Dichomitus squalens*, *Trametes versicolor*, *Heterobasidium annosum*, & *Irpex lacteus* etc. (**Dashtban *et al.*, 2010**). Although most of the laccase enzyme that is presently used for commercial applications, is produced using fungal systems, several factors such as faster growth rate, the ease genetic manipulation, wide ecological distribution, greater stress tolerance, ability to synthesize extremozymes, suggest greater potential for enzyme production using bacterial system.

Despite having several advantages over the conventional fuel ethanol, the second generation lignocellulosic bioethanol has not yet been implemented on an industrial or commercial scale according to the requirements. The major constrains in the production are high cost and availability of the feedstock, pretreatment process, high cost of the enzymes used for biological pretreatment and saccharification processes, cost effective enzyme production technology, and lack of the robust enzymes that can function efficiently at the broad temperature and pH range. The use of readily available fast growing lignocellulosic plant biomass, such as bamboo, that contains larger amount of cellulose and relatively low lignin content could be a potential feedstock for bioethanol industry. Considering all the factors and constrains in mind, the cost effective bioethanol production requires optimization of biological pretreatment strategies for effective delignification and conditions for maximizing lignolytic enzyme production in the best possible way. Moreover the characterization of the lignolytic enzymes for pH and temperature optima stability and shelf life needs to be optimized for the large scale application for delignification and saccharification processes.

Objectives: Keeping in view the above facts, present investigation was planned with following objectives:

1. Identification, characterization & selection of wood rot fungi for lignin degrading enzyme production under optimum culture conditions & characterization of lignolytic enzymes.

2. Optimization of delignification process using lignolytic enzymes for pretreated biomass of selected Bamboo sp. and monitoring for structural changes**.**
3. Bioconversion of processed delignified biomass into fermentable sugars through enzymatic saccharification for bio-ethanol production.

Review of Literature

Chapter 2 REVIEW OF LITERATURE

Over the last few decades, the negative impact on the environment due to extensive use of fossil fuel consequent is global warming, created by growing greenhouse gas (GHG) emission. Moreover, progressive demand of liquid transportation fuel for energy, inevitable depletion of the fossil fuels, and the unstable oil market have renewed the interest of society in searching for alternative fuels (**Perlack *et al*., 2005; Himmel *et al*., 2007; Solomon *et al*., 2007**). Thus climate change caused by carbon emissions using fossil fuels and energy security in the future are the two major constrains which drive us to search for alternative energy sources. The alternative fuels expected to clarify several requirements including substantial reduction of greenhouse gas emission to environment; capability of the alternative fuel is being produced from planty available, renewable available feedstocks, as well as worldwide availability of raw materials for their production (**Hahn-Hägerdal *et al*., 2007**). One such renewable feedstock, used to produce different forms of energy, is biomass. The world oil reserves projected to deplete in about 40 years, hence, emphasis has shifted towards the sustainable substitute of the liquid transportation fuel. The United State Biomass Research and Development Technical Advisory Committee have reported a 30% replacement of the current petroleum consumption with bio-fuels by 2030. This goal implies that the demand for cellulosic feedstock will increase to 1 billion dry tons annually, more than three-times the US corn production in 2011 (**Perlack *et al*., 2005; Perlack *et al*., 2011**).

India is a country with a positive outlook towards the production of renewable energy technologies and committed to use the plenty available renewable biomass resources to supplement its energy requirements for safe and bright future of the nation. Although it has 0.5% of the oil and gas resources but as per increase in population, there is a dependency on oil imports to meet the domestic demand (**Sukumaran and Pandey, 2009**). The country is one among the few nations to have a distinct ministry for renewable energy which address the technological development of bioenergies or biofuels along with the development and utilization of other renewable energy sources. In the year 2003, the Planning Commission of the Government of India presented a report on the development and utilization of native renewable resources of biofuels (**Planning Commission, 2003**).

As per the report, bio-ethanol and biodiesel were identified as the principal biofuels to be developed for the nation.

2.1 Biofuels and Their Benefits

In the recent years, research has been directed to find out alternate fuel for the substitution of petroleum derived transportation fuels to help address energy cost, energy security and global warming concerns (**Farrell *et al*., 2006; Ragauskas *et al*., 2006**). Biofuel is a type of fuel whose energy is derived from biological carbon fixation, generally by the plants. These include fuels derived from solid biomass (*e.g.* firewood, wood chips, pellets, charcoal, etc.), liquid biofuels (biodiesel, bioethanol, biobutanol, etc.) from biomass conversion, and various gaseous fuels (hydrogen, biogas etc.) (**Goldemberg, 2007**). Among different bioengrgy sectors, the liquid (transportation) biofuels have most rapid growth. The liquid biofuels can beclassified into 'first generation', 'second generation', and 'third generation' biofuels according to the biomass or feedstock used for their production.

2.1.1 First Generation Biofuels

First-generation biofuels include ethanol and biodiesel that are produced from the biomass which is more often edible. Ethanol is generally produced upon fermentation of monosaccharide (mostly glucose) by certain yeast strains such as *Saccharomyces cerevisiae*. Sugarcane and corn are the important feedstocks that are generally used for the production of first-generation biofuels (bioethanol). Brazil is one of the leading countries that is using sugarcane as a very common feedstock for bioethanol production (**McAloon *et al*., 2000; Larson *et al*., 2008; Brennana and Owendea, 2010).** Other feedstocks that can be considered to produce first-generation biothanol include whey, barley, sugarbeets, and potato waste. The three main kinds of first generation biofuels used commercially are biodiesel (bio-esters from available oil and fat), ethanol, and biogas. These first generation biofuels are being produced in large quantities using the production process that is considered 'completely established technology' (**Larson *et al*., 2008; Brennana and Owendea, 2010; Lavoie *et al*., 2011)**. But the demands of edibles oils are increasing trend for our society, moreover the food and feed competition while using the food crops for biothanol production so it difficult to use the agricultural food crop for biofuel production. Thus they pose a threat to food prices since the biomass used are food crops such as corn

and sugar beet. Again, first generation biofuel production has contributed to incrase in world prices for food and animal feeds. Additionally, biomass for first generation biofuels requires lots of fertile land to grow, and most available rainfed agricultural land is already in use (**Brennana and Owendea, 2010**). They only provide a small benefit over fossil fuels in regards to greenhouse gases since they still require high amounts of energy to grow, collect, and process. Thus use of first generation biofuels is a more expensive option than gasoline, making it economically unfavourable.

2.1.2 Second Generation Biofuels

The biomass sources for second generation biofuels include wood, organic waste, food waste and specific biomass crops. Fast growing trees such as poplar trees need to undergo a pretreatment step, which is series of chemical reactions that break down lignin, the "glue" that holds plants together, in order to make fuel. This pretreatment step involves thermochemical or biochemical reactions that unlock the sugars embedded in fibers of the plant. After this step, the process to generate plant ethanol resembles that of 1st generation ethanol production. Additionally, straw and other forest residues can go through a thermochemical step that produces syngas (a mixture of carbon monoxide, hydrogen and other hydrocarbons). Hydrogen can be used as a fuel and the other hydrocarbons can be used as additives to gas oil (**McAloon *et al*., 2000; Lynd *et al*., 2008**).

The goal of second-generation biofuels is to increase the amount of biofuel that can be produced sustainably by using biomass comprised of the residual non-food crops, as well as other crops that are not used for food purposes (**Greesel, 2008; Lynd *et al*., 2008**). Generally non edible Lignocellulosic biomass is the potential source of the second generation biofuels (bioethanol) and is expected to reduce net green house gas emission, increase energy efficiency of existing engines and potentially overcome the limitations of first-generation biofuels. Bioethanol can be produced by the fermentation of the lignocellulosic biomass from agricultural byproducts, forest residues, or energy crops are shows many potential advantages in comparison to sugar or starch derived first generation of biofuels (**Lin and Tanaka, 2006**). The problem for the production of second generation biofuels is to extract useful sugars from the cellulosic part of woody or fibrous biomass, but these sugars are locked in by lignin and hemicellulose in form of cellulose (**Inderwildi and King, 2009**). Lignocellulosic ethanol is made by liberating the sugar molecules from

lignocellulosic plant biomass using enzymes, or by other physical or chemical processes. These sugars can then be fermented by using native or genetically modified strains of yeasts to produce bio-ethanol in the same way as first generation bioethanol production. The by-product of this process is lignin which can be burned as a carbon neutral fuel to produce heat and power for the many other processing plants (**Peterson *et al.*, 2008**). However, conversion of these woody biomasses into fermentable sugars requires tough and costly technologies involving pre-treatment of lignocellulosic biomass with special enzymes or with the speacialized pretreatment technology, meaning that second generation biofuels cannot yet be produced economically on a large scale (**Brennan and Owende, 2010**).

2.1.3 Third Generation Biofuels

Microalgae offer great potential as a sustainable feedstock for the production of third generation biofuels, such as biodiesel and bioethanol. Third generation biofuels are derived from autotrophic microalgae/cyanobacteria and are considered to be a viable alternative energy resource that is also devoid of the major drawbacks associated with first and second generation biofuels. Microalgae are able to produce 15–300 times more lipid droplets or oil inside their cytoplasm for biodiesel production than that of the other traditional crops used for biodiesel production. Furthermore, the crop plants used for biodiesel production, are usually harvested once or twice a year, while, microalgae have very fast growth rate with very short harvesting cycle of 5-10 days or a maximum 15 days (depending on the process), allowing multiple or continuous harvests with significantly increased yields of the oil or lipid (**Dragone *et al.*, 2010; Nigam and Singh 2011**). However, several important scientific and technical constrains remain to be overcome before the large-scale production of microalgae derived biofuels. Technological developments, including advances in photobioreactor design, microalgae biomass production, microalgal biomass harvesting, drying, and processing are important and critical areas that may lead to enhanced cost-effectiveness and therefore, effective commercial implementation of the biofuel from microalgae (**Schenk *et al.*, 2008**).

2.2 Bioethanol: Properties, Production and Challenges

Bioethanol also called as ethyl alcohol (CH_3–CH_2–OH or ETOH) or sometimes grain alcohol because mostly produced from grains is a liquid transportation biofuel which

can be produced from several different biomass feedstocks and conversion technologies (**Hansen *et al.*, 2005**). Many properties of ethanol such as higher octane number than isooctane (an important component of gasoline) and methanol, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline, makes the bioethanol as a very good alternative to fossil based liquid transportation fuel (**Table 2.1**).

Table 2.1: Fuel properties of some liquid transportation fuels

Fuel property	Isoctane	Methanol	Ethanol
Cetane number	–	5	8
Octane number	100	112	107
Auto-ignition temperature (K)	530	737	606
Latent heat of vaporization (MJ/Kg)	0.26	1.18	0.91
Lower heating value (MJ/Kg)	44.4	19.9	26.7

Other properties of ethanol may be also higher compression ratio, shorter burn time and leaner burn engine, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (**Yu and Zhang, 2003; Farrell *et al.*, 2006; Hahn-Hägerdal *et al.*, 2006**). Ethanol has already been introduced for transportation on a large scale in India, Brazil, the US and some Asian and European countries, and as per many reports, it is expected to be one of the dominating renewable biofuels in the transport sector within the coming 20 years. Ethanol can either be blended with petrol or used as neat alcohol in dedicated engines, with the higher octane number and higher heat of vaporization. The cost effectiveness of ethanol production during current scenario can be exactly calculated as in the fuel market it is produced either from sugars (in Brazil) or from starch (in USA) at competitive prices (**Gray *et* al., 2006; Hahn-Hägerdal *et al.*, 2006**). However, the raw material that can be use for production of ethanol also has to be used for animal feed and human needs. Thus the feedstock will not be sufficient to meet increasing demand of transportation fuel ethanol; and the reduction of greenhouse gas production resulting from use of sugar or starch-based ethanol is not as high as desirable for the current conditions of environment (**Yu and Zhang, 2003; Farrell *et al.*, 2006**).These factors move us towards the exploitation of plant biomass based lignocellulose feedstocks, such as agricultural and forest residues, dedicated crops, unused forest trees and giant grasses (***e.g.*** Bamboo) etc.

for the production of biomass based ethanol (**Farrell *et al*., 2006; Gray *et* al., 2006; Hahn-Hägerdal *et al*., 2006**).

2.3 Lignocellulosic Biomass and Feedstock Availability

Lignocellulosic biomass is considered as the most feasible and sustainable feedstock for bio-ethanol production. Ethanol is an alternative, sustainable, cost-effective, convenient energy source, it is also known as an "oxygenate" because it contains 37% oxygen by weight, which enhances fuel combustion and therefore contributes to a reduction in exhaust emission of harmful gases and fossil fuel based petroleum use. Its property of atmospheric photochemical reactivity, further makes it a very convenient fuel due to the no effect on ozone. Ethanol is known to run combustion engines at higher compression ratios and thus provides better performance than other liquid fuels (**Bothast and Saha, 1997; Wheals *et al*., 1999**). Today, most of the ethanol is produced from renewable resources such as sugarcane and starchy grains, although, significant efforts are being made to produce ethanol from lignocellulosic biomass (lignocellulosic biomass comprises of almost 50% of all biomass in the biosphere) such as forest trees and other agriculture residues (**Bothast and Saha, 1997**). According to **USCB, 2008**, the world population is continuously increase day by day and it is estimated to increases from 6.7 billion to 8 billion by 2030. Further, **Campbell and Laherree (1998)** have reported that the global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050. Thus, the energy demands of the future are likely to play a key role for sustainable future of the human beings. Thus, nations around the world are investing in alternative sources of energy to sustain the human life which can include production of bioethanol from the readily available lignocellulosic plant or woody biomass. Today the leading countries in bioethanol production are Brazil and USA, of which USA is the world's largest producer of bioethanol (**Carere *et al*., 2008**). Now a days the production of ethanol can be achieved by various lignocellulosic biomass such as corn stover, wheat straw, sugarcane bagasse, rice straw, rice husk, corn cob, oat hull, corn fiber, woodchips and cotton stalk; energy crops such as switch grass and Alfa Alfa, and various weeds such as *Saccharum spontaneum*, *Lantana camara*, *Eichhornia crassipes* etc (**Sukumaran and Pandey, 2009; Limayem and Ricke, 2012)**. Thus lignocellulosic bioethanol become one of the best alternatives, because these sources have widespread abundance and the cost is relatively cheap (**Carere *et al*., 2008; Sukumaran and Pandey, 2009**). All these

substrates are of low cost and most of them available throughout the year as well as generated through less fertile land. According to a recent survey on the generation and availability of various biomass residues employed by the National Institute for Interdisciplinary Science and Technology India, the major agro-residues in terms of volumes generated (in million metric tons-MMT) were found to be rice straw (112), rice husk, (22.4) wheat straw (109.9), sugarcane tops (97.8) and bagasse (101.3) (**Sukumaran and Pandey, 2009**). Alone in U.S., 1.4 billion tons per year production of dry biomass is achieved through lignocellulosic materials and is estimated to be 30% of this biomass originating from forests. Most of the cultivated land in U.S. constitutes around 35% for the forest cultivation, approximately 27% for grazed and herbaceous cultivation and 19% is used as crop lands per approximately 2.25 billion acres (9.0 million km^2) (**Limayem and Ricke, 2012**). Municipal and industrial wastes are also potential recyclable cellulosic materials that can originate either from residential or non-residential sources such as food wastes and paper mill sludge (**Hu *et al*., 2008; Limayem and Ricke, 2012**).

2.3.1. Agricultural Residues, Herbaceous and Municipal Solid Wastes (MSW)

As per the data of U.S. Department of Energy Biomass Program, there are approximately 350 to 450 million tons per year crop residues harvested annually. Varying types of crop residues that can be used as feedstock are comprised of agricultural wastes such as corn stalks, corn stover, rice straw, wheat straws and sugarcane bagasse, and grasses, among there rice and wheat straw and corn stalks considered the potential bioethanol feedstocks in this category (**Knauf and Moniruzzaman, 2004; Limayem and Ricke, 2012).** Agricultural residues help to avoid dependency on forest woody biomass and thus reduce non-sustainable cutting of tree plants or deforestation. Moreover, unlike forest trees, crop residues are having a short growth period and short-harvest rotation period that renders them more consistently and voluntarily available for lignocellulosic bioethanol production (**Knauf and Moniruzzaman, 2004)**. Switch grass and *Miscanthus giganteus* (another fast-growing grass) are the primary herbaceous energy crops; perennial grasses, are having a great interest due to their low-cost investment for production as well as abundance, ability to resist diseases, and their high yield of sugar substrates per acre. In addition to these cellulosic feedstocks, municipal and industrial solid wastes are also potential raw materials for bioethanol production (**Demirbas, 2005)**. Although, over billion tons of agricultural residues, herbaceous and municipal solid wastes biomass per

year would be potentially available to meet 30% replacement of petroleum derived gasoline (**Perlack *et al.*, 2005**), the cost effectiveness of biomass could be a major problem if potentially available fertile soil containing lands and feedstocks are not utilised for production and managed efficiently. However, the problem arises from the composition of these biomasses as crop residues contain approximately 25 to 35% more hemicellulosic material than woody biomass (**Demirbas, 2005; Carere *et al.*, 2008; Limayem and Ricke, 2012**).

2.3.2. Forestry Feedstocks

Forest woody lignocellulosic feedstocks seem to be the most important and suitable feedstocks for bioethanol production. These feedstocks account for approximately 370 million tons per year (30%) of lignocellulosic biomass in the U.S. (**Perlack *et al.*, 2005**). Broadly the soft wood and hard wood are two types of woody materials. Poplar (cottonwood) and Bamboo, which includes 35 or more species, are the most abundant fast-growing plants suitable for bioethanol production. Unlike agricultural biomass, woody raw materials offer flexible harvesting times, abundant availability and avoid long latency periods of storage (**Carere *et al.*, 2008; Limayem and Ricke, 2012**). Additionally, the studies reported that woody feedstock possessed more lignin than agricultural residues and less ash content (close to zero). These unique characteristics of woody biomass including primarily high cellulose density and minimal ash content make woody raw material very attractive to cost-effective in conjunction to its lower content of pentoses (mainly xylan) over agricultural biomass and more favourable for bioethanol production, if the biomass recalcitrance is surmounted. Forestry wastes such as slashes, sawdust from sawmills, wood chips and branches from dead trees have also been used as bioethanol feedstocks (**Limayem and Ricke, 2012**).

2.4. Lignocellulosic Biomass Composition

The green biomass comprise about 85-90% lignocellulosic content with 20-50% cellulose, 20-35% hemicelluloses and 10-35% lignin (**Figure 2.1**). Cellulose, the most abundant component in lignocellulosics, is a linear, un-branched homo-polysaccharide, consisting of 10,000 to 15,000 D-glucose units linked through β-(1,4) glycosidic bonds. It is most amenable to the production of ethanol and other higher molecular weight alcohols by its enzymatic hydrolysis to glucose followed by fermentation to ethanol **(Kumar *et al.*,**

2009; Sannigrahi *et al*., 2010). Hemicelluloses are heterogenous in nature, mainly consist of different polymer like pentoses (xylose and arabinose), hexoses (glucose, mannose and galactose), and sugar acids. Broadly it is an amorphous and variable structure, that is composed of heteropolymers including hexoses (D-glucose, D-galactose and D-mannose) as well as some pentoses (D-xylose and L-arabinose) and sometimes may contain sugar acids (uronic acids) namely, D- glucuronic, D-galacturonic and methylgalacturonic acids (**Sun and Chang 2002; Czernik and Bridgwater, 2004; Elliott, 2007**). Its backbone chain is primarily composed of xylan β, 1-4 linkages that include nearly 90 % D-xylose and about 10 % L-arabinose. The glucomannans are hemicelluloses of softwood while hardwood hemicellulose is more frequently composed of xylans (**Sun and Chang 2002; Sannigrahi *et al*., 2010**). 70% of the entire biomass made up with cellulose and hemicelluloses, both components are tightly linked to third most recalcitrant and rigid component lignin through covalent and hydrogenic bonds that makes cementic structure (**Figure 2.1**). These bonding interactions make the structure of lignin highly tough, robust and highly resistant to any kind of treatment that is used to degrade it **(Sun and Chang 2002; Hendriks and Zeeman, 2009)**. Lignin, a non-fermentable molecule comprising of phenyl-propane units is the second most abundant class of organic polymeric substance on Earth after cellulose **(Saritha and Arora, 2012)**. This rigid, amorphous, hetero-polyphenolic polymer lacks a defined primary ultrastructure but consisting of three types of phenyl propeonic alcohols monolignols *viz p*-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (quaiacyl propanol), and sinapyl alcohol (syringyl propanol) (**Fig 2.2, a**). These molecules are held together by different kinds of linkages in the mess structure of lignin (**Fig 2.2, b**), and it occurs in vascular tissues of plants, specialized for liquid transport and mechanical strength **(Czernik and Bridgwater, 2004; Kotake *et al*., 2013)**. Some important lignocellulosic biomass resources and their composition is given in **Table 2.2**. Different feed-stocks that contain different amount of lignin can be used for bioalcohol production, but prior to use its lignin must be removed via any pretreatment technology to enhance biomass digestibility for obtaining reducing sugars (**Elliott, 2007; De Wild *et al*., 2011**).

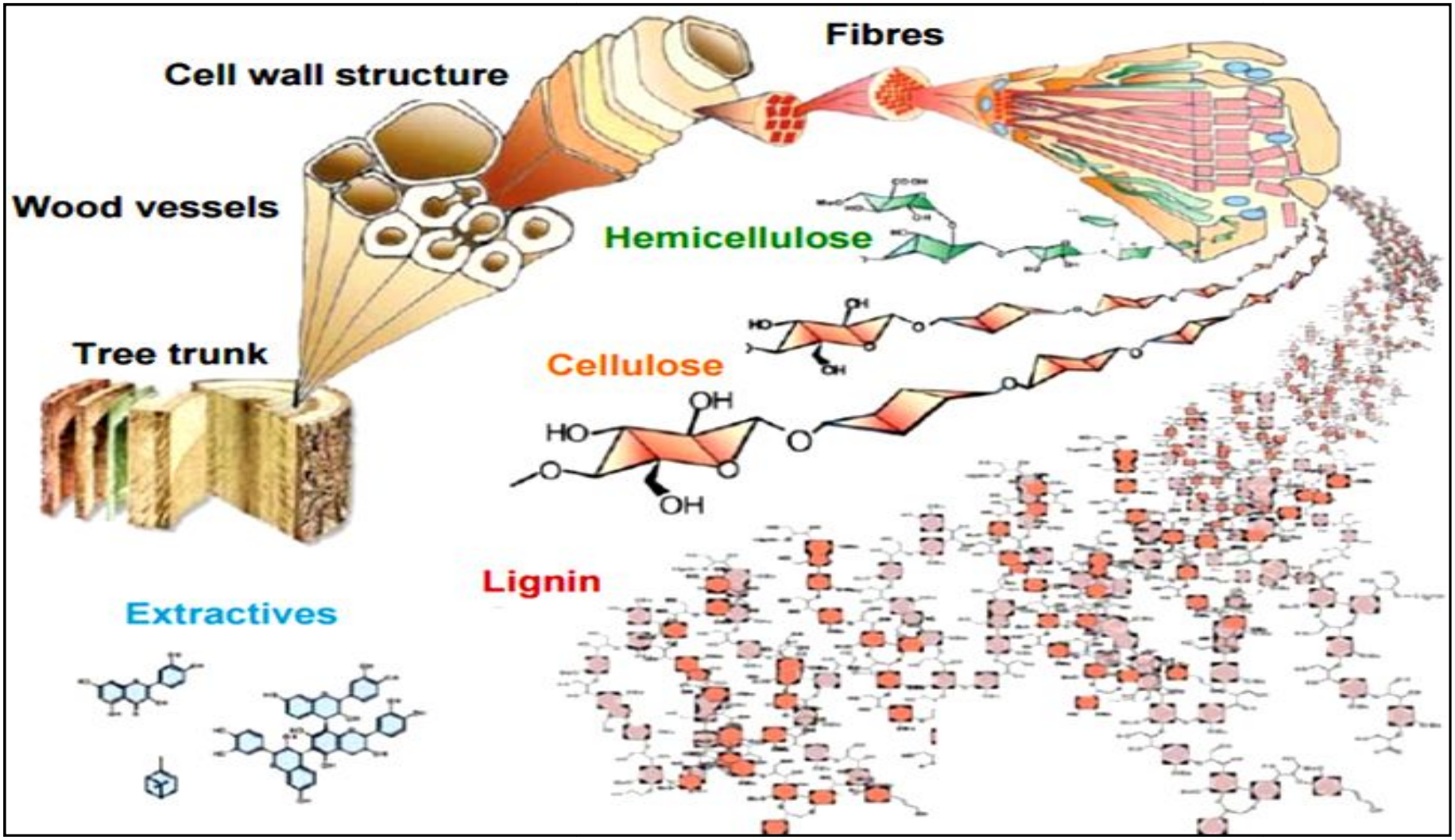
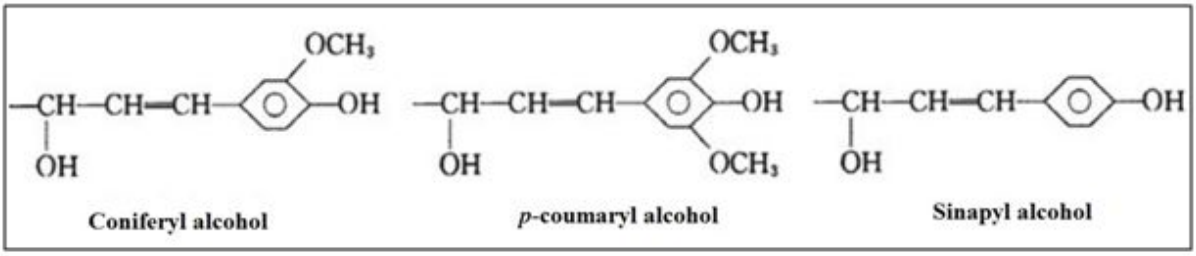
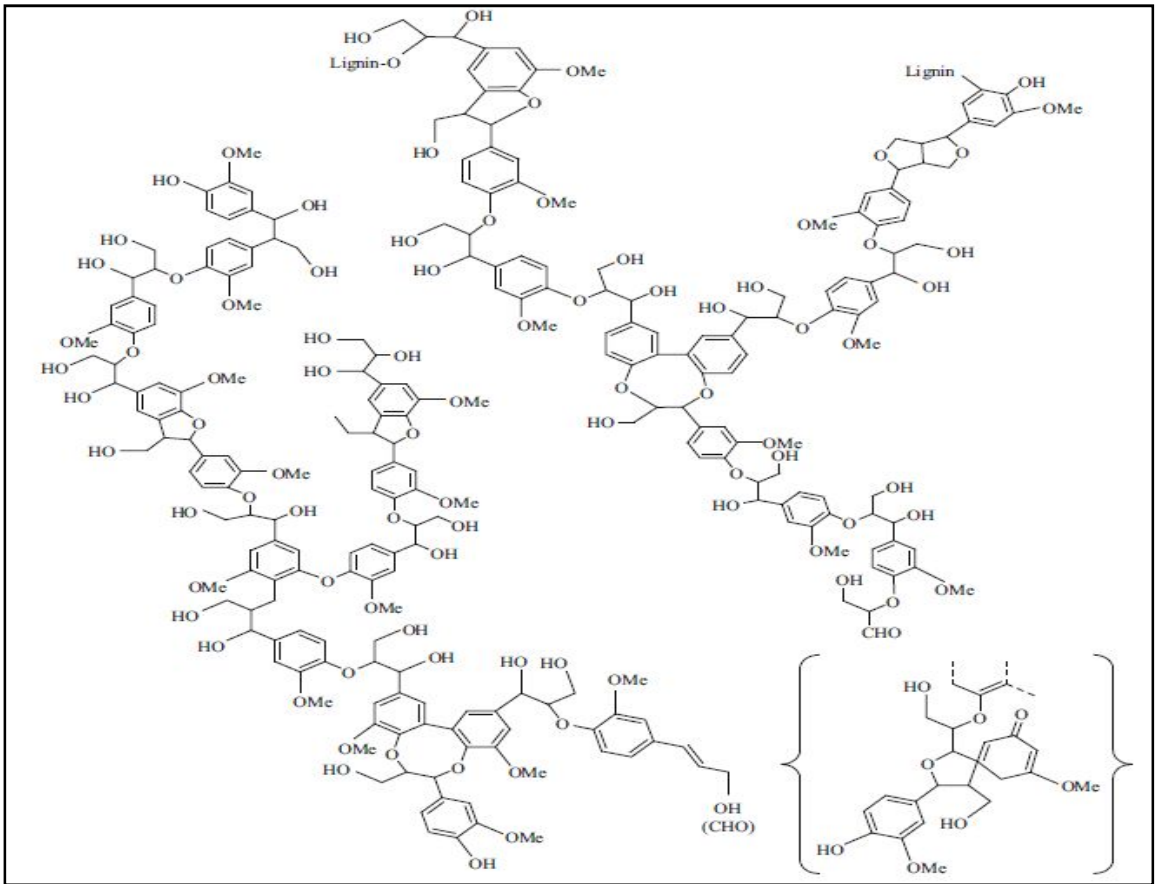


Fig 2.1: Ultra-structure of lignocellulosic biomass (Source: De Wild *et al*., 2011)



(a)



(b)

Fig 2.2: Basic structural units of lignin (a), and structural model of a section of cork lignin (b) (*Source:* Chen, 2014).

Table 2.2: Potntial lignocellulosic biomasses and their composition (% dry weight)

Raw material	Hemicelluloses	Cellulose	Lignin	Others
Agricultural residues	25–50	37–50	5–15	12–16
Hardwood	25–40	45–47	20–25	0.80
Softwood	25–29	40–45	30–60	0.50
Grasses	35–50	25–40	–	–
Waste papers from chemical pulps	12–20	50–70	6–10	–
Newspaper	25–40	40–55	18–30	–
Switch grass	30–35	40–45	12	–

2.5 Bamboo Feed Stock: Availability, Chemical Composition and Fuel Characteristics

Among the available lignocellulosic feedstocks, bamboos, are receiving a renewed interest due to their high growth rate and better reduction of carbon footprint of our environment compared to an equivalent area of woody plants. Taxonomically, bamboo falls to the family Poaceae, the grass family, subfamily Bambusoideae which contains approximately 1250 species. Despite of being a member of grass family, the bamboo still have "woody stem" or culm that can reach from 15-20 meters in height or even 40 meters with *Dendrocalamus giganteus*, the largest known species of bamboo (**Gratani *et al*., 2008; Shimokava *et al*., 2009**). Most of the species of bamboo are considered as the fastest growing woody grass plant on planet with the highest recorded growing tendency of 90cm in a day. The another important feature is its short harvestable time, for bamboo it is about 3-5 years in comparison to 10-20 years for other softwoods (**Scurlock *et al*., 2000; Gratani *et al*., 2008**). Bamboo also has high biomass production ability, self-regeneration power and can be able to tolerate or grow in nutrient deficient soils so that it can be cultivated in degraded or unused lands, which make it one of the best- and cost effective biomass resources on the earth (**Zhang *et al*., 2007; Gratani *et al*., 2008**). Normally all plants in a particular population of bamboo, flower at the same time and after that the plant will dies. Although, this is a unique feature but another physiological feature of bamboo is that flowering is very infrequent, approximately in all species. It is with intervals as long as 60 to 120 years. This phenomenal, called "mass flowering", although restricted the commercialization of many species but as per very long flowering time or a very rare flowering, it can be harvested several times for bioenergy generation (**Scurlock *et al*., 2000; Gratani *et al*., 2008; Shimokava *et al*., 2009**). *Dendrocalamus* sp. and *Bambusa* sp. are bamboo varieties common in India, of which large amounts of biomass is generated annually as by products of bamboo processing industries **(Lobovikov *et al*., 2007; Zhang *et al*., 2007)**. India has the largest bamboo forest area, therefore, the biomass projects are continuously developing fast in the country. India produces eight lakh cubic metres of wood annually and a fourth of this is bamboo, it would result in saving 8,000 hectares of forests which would also generate employment to the tune of 35 million man-days, particularly for rural, tribal and women folk and for in-house work **(Scurlock *et al*., 2000; Lobovikov *et al*., 2007)**. The catalogue of bamboo resource, available worldwide is illustrated in **Table 2.3**.

Table 2.3: Data representing the area of bamboo forest

Continent	Area of bamboo (1000ha)			
	1990	2000	2005	% of global total (in 2005)
Asia	21,230	22,499	23,620	65
Africa	2,758	2,758	2,758	28
Latin America	-	10,399	10,399	7
Global total	23,988	35,656	36,777	

Asia, Africa and South America are the three major continents of both natural and planted bamboo forests that cover more than thirty six million hectares worldwide. Moreover, bamboo sp. is most abundant in the monsoon areas of East Asia, especially in China and India with approximately 5.4 million ha and 11.4 million hectares covered, respectively **(Scurlock *et al.*, 2000; Lobovikov *et al.*, 2007)**. Over the last 10-15 years, the bamboo area in Asia has increased by 10 percent, mainly due to large-scale planting of bamboo in India and China (**Lobovikov *et al.*, 2007**). The data of bamboo resource, available in most important bamboo producing countries is illustrated in **Figure 2.3**.

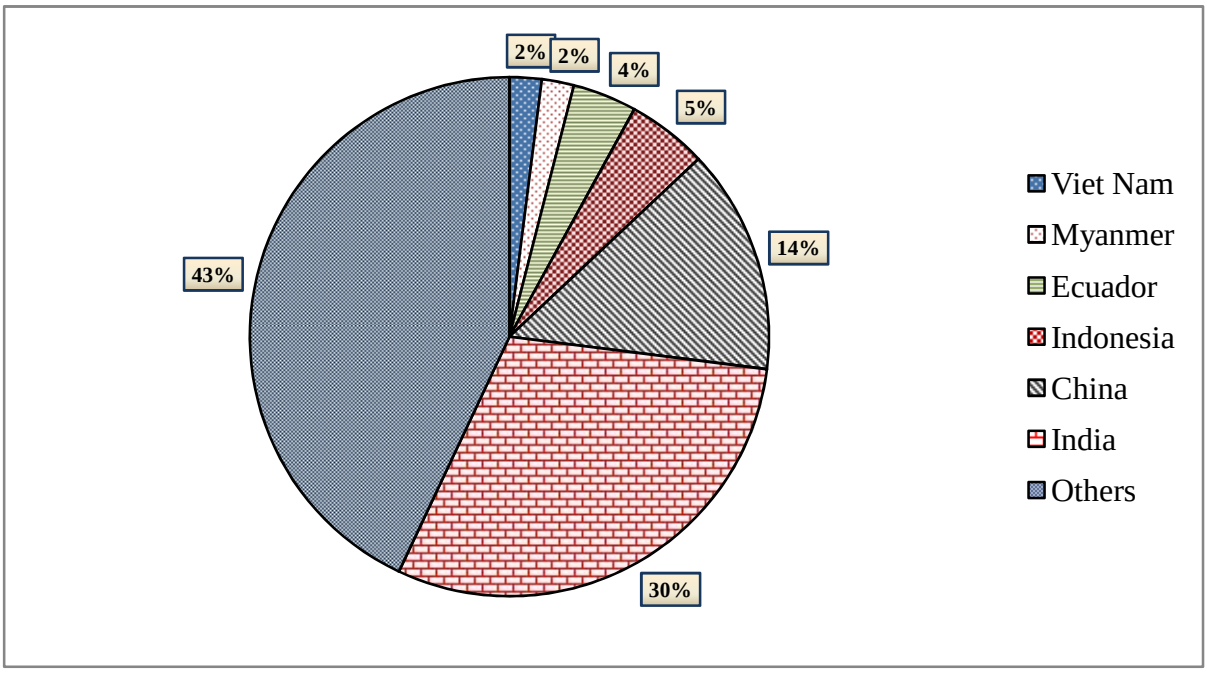


Figure 2.3: Countries with prominent bamboo resource (Source: Lobovikov *et al.*, 2007).

The anatomical, chemical and physico-mechanical properties of bamboo show no basic differences among bamboo genera and species compare to that of other woods. Also, variations in growth conditions and aging of the bamboo grass have apparently no significant effect on chemical composition, its structure and the structure of the tissue (**Liese, 1985; Liese, 1992; Wahab *et al*., 2009**). Like other biomasses, the chemical composition of bamboo cell walls is consisting of three basic polymeric substances that are cellulose, hemicelluloses and lignin. These polymers are composed of large chemical molecules and constitute 90-98% of the bamboo cell walls. The remaining 2-10 percent is called extractive, which is composed of low molecular weight compounds. As per **Tomalang *et al*.** (**1980**), the bamboo culms are made up of 60-70 percent of holocellulose, 20-25 percent pentosans, 20-30 percent hemicellulose and same 20-30 percent of lignin. The minor constituents such as resins, tannins, waxes and inorganic salts are also present. The chemical constituents of bamboo varied from species to species, and due to the location of cell within the tree (**Browing, 1975; Thomas, 1977**). Thus, there are some or few differences in these main constituents between species to species of bamboos, but these differences do not cause any influence on technological properties used to conversation of biomass to fuel or any hinderence in fuel production pathway (**Liese, 1992**).

Bamboo biomass has a number of desirable and important fuel characteristics such as low ash content; low alkali index with high heat value (HHV) than other most of the agriculture residues used for bio-energy production. This relatively higher heating value than other types of biomasses makes the bamboo is a good candidate for direct combustion or co-combustion in thermal power plants. **Scurlock *et al*. (2000)** reported another important aspects that make the bamboo biomass a potential feedstock for liquid fuel production, that is the moisture content, which is relatively low (8-23%) in comparison to other type of plant biomasses (**Scurlock *et al*. 2000; Sritong *et al*., 2012**). The moisture content of bamboo is nearly similar to that of rice husk and rice straw but much less than bagasse and corn stalk. The low moisture content of any biomass is very important becauseit reduces the energy input to dry the biomass resulting in better efficiency of utilization. All the details with comparison of bamboo biomass and other type of biomass as per utilization for fuel production are discussed in **Table 2.4**.

Even though the lignocellulosic biomass is highly available on the planet earth, the commercialization of the production process to produce bioethanol from it is limited due to insufficient research, less technology development and the less intensive research related to minimization of production cost (**Kaya *et al*., 2000; Aswathy *et al*., 2010**).

Table 2.4: The fuel characteristics of some common crop based lignocellulosic biomass as feedstocks for fuel production

Types of biomass	Moisture (%)	Ash(%)	Volatile matter (%)	Fixed carbon (%)	Higher heating value kJ/kg
Rice husk	12.05	12.73	56.98	18.88	14.638
Rice straw	10.12	10.42	60.87	18.80	13.275
Bagasse	50.76	1.75	41.99	5.86	9.664
Palm shell	12.12	3.66	68.31	16.30	18.446
Corncob	40.11	0.95	45.55	13.68	11.198
Corn stalk	41.69	3.80	46.98	8.14	11.634
Bamboo (*Bambusa Deecheyama*)	14.30	3.70	63.10	18.90	15.700
Bamboo (*Dendrocalamus asper*)	5.80	2.70	71.70	19.80	17.585

2.6 Lignocellulosic Bioethanol Production Pathway

Development of second generation biofuels from lignocellulosic plant biomass has many advantages over first and third generation biofuels in view of energy and environmental concerns. Efficient conversion of lignocellulosic materials to ethanol/ other value added products is still today a challenging process. In contrast to fossil fuels, lignocellulosic ethanol is produced through fermentation of sugars from renewable energy resource (**Hahn-Hägerdal *et al*., 2007**). The conversion process relies heavily on three basic steps *viz*. pretreatment of biomass, saccharification of pretreated substarate and fermentation of sugar to ethanol (**Fig 2.4**). Major technological innovations for lignocellulosic bioethanol production centered on effective and low cost production of

lignocellulolytic and saccharifying enzymes, proper processing of feedstocks for lignin removal and efficient process design for saccharification and fermentation processes (**Arantes *et al*., 2011; Menon and Rao, 2012**).

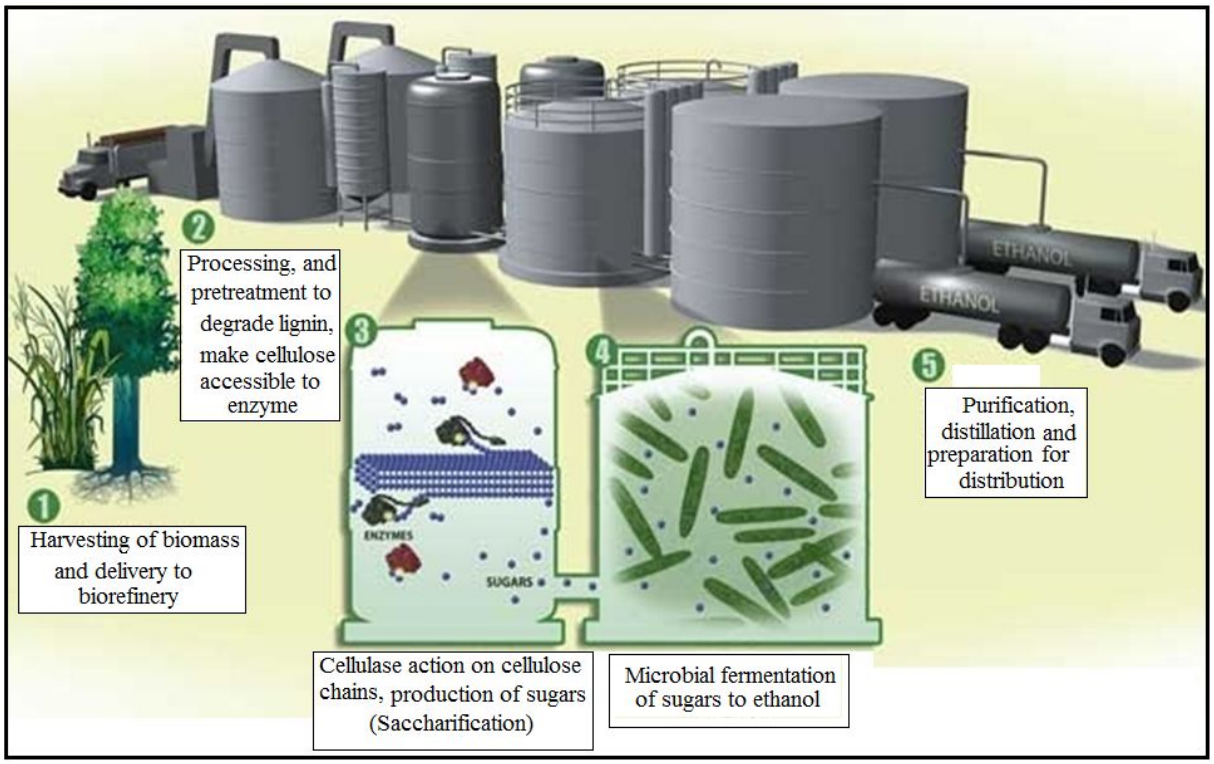


Fig 2.4: Major Steps in Lignocellulosic Bioethanol Production Pathway (*Source*: https://i2.wp.com/www.bioenergyconsult.com)

After delivering any potential lignocellulosic feedstock to the ethanol plant, the first need is to store it carefully to prevent early contamination, degradation, detoriation, and fermentation from its own normal microbial flora or contaminants. Most of the potential cellulosic substrates are heavily lignified and are thus unsuitable for ethanol production unless subjected to some effective and economical pretreatment(s) to remove or modify lignin to yield highest exposed cellulose fibre material. Through suitable pretreatment (physical/ physicochemical/ chemical/ biological), cellulosic part is made available in different proportions depending on the biomass used and the pretreatment method. (**Kang *et al*., 2014**). Pretreatment mainly involves maximum lignin degradation (delignification) and minimum cellulose or sugar loss of the feedstock in order to make exposed cellulosic material more accessible in the next hydrolysis or saccharification step. Generally carbonic acids and alkali based pretreatment methods have observed to perform best for delignification (**Sun and Cheng, 2002**). In the second stage of hydrolysis of

delignified biomass, the exposed cellulose gets converted into glucose (so called saccharification process), which is catalysed using various methods. Dilute acid, concentrated acid, and microbial cellulase enzymatic conversation are preferably used. The best effective method is use of cellulase enzyme cimplex (**Cui *et al.*, 2014; Singh *et al.*, 2014; Maitan-Alfenas *et al.*, 2015; Zhang *et al.*, 2015**). Saccharification process involves catalytic breakdown of cellulose/hemicellulose polymers into monomeric units that can be represented by following reactions:

$$(C_6H_{10}O_5)_n + nH_2O \longrightarrow nC_6H_{12}O_6$$

$$(C_5H_8O_4)_n + nH_2O \longrightarrow nC_5H_{10}O_5$$

The lignocellulose-to-ethanol processes at major final step of fermentation of released sugars (hexoses and pentoses) into ethanol can be done by a variety of microorganisms, such as, wild type or mutant yeast, or ethanologenic bacteria and fungi. Following reactions represents bioconversion of hexoses (C6) and pentoses (C5) into ethanol:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

$$3C_6H_{10}O_5 \longrightarrow 5C_2H_5OH + 5CO_2$$

Thus, cellulosic bioethanol production process involves mainly four steps: delignification, saccharification, fermentation of sugars and finally recovery of the product (ethanol) from fermented liquid. But selection of fermenting organism, production of chemical inhibitors during fermentation process are still need to be investigated (**Kang *et al.*, 2014; Singh *et al.*, 2014**). Use of genetically engineered *Saccharomyces cerevisiae* or *Zymomonas mobilis* is promising for higher alcohol tolerance and production capacity of the organism during bioethanol production process (**Cui *et al.*, 2014; Kang *et al.*, 2014; Singh *et al.*, 2014**).

2.7 Factors Limiting Hydrolysis of Biomass

Crystalinity of cellulose has been reported as one of the factors limiting enzymatic hydrolysis of lignocellulosic biomass (**Puri, 1984; Chang and Holtzapple, 2000)**. Besides the crystallanity of cellulose, other factors are also involved that may limit the proper hydrolysis of lignocellulosic biomass at any step of the production pathway (**Chang and**

Holtzapple, 2000). These include moisture content of biomass, degree of polymerization of mainly lignin and cellulose, available surface area and lignin content (**Puri, 1984; Chang and Holtzapple, 2000; Laureano-Perez *et al*., 2005). Caulfield and Moore, (1974**) reported that decreased particle size and increased available surface area rather than crystalinity of cellulosic part mainly affect the rate and extent of the hydrolysis of lignocellulosic biomass. The particle size of the substrate in relation to the size of the enzyme is also the main limiting factor in the enzymatic hydrolysis **(Grethlein, 1985; Thompson *et al*., 1992)**. Increase in pore size in lignocellulosic biomass can be achieved by removal of hemicellulose and therefore it increases the probability of hydrolysis of cellulosic part by cellulase enzyme. After the pretreatment, drying of pretreated lignocellulosic substrate could be the reason of degradation or shrinkage in pore structure, resulting in a decreased cellulase accebility and thus hydrolysability of exposed cellulose. Thus drying after pretreatment should be avoided (**Grous *et al*., 1986; Palonen *et al*., 2004**). Lignin totally limits the rate and extent of enzymatic hydrolysis of cellulose by acting as a shield or barrier, which prevents the exposure of cellulose to cellulase enzyme complex thereby preventing its hydrolysisn (**Chang and Holtzapple, 2000**).

2.8 Processing of Lignocellulosic Biomass

Before proceeding for any pretreatment step, the woody substrate firstly dried and converted to small pieces. The substrate should further proceesed to fine powder using various physical proceesing technologies, collectively comes under Milling or Comminution. Comminution is the process utilized to reduce particle size of the biomass, typically applied to almost all the biofuel production processes prior to pretreatment or hydrolysis step to improve feedstock handling and treatment (**Zheng *et al*., 2014)**. Thus, first of all drying (sun drying followed by oven drying at 60-70^{0}C) of biomass is carried out to remove total moisture. After drying, particle size reduction of the biomas is often needed to make material handling easier, to increase surface/volume ratio and to make biomass easy for hydrolysis. This can be done by chipping, milling or grinding before following bioethanol production pathway. Comminution of biomass can be accomplished by using specific type of milling or grinding machines, including vibro, ball, hammer, two-roll, knife, colloid, and attrition mills etc. sometimes electronic wheel saw can also be used to make fine powder. For mechanical pretreatment, some factors such as resources and their costs, costs in operation and energy input, scale-up possibilities and depreciation of

equipment are very important (**Kim *et al*., 2013**). **Schell and Harwood, (1994)** reported that particle size must be reduced to or below 1-2 mm to eliminate such limitations for hydrolysis of lignocellulosic biomass; however, sometimes size reduction is a very expensive operation and may be a limitation for hydrolysis. As according to **Zheng *et al*. (2014)**, it consumes about 33% of the total electricity demand for the whole bioethanol production process.

2.9 Pretreatments for Delignification and Structural Modifications of Feedstock

Pre-treatment of lignocellulosic biomass prior to enzymatic saccharification is a very important step for the recovery of cellulosic fibres from lignin based biomass, as compared to the starchy materials (**Barl *et al*., 1991; Wyman, 1996; Mosier *et al*., 2005**). The pre-treatment process requires breaking down the cementic lignin barrier from other components of the biomassso as to recover cellulose, which is then subjected to hydrolysis by cellulase enzyme complex for its bioconversion into convert into fermentable or reducing sugars (**Figure 2.5**).

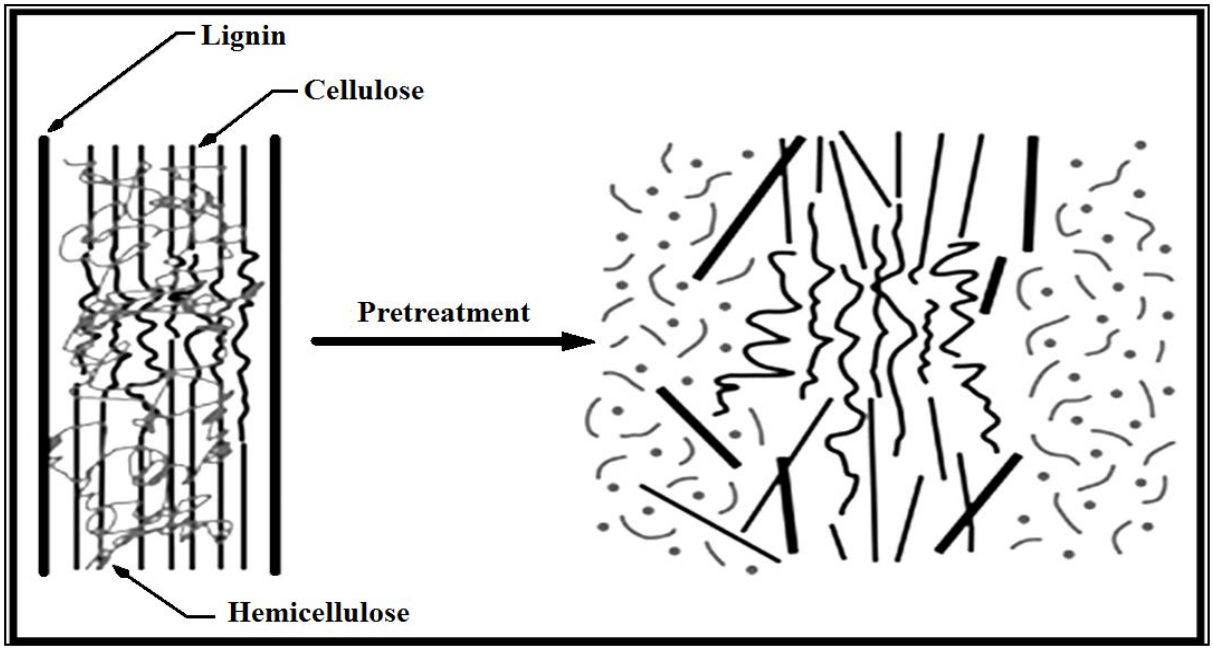


Figure 2.5: Schematic representation of influence of pretreatment on lignocellulosic biomass (*Source:* Mosier *et al*., 2005)

During the past few decades, several groups of pre-treatment approaches as well as combinations of these have been studied to obtain cost-effective yield of fermentable

sugars from most of the agricultural, forestry, and other lignocellulosic materials (**Yang & Wyman, 2008**). An effective and economical pretreatment should meet the following requirements: (a) removal of lignin fractions, and crystalinity of cellulose, to yield maximum reducing sugar contents (b) production of reactive cellulosic fiber for enzymatic attack, (c) avoiding destruction of hemicelluloses and cellulose, (d) minimization of the formation of toxic chemical inhibitors during degradation processthat inhibit hydrolytic enzymes and fermenting microorganisms, (e) minimizing the energy demand, (f) process should be economically and environmentally efficient, (g) producing less residues, (h) consumption of little or no chemical and using a cheap chemical (**Wyman, 1996; Karimi *et al*., 2006).** Several methods have been introduced for pretreatment of lignocellulosic materials prior to enzymatic hydrolysis or digestion. These methods broadly classified into "Physical pretreatments", "Chemical pretreatments", and "Biological pretreatments" **(Barl *et al*., 1991; Wyman, 1996; Berlin *et al*., 2006).** Pretreatment of lignocellulosic materials is very complicated. The optimum method and conditions for biomass pretreatement greatly depend on the type of lignocellulosics. For instance, pretreatment of bark from poplar trees or corn leaf with a dilute-acid process seems to be promising, but this method is not effective for treating the bark from sweetgum (*Liquidambar styraciflua*) or corn stalks (**Torget *et* al., 1991; Donghai *et al*., 2006)**.

2.9.1 Physical pretreatments

Physical pretreatment refers to the pretreatment methods that do not involve use of any chemical or microorganisms/ their enzymes during the processe (**Table 2.5**). Most physical pretreatment technologies use water; in either liquid or gaseous state, if used in pretreatment, it is not considered as a chemical (**Zheng *et al*., 2014)**. Previously developed physical pretreatment techniques are briefly described below:

(a) Steam Explosion

In autohydrolysis or steam explosion, the milled biomass using comminution are heated with high-pressure in presence of saturated steam (160-260^{0}C, 0.69-4.83 MPa), for a short period of time (several seconds to a few minutes) and the pressure is slowly reduced to terminate the reactions. The steam explosion causes the biomass to undergo an explosive decompression. Under these conditions, hemicellulose is hydrolyzed and lignin is transformed to monolignols upto certain extent, thus making pretreated biomass more

accessible to saccharifying enzymes. Organic acids such as acetic acids and other acids formed during hydrolysis from acetyl or other functional groups facilitate the hydrolysis of hemicellulose and lignin. In addition, water, which possesse certain acid properties at high temperature, further catalyzes hemicellulose hydrolysis and removal of cellulose crystalanity (**Weil *et al*., 1997**). Although, most important limiting factor during autohydrolysis is degradation of reducing sugars due to acidic conditions. Steam-explosion is one of the most common pretreatment methods for lignocellulosic biomass (**Cantarella *et al*., 2004; García-Aparicio *et al*., 2006**).

(b) Irradiation

Microwave, ultrasound, gamma-ray, and electron beams are different kinds of irradiation pretreatements. Microwave heating was the most studied irradiation pretreatement method (**Eskicioglu *et al*., 2007**). The electromagnetic field generates energy and delivers it directly to the powdered biomass for rapid heating. The entire material heated with reduced thermal gradients (**Thostenson *et al*., 1999; Eskicioglu *et al*., 2007**). Microwave technology is an effective alternative to other heating methods that used conventionally, reduces heating time, spread more rapidly to larger volumes of biomass, thus can lead to energy savings. (**Jackowiak *et al*., 2011, a; Jackowiak *et al*., 2011, b**). Microwave radiation at 150^0C was used by **Jackowiak *et al*. (2011, b)** to pretreate wheat straw and they found the enhanced methane yield from by 28%, compared with untreated wheat straw. The limitatrions of this method of pretreatement are production of heat-induced inhibitors such as furfurals and some phenolics (**Eskicioglu *et al*., 2007**). Thus by controlling the pretreatment conditions for avoiding the formation of these inhibitors, method should be improved. For which, microwave has been used with combinations with alkali to enhance the effectiveness at relatively low temperatures without compromising pretreatment effects (**Qi *et al*., 2007).**

Gamma-ray and electron beam radiation have also been used to improve biofuel production from various materials, but then application of them for lignocellulosic biomass, clearly has not been explored yet. These irradiation methods are highly expensive and have many difficulties in use for industrial application purposes (**Zheng *et al*., 2014)**.

(c) Sonication or Ultrasonic Pretreatment

Sonication is the act of applying sound energy to agitate particles, mainly for degradation of any part or component of sample, for various purposes (**Bochek, 2003**). Although, ultrasonication for pretreatment of lignocellulosic biomass has only investigated at laboratory scale, it is a well known technique for sludge pretreatment before releasing it to water stream from waste water treatment plants, where lignin and related compounds are present as xenobiotics in sluge (**Bochek, 2003; Imai *et al*., 2004**).

(d) Liquid Hot Water Pretreatment

Hydrothermolysis or liquid hot water (LHW) pretreatment is one of the best studied hydrothermal pretreatment processes, that does not require addition of chemicals (**Van Walsum *et al*., 1996**). It is mostly used in pulp industry and for bioethanol production from woody biomass. In LHW pretreatment, 100-103^{0}C temperature is avhieved for delignification process. Pressure is utilized to maintain liquid state of water for high temperature cooking/ boiling of lignocellulosic biomass throughout the processs (**Van Walsum *et al*., 1996; Qiao *et al*., 2011**). During pretreatment, water can penetrate into the biomass deeply and the cell structure, cellulose crystalinity, hemicellulose integrity and lignin content gradually removed. LHW pretreatment is highly effective for enlarging surface area for optimum cellulose digistibility using microbes and/or enzymes. Lower concentrations of inhibitors, were obtained using liquid hot water pretreatment compared with low acidic pretreatment methods such as dilute acid pretreatment, where inhibitors are produced in larger amounts (**Badshah *et al*., 2012; Li *et al*., 2012; Monlau *et al*., 2012**).

Table 2.5 Physical pretreatment conditions for potential lignocellulosic feedstocks used for bioethanol production

Physical pretreatment	Feedstock	Pretreatment conditions
Comminution	• Agricultural residuals: wheat straw, rice straw, oat, clover, bagasse, coconut fiber, hemp, banana peelings, cauliflower leaves, and digested biofibers • Forest residuals: mirabilis leaves • Grass: dump grass and grass hay • Municipal solid waste (MSW): organic fraction of MSW (OFMSW)	Particle size: 0.003–30 mm
Steam-explosion	• Agricultural residuals: wheat straw, corn stalks, corn straw, citrus waste, potato pulp, rape straw, and digested biofibers • Hardwood: Japanese cedar, willow, and birch • Grass: bulrush, *Miscanthus*, and seaweed • MSW: OFMSW and paper tube residuals • Softwood: bamboo	160–260 °C, 0.7–4.8 MPa, and seconds to a few minutes
Liquid hot water (LHW)	• Agricultural residuals: Wheat straw, rice straw, oil palm empty fruit bunches (OPEFB), sunflower stalks, and sugarcane bagasse • Grass: *Miscanthus* and hybrid grass	100–230 °C (0.1–2.8 MPa) for a few minutes to hours
Irradiation	• Agricultural residuals: wheat straw, barley straw, spring wheat, winter wheat, oat straw, and rice stalks • Grass: switchgrass and hybrid grass	115–300 °C for a few minutes to hours

2.9.2 Chemical Pretreatments

Use of various chemicals such as as acids, bases, and ionic liquids has been proposed to alter the physical and chemical characteristics and structure of lignocellulosic biomass. Among the pretreatments, chemical pretreatment methods have received more attention and research interest for delignification of biomass.

(i) Alkaline Pretreatment

In alkaline pretreatement, dilute bases are used generally for delignification of lignocellulosic biomass. These bases include sodium hydroxide, calcium hydroxide, potassium hydroxide and wet or absolute ammonia (**Zheng *et al*., 2014**). The use of base effectively removes lignin, hemicellulose, and cellulose crysytalinity, rendering cellulosic part of biomass more accessable to microbes and their oxidoreductive enzymes. Alkaline treatment using NaOH has been extensively used in the bioethanol and pulp and paper industries. NaOH is a strong base with good saponification ability, thus, the function of alkaline pretreatment is believed to be the saponification of lignin-carbohydrate linkages, and then cleavage of the bonds. The cleavage ultimately leads to degradation/ separation of biomass components (**Tarkow and Feist, 1969; Zheng *et al*., 2014**). Alkaline pretreatment leads to an increase of porosity of substrate by removing cross links present in biomass. It also lead to an increase in internal surface area, structural swelling, a lowering of degree of polymerization, crystalinity of other components with cellulose, disruption of lignin structure, and a breakdown of links between lignin and other polymers. However, disposal of sodium ions containing effluent from lignocellulose processing industries could lead to negative environmental impacts such as increase in soil salinity and ground water pollution. **Chen, (2012)** has developed an integrated NaOH pretreatment process that recycles the NaOH used during the bioprocessing for biogas production. This process could help to reduce the sodium ion disposal to environment, but yet not been fully applied to bioethanol industry. Calcium oxide (CaO), or calcium hydroxide $Ca(OH)_2$ are also used for lime pretreatment (**Monlau *et al*., 2012**). The KOH pretreatment also gives good results for hydrolysis of lignocellulose. Compounds of ammonia, such as ammonium hydroxide and ammonia in watery and absolute form, was also reported for adequate pretreatment (**Himmelsbach *et al*., 2010; Song *et al*., 2012**). These pretreatement processes have mostly been applied for biogas production. Thus, based on many studies,

NaOH pretreatment seems to be the most costeffective and widely used alkali for structural modification of lignocellulosics for fuel production (**Monlau *et al*., 2012**).

(ii) Acid Pretreatment

Concentrated acids can be used at low temperature (30-70% acid at 30-40^{0}C) while dilute acids coan be used at high temperatures (0.1%-2% at 120-230^{0}C). Most commonly used inorganic and organic acids for pretreatment of lignocellulosic biomass are sulfuric acid, hydrochloric acid, nitric acid, phosphoric acid, and acetic acid, with HCl and H_2SO_4 most commonly used acids. Cocentrated acids have great effect on biomass hydrolysis but, extreme toxicity, corrosiveness, tough handling, and problem in reactor construction for hydrolysis are some of the constrains that limit their use (**Badshah *et al*., 2012**). In addition, the acid used during the pocessmust be recovered after treatment because of environmental and economic issues. Therefore, dilute acid pretreatemnet is favored over use of concentrated acid for lignocellulosic biomass pretreatment. The main consequences of pretreatment of lignocellulosic material with dilute acid are increasing the accessibility of cellulose to enzymatic degradation and delignification of biomass (**Monlau *et al*., 2012; Us and Perendeci**, **2012**).

(iii) Steam Explosion with Chemical Catalyst

Acid or alkali catalyzed steam-explosion is a very effective steam pretreatment that can be carried out with the addition of varying amount of one of the chemical catalysts such as hydrochloric acid, sulphur-di-oxide and other sulphur compounds, sodium hydroxide, etc. (**Morjanoff and Gray, 1987; Zheng *et al*., 2014**) The presence of a catalyst resulted in improved biodegradability of recalcitrant parts of lignocellulosic biomass, also the use of low amount of catalysts the reduction in production of inhibitory compounds can occur. As well as the results are again moved to complete solubilization/recovery of hemicellulosic part of biomass (**Morjanoff and Gray, 1987; Zheng *et al*., 2014**). Although several studies have reported the improvement of enzymatic hydrolysis and cellulosic ethanol production using acid or alkali catalyzed steam-explosion but the pretreatment is very effective and efficient when sulphuric acid or SO_2 is used. However, use of high sulphuric acid concentration is still not desirable as it will result in high sulphate concentration which can compromise with value added product production. (**Morjanoff and Gray, 1987; Nkemka, and Murto, 2013**).

(iv) Ozonolysis Pretreatment

Ozone is a powerful oxidizing agent, is also used to pretreat the ignocellulosic biomass by the method named as ozonolysis. Primarily, ozonolysis pretreatment results in ozone mediated degradation of lignocellulosic biomass, mainly via lignin degradation, with some alteration to hemicellulose, but almost negligible effect on crystalline or amorphous cellulosic part of lignocellulose (**Appels *et al*., 2012**). The limiting parameters for ozonolysis pretreatment of biomass are solid to liquid ratio in the reactor, biomass particle size, and concentration of ozone concentration in the gaseous form. Among these parameters, the water content or soilid liquid ratio is most important factor affecting the degradation and solubilization of feedstocks (**Taherzadeh and Karimi, 2008**). Formation of hydroxyl redicals from ozone in presence of water, resulting in a combined oxidation of lignin by both hydroxyl redicles and ozone itself when ozone is applied during pretreatment (**Appels *et al*., 2012)**.

(v) Oxidative Pretreatment with Peroxides

Oxidative pretreatment of lignocellulosic biomass with peroxides is very common method for enhancing the biological conversion to bioethanol. Mostly peroxides are transformed into hydroxyl radicals ($OH^{\cdot}$), which are more powerful for degradation as compared to ozone redicals of peroxide itself. Mostly hydrogen peroxide, dimethyldioxirane, and peroxymonosulphate are used as peroxide redical producers (**Zheng *et al*., 2014**). The commonly used peroxide is hydrogen peroxide. During hydrogrn peroxide pretreatment, inhibitors might be generated due to the oxidation of lignin to monolignolic aromatic compounds (**Hendriks and Zeeman, 2009**). Therefore, concentration of hydrogen peroxide should be critically optimised. Several workers have also reported the high concentration of hydrogen peroxide at or about 4% was found to inhibits thepretreatment process due to the toxicity of excess of hydroxyl ions (**Hendriks and Zeeman, 2009; Song *et al*., 2012**). But, sometimes due to the low toxicity index and easily degradable nature of hydrogen peroxide, it can be considered as a strong but good oxidant and has been extensively used for biomass pretreatment for bio-ethanol industry as well as for biogas production. However, while treating some other complex biomaases, H_2O_2 alone may not be able to degrade complexity as per industrial requirements. Therefore it is used in combination to other pretreatement methods is mostly used. The use

of acidic or alkaline hydrogen peroxide, and microwave assisted hydrogen peroxide pretreatment are such type of methods that can be used for hydrolysis of lignocellulosic plant biomass, to achieve better results (**Teghammar *et al*., 2010)**.

2.9.3 Biological Pretreatments: Enzymology in Bio-convesion of Lignocellulosic Biomass

Most of the pretreatment technologies explained during above sections generally have expensive instruments/equipments of high energy requirements, particularly physical and thermochemical processes of pretreatment. Moreover, use of certain chemicals causes environmental and health hazards also. Therefore, biological pretreatment is now been in focus that carried out by using various types of wood decaying bacteria and fungi, espicelly rot fungi, can be more safe and ecofriendly method for lignocellulose structure modification (**Okano *et al*., 2005; Kumar *et al*. 2009**). In biological pretreatment processes, the rot fungi such as brown-, white-, and soft-rot fungi or their lignocellulose degrading enzyme systems can be utilised to degrade lignin and hemicellulose. (**Galbe and Zacchi, 2007**). Brown rot fungi generally attack on the cellulosic part therefore, not preferable for biofuels industries. While, white rot and soft rot fungi attack both cellulose and lignin, thus are very important for lignocellulosic bioethanol production process. *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Phanerochate sordid*, *Pycnoporus cinnabarinus*, *Ceriporiopsis* sp., *Ceriporia lacerata*, *Stereum hirsutum*, *Polyporus brumalis,* and *Cyathus stercoreus* are some important white rot fungi that are utilised for lignocellulosic biomass degration for bioethanol production (**Lee *et al*., 2007; Alvira *et al*., 2010)**. Some of the biological pretreatment processes that used for pretreatment of lignocellulosic biomass are summerized in **Table 2.6.**

In fungal pretreatment, mainly fungi directly inoculated to lignocellulosic biomass that selectively degrade lignin and hemicellulose, without utilizing or utilizing very little cellulose. Lignin degradation and hemicellulose removal result in increased surface area thus increased digestibility of cellulose via cellulolytic saccharifying enzymes (**Sun and Cheng, 2002; Zheng *et al*., 2014**). Microbial consortium pretreatment is another type of pretreatment, that can be conducted using a mixture of two or more microbes (generally fungi) screened from natural environments, where they selectively degrade lignocellulose. Sometimes, the hydrolysis of cellulose and hemicellulose is a rate-limiting step for further progress of process (**Romano *et al*., 2009)**. After proper delignification process, enzymes

with hydrolytic activity such as cellulases and hemicellulases were applied to increase fermentable sugar production from lignocellulosic biomass. Although ligninases are highly effective in biodegradation of lignin, but practically the application of purified laccase and peroxidase enzymes in lignin degradation for bioethanol production is not well characterized. It may be because of less effectiveness in compared to fungal pretrteatment and high production and purification cost of enzymes. The low energy requirements for pretreatment and the use of mild environmental conditions during pretreatment are most important advantages of biological pretreatment methods. However, the rate of hydrolysis in any type of biological pretreatment processes is very slow (**Kumar *et* al. 2009; Romano *et al*., 2009**).

(i) Lignin Degradading Enzyme: Biological Delignification

Degradation of lignin by white-rot fungi occurs through the action of the groups of lignin-degrading enzymes so called ligninases and are include various peroxidases and most importantly laccase (**Lee *et al*., 2007**). Most of these enzymes are secondary metabolites that are regulated by mainly carbon and nitrogen sources, and their concentrations present in production medium (**Hatakka *et al*., 1983; Kumar *et* al. 2009**). Peroxidases include lignin peroxidase (degrades non-phenolic lignin units) and manganese (dependent) peroxidase (degrades both phenolic and non-phenolic lignin units) forms important lignolytic enzyme system (**Binod *et al*., 2011**). These enzyms produced by white-rot fungi during secondary growth phase of their growth, in response to carbon or nitrogen limitation. Manganese peroxidases (MnP) are the most common ligninolytic peroxidases that produced by almost all white-rot basidiomycetus fungi and by other litter-decomposing fungi (**Nie *et al*., 1999**). Peroxidases are heme-containing glycoproteins that strictly require hydrogen peroxide (H_2O_2) for their mode of action (**Fig 2.6**). *Phanerochaete chrysosporium* is the standard white rot fungi that produce peroxidases which oxidizes Mn^{2+} to Mn^{3+} (**Fig 2.6**). This conversion oxidizes phenol rings to phenoxy radicals resulting in decomposition of phenolic and non-phenolic compounds of lignin (**Sindhu *et al*., 2016**). Laccases are other most important copper containing oxidative enzymes that are involved in lignin degradation. Complete biodegradation of lignocellulosic biomass accomplished by laccase along with lignin and manganese peroxidase leading to complete degradation of lignin. Oxidation of phenolic units in lignin, phenolic compounds, and aromatic amines catalysd by laccase. According to **Saloheimo *et***

***al.* (2002),** redox mediaters such as phenolic compounds like 3-hydroxyanthranilic acid and 2, 2 azino-bis (3-ethylthiazoline-6-sulfonate) play vital role in laccase catalysis. Without the action of redox mediators, laccases have a very limited lignin degrading effect. The laccse enzymes are N-glycosylated blue oxidases, mostly extracellular with the molecular mass ranging from 60–390 kDa, contain four copper atoms in their active site (as Cu^{2+} in the resting enzyme) (**Call and Mücke, 1997**). Activity of laccase is characteterised by the oxidation of a variety of aromatic compounds (hydrogen donors/phenolics) with concomitant reduction of oxygen to water (**Fig. 2.7**). Laccases not only oxidize the phenolic and methoxyphenolic acids, but also having decarboxylation property that is used to their methoxy groups (demethylation). Thus, laccases have been intensively studied by many researchers with a focus on their versatile industrial and environment protection applicability (**Yaropolov et al., 1994; Bajpai, 1999; Gianfreda *et al.*, 1999**).

The induction of ligninolytic system (especially lignin peroxidase and laccase) of most of fungi happens under nutrient deprivation (stress response), mainly when nitrogen, carbon and sulphur are limited in medium (**Gold and Alic, 1993**). Furthermore, presence of Mn^{+2} in medium is required for induction of manganese dependent peroxidasegenes in the standard white-rot fungi *P. chrysosporium* (**Gold and Alic, 1993; Gettemy *et al.*, 1998**). Besides these two factors, heat shock in nitrogen limiting conditios, presence of hydrogen peroxide, and chemical stress of molecular oxygen were also orher important factors for induction of lignolytic MnP genes in fungi (**Mayfield *et al.*, 1994; Li *et al.*, 1995)**. Laccase enzymes are multicopper oxidase proteins, thus can be induced by copper, but other metal ions such as manganese and cadmium, and other xenobiotic/ cyclic compounds that often mimic the chemical structure at even a very less extent can induce the expression of laccase genes as well in wood rot fungi (**Scheel *et al.*, 2000; Soden, D., M., and Dobson, 2001; Giardina *et al.*, 2010**). Laccases are reported to be the first enzymes that degrade lignin, and it is said that degradation products from lignin polymer again could act as potential inducers to amplify laccase genes expression, and surprisingly induce other lignocellulolytic genes (**Scheel *et al.*, 2000**).

Table 2.6: Biological Pretreatment Methods Used to Modify Lignocellulosic Biomass

Biological pretreatment	Microbes and enzymes	Feedstocks	Pretreatment conditions
Fungal pretreatment	White-, brown-, and soft-rot fungi (e.g. *Ceriporiopsis subvermispora*, *Auricularia auricula-judae*, *Trichoderma reesei*), and basidiomycete fungi (e.g. *Ischnoderma resinosum* and *Fomitella fraxinea*)	• Agricultural residuals: sweet chestnut leaves/hay and sisal leaf decortications residue (SLDR) • Hardwood: Japanese cedar wood chip	28–37 °C for 12 days to 8 weeks; Autoclaving or no autoclaving of feedstock before inoculation; Aerobic condition
Microbial consortium	Complex microbial agents containing yeast and cellulolytic bacteria, heat-treated sludge, *Clostridium thermocellum*, and mixture of fungi and composting microbes	• Agricultural residuals: corn straw, corn stalks, cotton stalks, cassava residues, and manure biofibers	20–55 °C for 12 h–20 days; Autoclaving or no autoclaving of feedstock before inoculation; Aerobic condition
Enzymatic pretreatment	Laccase; pectinase; mixture of cellulase and hemicellulases; mixture of cellulase, hemicellulase and β-glucosidase; and crude *Trichoderma* enzyme complex	• Agricultural residuals: Sugar beet pulp, spent hops, and manure biofibers • MSW: pulp and paper sludge • Grass: jose tall wheatgrass	37 °C for 4–24 h; No sterilization before loading of enzymes; Aerobic (if pretreatment is separated from AD) or anaerobic (if enzymes were loaded into AD reactors directly) condition

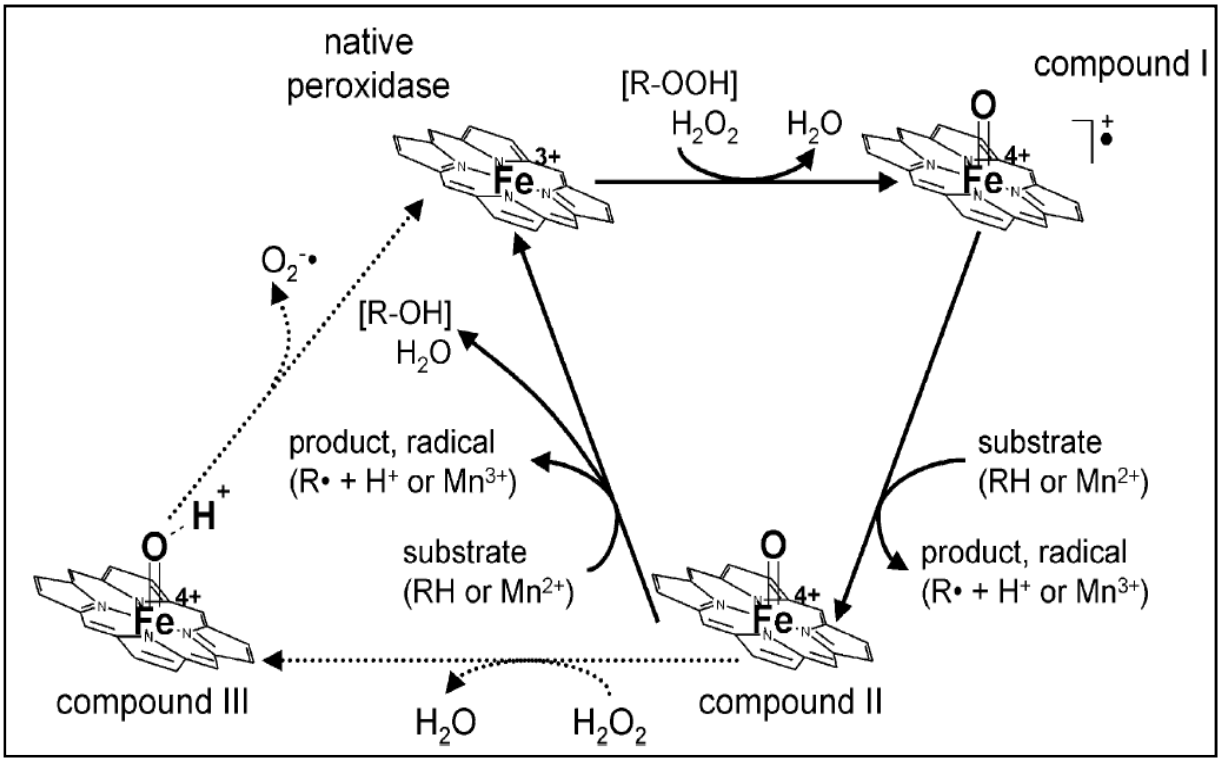


Fig 2.6: General scheme of the catalytic cycle of peroxidases during oxidation of any compound (*Source*: Wesenberg *et al*., 2003)

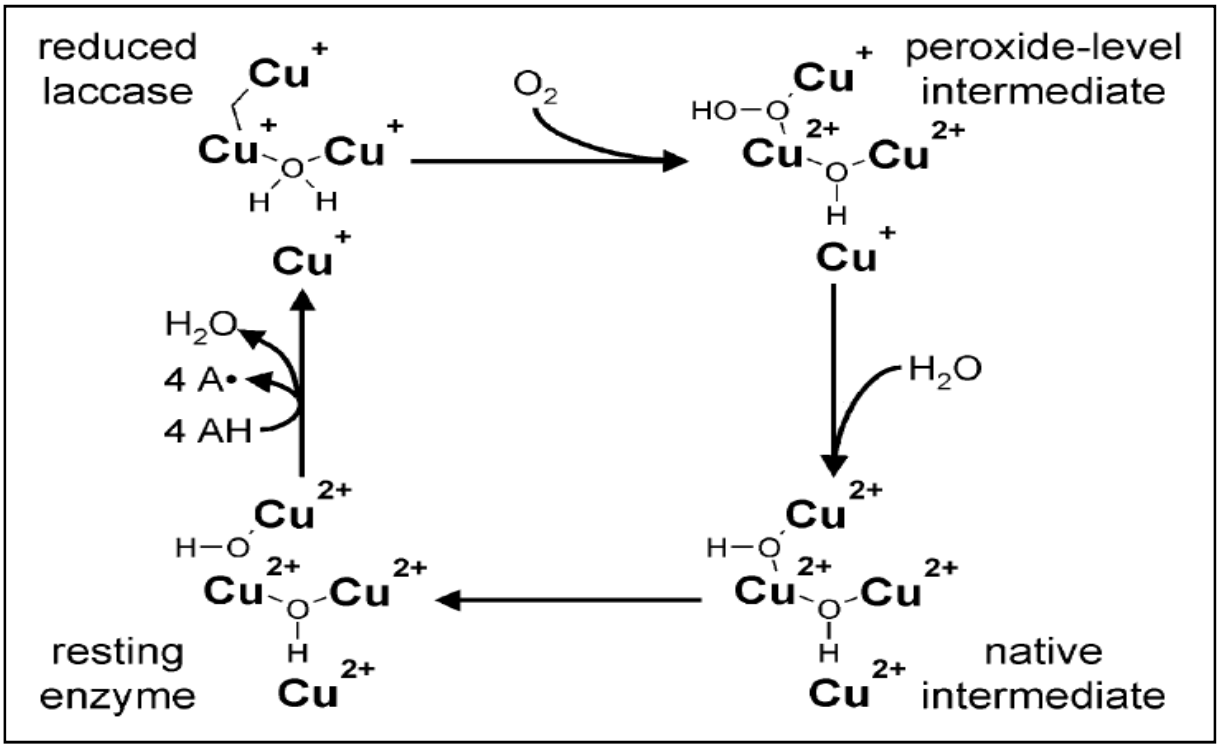


Fig 2.7: The catalytic cycle of laccases (*Source*: Wesenberg *et al*., 2003)

(ii) Cellulose Degradation: Sacchrification of Pretreated Biomass

Cellulases are the mixture of three different enzymes, thus called cellulase complex enzyme or cellulase enzyme complex, able to catalyze the hydrolysis of β-1, 4 linkages in amorphous cellulose, ultimately to reducing sugar, glucose (**Saini and Tewari, 2012).** Cellulase enzyme system include mixture of three different kinds of enzymes, (i) endo-β-D-glucanase (EG) (EC 3.2.1.4) or endoglucanase, that randomly hydrolyze internal β-1,4-glucosidic bonds in linear chains of glucose in cellulose polyme; (ii) cellobiohydrolase (CBH) (EC.3.2.1.91) or exoglucanase, which trevels progressively along with the linear glucose chains in cellulose molecule and cleaves off it from the ends leave cellobiose units; and (iii) β-glucosidase (BGL) (EC.3.2.1.21), which converts cellobiose units and soluble cellodextrins into glucose molecules (**Das *et al.*, 2013; Sindhu *et al.*, 2016**). The conversion of cellulose into fermentable glucose molecules (saccharification process) requires step by step action all the three enzymes. Mechanism of Action of Cellulase Enzyme Complex on cellulose complex is represented in **Fig 2.8**. Endglucanases perform random hydrolysis of β-1, 4 glycosidic linkages in the cellulose chain, thus the short chain of glucose molecule formed. With the action of second and very important enzyme (cellobiohydrolase), the randomly cleaved glucose chains converted to cellobiose units by making cleavage from the end of the chain and β-glucosidase releases glucose units by acting on cellobiose (**Himmel *et al.*, 1996; Kumar and Wyman, 2009**). Several fungal species especially *Trichoderma viride*, *Aspergillus niger*, *Penicillium funiculosum*, *Rhizopus oligosporus* and few mushrooms (**Sukumaran *et al.*, 2009; Gautam *et al.*, 2010**); and bacteria like *Cellulomonas fimi*, *Clostridium thermocellum*, and Bacillus subtilis stand out among the cellulose-producing microorganisms (**Bisaria, V.S. and Martin, 1991; Akhtar *et al.*, 2001**). The most studied filamentous fungi for extracellular cellulase production with high enzyme activities are *Trichoderma* sp **(Bayer *et al.*, 1998; Rawat and Tewari, 2012)**. There is a synergistic action mechanism between the three different types of enzyme of cellulase complex. It is proposed that EGs attack the amorphous regions of cellulosic fibers, thereby creating different sites for action of CBHs which would then proceed into the crystalline regions of the fiber. BGLs perform the last step of saccharification/cellulose hydrolysis and also prevent the build-up of cellobiose that inhibits the activity of CBHs (**Highley, 1980**). The interpretation of these observations is still debated.

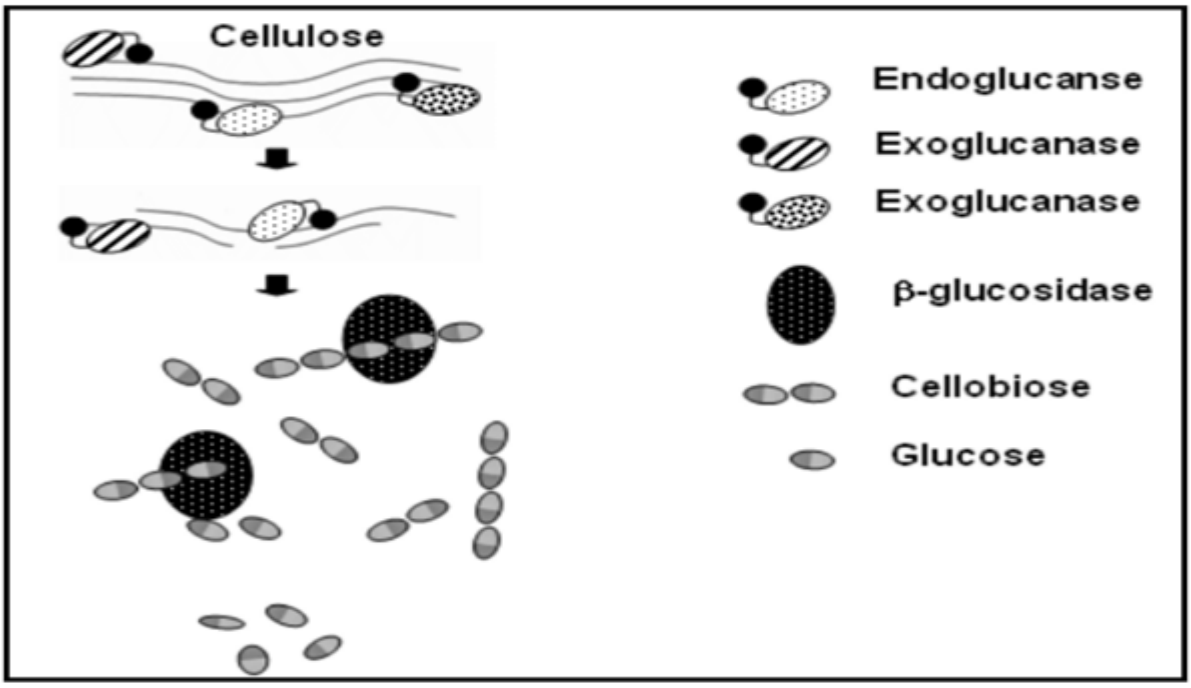


Figure 2.8: Basic mechanism of action of cellulase enzyme complex on cellulose

(iii) Fungal Hemicellulases

Hemicellulose is a complex polysaccharide that composed of different residues branched in three kinds of structures, namely xylan, xyloglucan and mannan. The most important constituent of hemicellulose is the polymer of pentose sugar called xylan which is most abundant component of hemicellulose (70% of total hemicellulose). Same as cellulose degradation, hemicellulose degradation requires a combined action of endo-enzymes (that cleve internally in main chain), exo-enzymes (that release monomeric sugars), and accessory enzymes (that cleave at the side chains so that lead to the release of various mono- and disaccharides) (**De Souza, 2013**). Thus, xylan (β-1, 4-linked D-xylose units) degradation achieved through the action of β-1, 4-endoxylanase, which cleaves complex xylan backbone into small oligosaccharides, and and then with the help of β-xylosidase, produced oligosaccharides further hydrolyzed into xylose. β- xylosidases are very specific for small unsubstituted xylose olygosaccharides and, thus are very important for the complete degradation of xylan oligomers. Ofcourse, other additional/accessary enzymes such as β- mannanases, and α-L-arabinanases are also required depending on the composition of hemicellulose in lignocellulosic biomass (**Hovel *et* al., 2003; Saha and Cotta, 2006**). Further, as reported by **Van den Brink and de Vries (2011),** the complete

degradation of hemicellulose is only achieved after release of all substitutions present on main backbone of the polymer. Moreover, high degree of substitution in the hemicellulose polymers requires the action of at least nine different other enzyme activities distributed along 12 GH and 4 CE families (**Fig 2.9**). A wide variety of hemicellulases are produced and secreated extracellularly in high concentrations by aerobic fungal species such as *Trichoderma* and *Aspergillus*, and these various hemicellulase enzymes work in a synergistic manner for complete degradation of hemicellulose polymer.

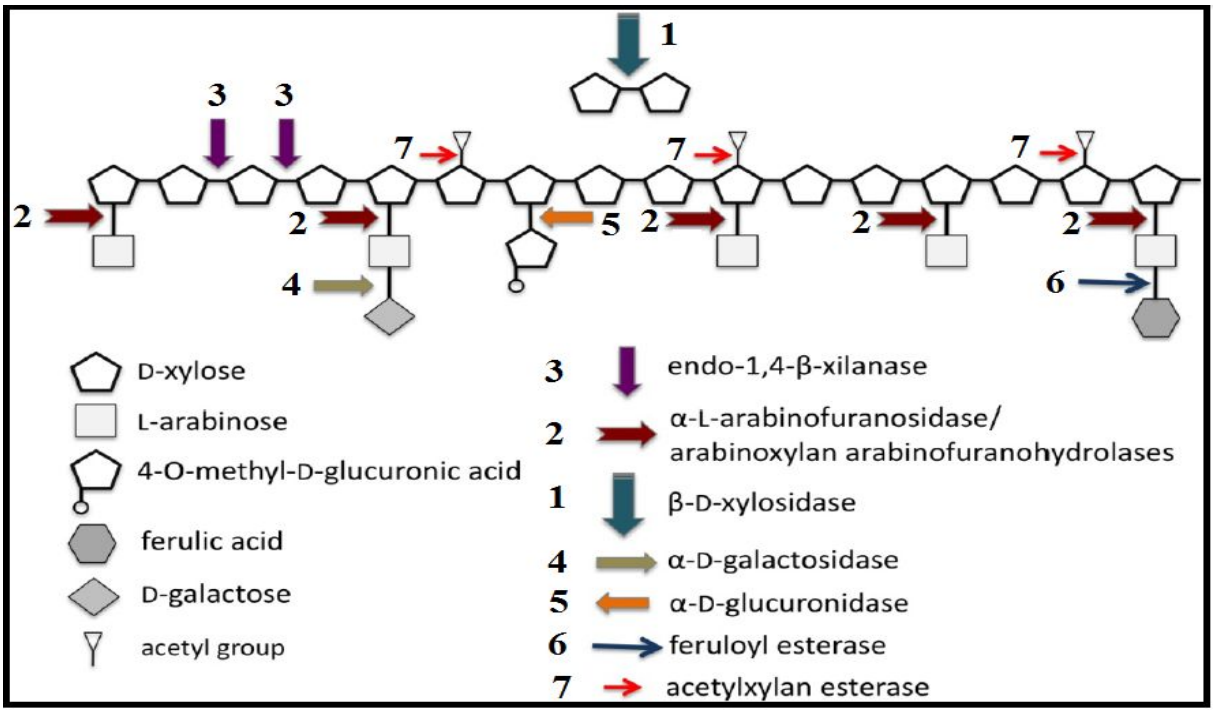


Fig 2.9: Schematic view of degradation of arabinoxylan; arrows represent each enzyme active at specific site (*Source*: Van den Brink and de Vries, (2011).

Thus, fungal lignin degrading enzymes, cellulase enzyme complex, and, if necessary, the hemicellulolytic enzymes play a vital role in ecofriendly bioethanol production from lignocellulosic plant biomass. Use of whole cell as biocatalyst or its active extracellular enzymes either for delignification or saccharification process could be a safe and cost effective method than that of physical/chemical methods. Although there are both advantages and constrains in each of the methods explained during above sections (**Table 2.7**), there is an urgent need for development of proper microbial technology, either using native or genetically modified microbial strains for industriual and ecofrendly pathway for bioethanol production.

Table 2.7 Summary of Various Processes Used for Pretreatment of Lignocellulosic Biomass

pretreatment process	advantages	limitations and disadvantages
mechanical comminution	reduces cellulose crystallinity	power consumption usually higher than inherent biomass energy
steam explosion	causes hemicellulose degradation and lignin transformation; cost-effective	destruction of a portion of the xylan fraction; incomplete disruption of the lignin−carbohydrate matrix; generation of compounds inhibitory to microorganisms
AFEX	increases accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes	not efficient for biomass with high lignin content
CO_2 explosion	increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds	does not modify lignin or hemicelluloses
ozonolysis	reduces lignin content; does not produce toxic residues	large amount of ozone required; expensive
acid hydrolysis	hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	high cost; equipment corrosion; formation of toxic substances
alkaline hydrolysis	removes hemicelluloses and lignin; increases accessible surface area	long residence times required; irrecoverable salts formed and incorporated into biomass
organosolv	hydrolyzes lignin and hemicelluloses	solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
pyrolysis	produces gas and liquid products	high temperature; ash production
pulsed electrical field	ambient conditions; disrupts plant cells; simple equipment	process needs more research
biological	degrades lignin and hemicelluloses; low energy requirements	rate of hydrolysis is very low

2.10 Fermentation of Saccharified Fluid into Bioethanol

The conversion of lignocellulose to bio-ethanol requires challenging biological pretreatment/ conversion processes that includes: (i) delignification of biomass in order to dissolve lignin and release free cellulose and hemicellulose from the lignocellulosic material; (ii) saccharification of the carbohydrates polymers (mostly cellulose) generate free sugars (mostly hexoses and pentoses); and (iii) microbial fermentation of saccharified syrup of mixed hexose and pentose sugars to bio-ethanol (**Lee, 1997**). As dscribed previously, glucose (a fermentable reducing sugar) presents ≥ 60% of the total sugars available from pretreated and saccharified lignocellulosic biomass. For production of bio alcohol form that saccahrified syrup, use of potential strains of yeast (*Saccharomices cerevisiae*) is the most important, classical and widely used method to ferment glucose (hexose), generating ethanol (**Galazka *et al*., 2010**). However, pentose sugars such as xylose and arabinose, if present in saccharified fluid, represents one of the major challenges for the production of bioethanol from lignocellulosic biomass because for fermentation of such sugars, most of ethanolagenic *S. cerevisiae* strains are not able to efficiently ferment C5 (pentose) sugars. Several examples of naturally occurring microorganisms that are able to ferment pentose sugars include *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* (**Hahn-Hagerdal *et al*., 2007**); of wihich the yeast *P. stipitis* has the highest ability to ferment xylose under limited aeration conditions. It was reported that the ethanol production from lignocellulosic xylose fermentation using potential strain of *P. stipitis* is significantly lower than lignocellulosic glucose fermentation using ethanolagenic *S. cerevisiae* strains (**Chu and Lee, 2007**). Therefore, it could mendatory to genetically improve the potential yeast strains to obtain enhanced fermentation ability with them to ferment pentoses, and especially hexoses. In addition to this, enhanced ethanol production and most importantly a high ethanol tolerance to organism for continuous ethanol yield is required.

2.11 Recovery of Ethanol from Fermented Broth

During ethanol fermentation using potential *Saccharomyces* sp., various other by-products also formed in variying concentrations (**Sree *et al*., 1999**). Thus, there is a need to remove these by-products to obtain pure ethanol. There may be contamination of esters, organic acids, and higher alcohols; as well as cyclic and heterocyclic compounds (**Onuki**

***et al.,* 2008**). Therefore, most widely used methods for pure ethanol recovery are distilaltion, adsorption, ozonation, and gas striping.

2.11.1 Distillation

Distilaation is the most used method to remove fermentation by-products and is most recognized industrial purification technique for ethanol. It utilizes principle of differences in volatilities of components present in a mixture. By heating a mixture, the components with low boiling point goes in the vapor phase and after condensing this vapour (using cold water), concentrated and less volatile compounds are recovered in liquid phase (**Pontes *et al.*, 2006; Onuki *et al.*, 2008**). A distillation tower is designed for separation of water and ethanol efficiently. Water is recovered from bottom and ethanol obtained from top of distillation tower. However, volatile by-products such as several acids and other alcohols stay in ethanol, therefore, further distillation is not cost and energy effective. Many studies have reported the need of some modifications in distillation or to find a new purification technique of ethanol which can be used at the place of distillation (**Pontes *et al.*, 2006; Kim *et al.*, 2008; Onuki *et al.*, 2008**).

2.11.2 Adsorption

In adsorption, adsorbent is utilised that simply adsorb the desired compound depending on their physical and chemical properties on its large surface area (**Tripathi and Raichur, 2008**). In general, bigger particles and/or particles having same polarity tend to be adsorbed more on respective adsorbant. During ethanol purification, non-polar surface and pores with various rang are used, because of polarity of ethanol and varying particle sizes of impurities. Activated carbon and alumina are most favourable adsorbants (**Demirbas *et al.*, 2008; Tripathy and Raichur, 2008**).

2.11.3 Ozonation

Tri-atomic molecule, Ozone, with its strong oxidation potential, could decompose various kinds of compounds. Decomposition of impurities occour while mixture treated with ozone, result in the change in physical/ chemical properties (such as increases in volatility, biodegradability, decrease in toxicity, etc.) of compounds. Oxidation of ethanol could not be take place under the atmospheric condition (**Bailey, 1982**), therefore, ozone can only remove impurities without damaging the ethanol. Although, non-oxidizable

impurities and by products of ozonolysis are further constraints to use this method. Furthermore, physically compounds are not removed by ozone treatment. These impurities should be removed after the process using some other post-ozonation treatments (**Bailey, 1982; Onuki *et al*., 2008**).

2.11.4. Gas stripping

In gas stripping separation method, separation of desired compound from the mixyure is based on differences of volatilities among compounds (**Egan *et al*., 1988; Onuki *et al*., 2008**). The Henry's law constant is used to govern the separation efficiency (**Alley, 2007**). Accoring to Henry's law:

$$H = \frac{P_{vap}}{C_{sat}}$$

Where, H= Henry's constant

P_{vap}= Partial pressure pure compound (atm), and

C_{sat}= Saturation concentration of pure compound in liquid state (moles/ or mg/L)

Henry's law constant varies depending on vapor and liquid phases. It is easily considered that compounds that are haing low boiling points can be stripped more easily. Examples include acetaldehyde that is found as one of the major impurities in ethanol fermentation broth.

Thus, there is an urgent need to develop proper microbiological pretreatment, saccharification and fermentation technique; as well as efficient separation method for ethanol recovery from fermented liquor. Any of the techniquies described above does not completely fulfil the demand for industrial productin of bioethanol from lignocellulosic plant biomass. Selection of potential biomass, optimization for stable enzyme production and pretreatment conditions, minimum sugar loss during pretreatment process, and efficient and cost effective recovery of bioethanol from fermented broth are some major constrains for proper technology development. However, the wood rot fungi (especially mushrooms) are having a great efficiency to produce biomass degrading enzymes, and moreover their use for bioconversion at industrial level can be a good choice for technology development.

Materials and Methods

Chapter 3 MATERIALS AND METHODS

3.1 Cultures Used

Various wood rot fungal and bacterial cultures used during present investigation, were isolated from diverse sources (decaying woods, and infected trees) collected from different locations of Tarai (Pantnagar) and Kumaun (Pithoragarh) regions of Uttarakhand. Standard culture of *Phanerochaete chrysosporium* MTCC-787 was procured from Microbial Type Culture Collection, Chandigarh, India. Besides these some non wood rot fungal cultures used during present investigation (*T. harzianum, Aspergillus niger, Rhizoctonia solani, Fusarium* sp.) and some fungal phytopathogens *viz Fusarium oxysporum, Helminthosporium maydis*, and *Alterneria solani*, used in the study were obtained from Department of Microbiology and department of Plant Pathology, G. B. Pant University of Agriculture and Technology Pantnagar, U.S. Nagar Uttarakhand. Detailed description of the cultures is given in **Table 3.1.**

3.2 Isolation and Conservation of Microbial Gene Pool

3.2.1 Culture Media Used

All fungal cultures were isolated and maintained on Potato Dextrose Agar (PDA) medium (gL^{-1}; potato peeled 200.0, dextrose 20.0, and agar 15.0, pH 5.6±0.2). All bacterial cultures were isolated and maintained on Nutrient Agar (NA) medium (gL^{-1}; beef extract 3.0, peptone 5.0, NaCl 5.0, and agar 18.0, pH 7.4±0.2).

3.2.2 Isolation of Microbial Cultures

The decaying wood samples and mushroom fruiting bodies were used for isolation of wood rotting microbes. For fungi, small pieces of fruiting bodies were surface sterilized with 70% ethanol, followed by several washings with sterile distilled water. The sterilized pieces were than inoculated in triplicate in the centre of PDA plates and incubated at 28 ± 2^0C for 5-10 d. For isolation of bacterial cultures, the decaying wood samples were crushed aseptically using sterile mortar-pestle and serially diluted in sterile normal saline (0.85%) solution, and then pour plated with the nutrient agar medium. The plates were incubated at 35 ± 2^0C for 48 h for bacterial growth. The purified cultures were preserved at 4^0C in refrigerator as agar slants and at -20^0C as glycerol stocks in deep freeze for short and long term storage, respectively.

Table 3.1 Detailed description of the fungal and bacterial isolates/cultures used during present investigation

S.N.	Location	Source	Number of Isolates	Fungal/ Bacterial Cultures
A. Fungal Isolates:				
1.	Pithoragarh, Uttarakhand, India	Decaying wood/ forest tree trunk (Fruiting bodies or Mushrooms)	09	WFPi1, WFPi2, WFPi3, WFPi4, WFPi5, WFPi6, WFPi7, WFPi8 and WFPi9
2.	AFRC, GBPUA & T, Pantnagar, Uttarakhand	Decaying wood/ forest tree trunk (Fruiting bodies or Mushrooms)	39	WFPn1, WFPn2, WFPn3, WFPn4, WFPn5, WFPn6, WFPn7, WFPn8, WFPn9, WFPn10, WFPn11, WFPn12, WFPn13, WFPn14, WFPn15, WFPn16, WFPn17, WFPn18, WFPn19, WFPn20, WFPn21, WFPn22, WFPn23, WFPn24, BAFP1, TAFP2, BAFP3, TAFP4, AAFP5, BAFP6, CAFP7, MAFP8, MAFP9 PAFP10, PAFP11, TAFP41, TAFP42, WDP1, WDCP2
3.	GBPUA & T, Pantnagar, Uttarakhand	Department of Microbiology and Department of Plant Pathology	12	KT18, SE6, *Fusarium oxysporium*, *Fusarium* spp. *Rhizoctonia solani*, *Helminthosporium maydis*, F36, *Alterneria* spp., *Trichoderma harzianum*, *Aspergillus niger*, ST1, PCF1
4.	Tanakpur, Uttarakhand, India	Decaying Wood	12	DWFP1, DWFP2, DWFP3, DWFP4, DWFP5, DWFP6, DWFP7, DWFP 8, DWFP9, DWFP10, DWFP11, DWFP12
5.	Standard cultures		01	*Phanerochaete chrysosporium* MTCC-787
B. Bacterial Isolates:				
6.	GBPUA & T, Pantnagar, Uttarakhand	Decaying Wood	41	WDBP1, WDBP2, WDBP3, WDBP4, WDBP5, WDBP6, WDBP7, WDBP8, WDBP9, WDBP10, WDBP11, WDBP12, WDBP13, WDBP14, WDBP15, WDBP16, WDBP17, WDBP18, WDBP19, WDBP20, WDBP21, WDBP22, WDBP23, WDBP24, WDBP25, WDBP26, WDBP27, WDBP28, WDBP29, WDBP30, WDBP31, WDBP32, WDBP33, WDBP34, WDBP35, WDBP36, WDBP37, WDBP38, WDBP39, WDBP40, WDBP41

3.3 Qualitative Screening for Enzyme Production: Plate Assay

3.3.1 Ligninase Production

A. Preliminary Screening for Overall Lignin Modifying Activity

In view of selection of lignolytic cultures, all the fungal and bacterial cultures/isolates were first screened for their overall lignin modifying activities (any type of ligninases production) using lignin modifying enzyme basal medium (LBM) (**Pointing, 1999).**

Composition of Lignin Modifying Enzyme Basal Agar Medium

Components	Concentration (gL^{-1})
KH_2PO_4	1.0
$C_4H_{12}N_2O_6$	0.5
$MgSO_4.7H_2O$	0.5
$CaCl_2.2H_2O$	0.01
Yeast Extract	0.01
$CuSO_4.5H_2O$	0.001
$Fe_2(SO_4)_3$	0.001
$MnSO_4.H_2O$	0.001
Agar	16.0
Volume made up to 1000 ml with distilled water.	

LBM was autoclaved at 15 lbs psi pressure for 15 minutes. Then 1 ml of separately sterilized 20% (w/v) glucose solution and 1 ml of 1% (w/v) aqueous tannic acid solutions were added to 100 ml of the medium; it was then pour plated in sterilized petri plates. Active fungal and bacterial cultures were inoculated in LBM plates in triplicates, un-inoculated plate served as control. The inoculated plates were incubated at 28 ± 2^0C for 5-10 d for fungus and at 35 ± 2^0C for 1-4 d for bacterial cultures. The qualitative measure of extracellular overall lignin modifying activity is the presence of brown oxidation zone around the colony and is reported as the index of relative enzyme activity (I_{lig}). The following formula was used for calculating the (I_{lig}=zone diameter/colony diameter).

Out of 114 microbial (fungal and bacterial) isolates/cultures, only nine fungal isolates showing overall lignin modifying potential were screened further qualitatively for production of lignin degrading enzymes *viz.* lignin peroxidase (both lignin peroxidase, and manganese dependent peroxidase) and laccase and compared with those of the standard culture (*Phanerochaete chrysosporium*).

B. Lignin Peroxidase Activity

Screening of the cultures for lignin peroxidase production was done using two methods: (i) Glucose Malt Extract Salt Agar Medium (**Thiyagarajan *et al*., 2008**) and (ii) using Azure B plate assay (**Pointing, 1999**).

Composition of Glucose Malt Extract Salt Agar Medium

Components	Concentration (gL^{-1})
Glucose	20.0
Malt Extract	20.0
$NaNO_3$	2.0
KH_2PO_4	2.0
KCl	2.0
$MgSO_4.7H_2O$	1.0
$FeSO_4.7H_2O$	0.02
pH	6.5
Agar	16.0
Volume made up to 1000 ml with distilled water.	

The test cultures were inoculated on glucose malt extract salt agar plates and incubated at 28 ± 2^0C for 5-10 d for fungal and at 35 ± 2^0C for 1-4 d for bacterial growth. Un-inoculated plate served as control. After incubation, 1.7mM ABTS (3 ml) & 2.5mM hydrogen peroxide (3 ml) overlapped on the plates and the plates were kept in dark at 25^0C for 5 minutes. The qualitative measure of extracellular peroxidase activity was recorded as appearance of clear bluish green zone around the colony. The peroxidase production was reported as the index of relative enzyme activity (I_{per}=zone diameter/colony diameter).

For determination of peroxidase activity by azure B agar plate assay, separately sterilised 20% (w/v) glucose solution (1ml) was added to pre-sterilised basal medium (LBM, 100ml) supplemented with 0.01% (w/v), Azure B dye. The medium was transferred to petri plates and plates were incubated with test microorganism at 28 ± 1^0C for 5-10 d for fungus and at 35 ± 1^0C for 1-4 d for bacterial growth in dark. Production of lignin peroxidase was recorded as clearance of blue coloured medium around the colonies.

C. Laccase Activity

Qualitative screening for laccase enzyme production was done using potato dextrose agar (PDA) for fungus and nutrient agar (NA) medium for bacteria amended with 0.01% (v/v) of guaiacol (**Kiiskinen *et al.*, 2004**). Plates were inoculated with test microorganisms and incubated at 28 ± 1^0C for 5-10 d for fungus and at 35 ± 1^0C for 1-4 d for bacterial growth. The presence of brick red colour around the fungal mycelium or bacterial colony was considered as positive test for guaiacol oxidizing laccase secreting microorganisms. The extracellular laccase enzyme production was reported as the index of relative enzyme activity (I_{lac}=zone diameter/colony diameter).

3.3.2 Cellulase Production

Qualitative screening of selected cultures for cellulase enzyme production was done using carboxy-methyl cellulose (CMC) agar media (**Devi & Kumar, 2011**).

Composition of carboxy-methyl cellulose (CMC) agar media

A. For Bacterial Cultures

Components	Concentration (gL^{-1})
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
NaCl	0.5
$FeSO_4.7H_2O$	0.01
$MnSO_4.H_2O$	0.01
NH_4NO_3	0.3
CMC	10.0
Agar	16.0
pH	7.0

B. For Fungal Cultures

Components	Concentration (gL^{-1})
$NaNO_3$	2.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
KCl	0.5
CMC Sodium Salt	10.0
Peptone	0.2
Agar	17.0
pH	6.5

The test cultures were inoculated on CMC agar plates and incubated at 28 ± 2^0C for 4-5 d (fungus) and at 35 ± 2^0C for 1-3 d (bacterial growth). Un-inoculated plates served as control. After incubation, plates were flooded with 1% congo red solution for 15 minutes and then de-stained with 1M NaCl solution for 15 minutes. The diameter of the zone of decolorization around each colony was measured. The extracellular cellulase enzyme production was reported as the index of relative enzyme activity (I_{cel}=zone diameter/colony diameter).

3.4 Testing of Lignolytic Cultures for Synthetic Dye Decolourization

Since wood rotting lignolytic enzymes have also been reported to be actively involved in synthetic textile dye decolourization in various studies, therefore, the study was undertaken for decolorization of various synthetic toxic dyes such as congo red, crystal violet, fuchsin basic, malachite green and brilliant green using selected wood rot fungal isolates.

A. Qualitative Test

Dye degrading ability of selected fungal cultures was assayed in low nitrogen basal medium as described by **Murugesan** (**2006**).

Composition of Low Nitrogen Basal Medium (LNBM)

Components	Concentration (gL^{-1})
Glucose	1.0
$CaCl_2$	1.5
$MgSO_4$	2.0
KH_2PO_4	1.5
NH_4Cl	0.15
Volume made up to 1000 ml with distilled water	

The LNBM agar plates supplemented with textile dyes (congo red, crystal violet, fuchsin basic, malachite green and brilliant green) (100 mg/L) were individually inoculated with cultures and incubated at 30 ± 2^0C under dark condition. The plates were regularly monitored at every 24 h for growth and decolorization activities.

B. Quantification of Dye Decolorization

The cultures selected on the basis of qualitative screening, were used to quantify dye decolorizing potential *in vitro*. The five active fungal discs (5mm diameter) were grown in low nitrogen basal broth medium (**Section 3.4, A**) supplemented with dye at the concentration of 100 mg/L. Samples were withdrawn periodically at an interval of 72 h and observed for colour change by measuring optical densities, spectrophotometrically, at the λ_{max} of each dye (congo red, 497 nm; malachite green, 619 nm; brilliant green, 630 nm; fuchsin basic, 545 nm, and crystal violet, 588 nm). The cultures showing bio-sorption of the dye into the mycelium, were also checked visually and microscopically. The percent decolorization (*D*) of each dye (%) was calculated using the following formula:

$$D(\%) = \frac{Initial\ OD - Final\ OD}{Initial\ OD} \times 100$$

The completely decolorized broth cultures of selected isolate were also checked for the extracellular laccase enzyme production as described in following **Section 3.5.1, II, c**.

3.5 Determination of Extracellular Enzyme Activities

3.5.1 Lignolytic Enzymes

The selected wood rot fungal cultures showing positive results for overall lignin modifying activities, peroxidase activities, and laccase activities during qualitative testing, were further evaluated quantitatively for production of of lignin degrading enzymes, laccase, Lac (EC 1.10.3.2) and two different lignin degrading peroxidases *viz*. lignin peroxidase, LiP (EC 1.11.1.14), manganese peroxidase, MnP (EC 1.11.1.13), using carbon limited basal liquid medium as described by **Ahammed (2002)**, with slight modifications**.**

Composition of Carbon Limited Basal Liquid Medium

Components	Concentration
Glucose	3.0 gL^{-1}
KH_2PO_4	5.0 gL^{-1}
NH_4NO_3	12.5 mM
$MgSO_4.7H_2O$	1.0 gL^{-1}
Tween 20	0.2 gL^{-1}
KCl	0.5gL^{-1}
FeSO4	0.001gL^{-1}
Veratryl Alcohol	1mM
Trace metal solution	0.1%
pH	5.0
Volume made up to 1000 ml with distilled water.	

Composition of Trace Metal Solution

Components	Concentration (gL^{-1})
$B_4O_7Na_2$	0.1
$MnSO_4$	0.01
$(NH_4)_6Mo_7O_{24}$	0.01
$ZnSO_4$	0.01
$CuSO_4$	0.05

For determination of MnP activity, the trace metal solution was prepared without $MnSO_4$ and the basal medium was supplemented with a high concentration (0.05%) of $MnSO_4$. The basal liquid medium (100ml) was inoculated with the active culture discs (five discs, 5mm diameter each) (**Shivakumar *et al*., 2010**).

(I) Preparation of Crude Enzyme

Starter culture was prepared by reviving the cultures on potato dextrose agar plates at 30 ± 2^0C under dark condition for 5-7d. The five active fungal mycellial discs (5mm each) were evenly inoculated into the production medium (100 ml) in 500 ml Erlenmeyer flasks. The inoculated flasks along with un-inoculated controls were incubated at 30 ± 2^0C under dark in static conditions. The aliquots (2.0 ml) were withdrawn periodically up to 24d at an interval of 72h and centrifuged at 10,000 rpm for 10 min at 4^0C. The cell-free supernatant was used as the source of crude enzyme for quantification of lignin degrading enzymes.

(II) Measurement of Enzyme Activities

(a) Lignin peroxidase, LiP (EC 1.11.1.14)

LiP activity was assayed by measuring the rate of H_20_2-dependent oxidation of veratryl alcohol to veratraldehyde, spectrophotometrically following the method given by **Tien & Kirk, (1988)** with slight modifications. The reaction mixture (2.05 ml) contained 0.8 mM veratryl alcohol in 0.1 M citrate buffer (pH 3.0) and 1 ml of culture supematant. The reaction was started by addition of 150 mM H_20_2 and the linear increase in absorbance at 310 nm was monitored for one minute at 30°C. One unit of LiP activity was defined as "the amount of enzyme required for conversion of 1µM of substrate (veratryl alcohol) into 1µM of product (veratryldehyde) in one minute at 310 nm" and it was expressed as Uml^{-1} (ε= 9300 $M^{-1}cm^{-1}$). The enzyme activity was calculated using the following formula:

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

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

V = Volume of assay solution in cuvette

ε = Extinction coefficient

v = Volume of crude sample

t = Time in minutes

(b) Manganese peroxidase, MnP (1.11.1.13)

Manganese peroxidase activity was determined by monitoring the oxidation of Mn^{+2} to Mn^{+3}following the method of **Paszczynski *et al*. (1988)** with slight modifications. The assay solution (3.06 ml) contained 0.1 mM guaiacol & 0.1 mM $MnS0_4$ in 0.1 M citrate buffer (pH 5.0) and 1ml of culture filtrate. The reaction was started by addition of 0.1 mM H_20_2. One unit of MnP enzyme activity was defined as "the amount of enzyme required for conversion of 1µM of substrate into 1µM of coloured product in one minute at 465 nm" (ε= 12.1 x 10^3 $M^{-1}cm^{-1}$), and calculated using the formula mentioned in section 3.5.1, II (a).

(c) Laccase, Lac (1.10.3.2)

The laccase enzyme activity was determined according to **Niku-Paavola *et al*. (1990)** with slight modifications. It was monitored by oxidation of 500 µM ABTS {2,2-azinobis (3-ethylbenzthiazoline-6- sulfonic acid)} disodium salt buffered with 50 mM citrate buffer (pH 4.5). The reaction mixture (3 ml) contained 1 ml of culture filtrate and one unit of enzyme activity was defined as "the amount of enzyme required for conversion of 1µM of colourless substrate into 1µM of oxidised coloured product in one minute at 420 nm" (ε= 3.6 x 10^4 $M^{-1}cm^{-1}$), and calculated using the formula mentioned in section 3.5.1, II (a).

Among the three lignolytic enzymes studies, the laccase enzyme produced by all the selected lignolytic cultures, was studied for further experiments.

3.5.2 Cellulase Enzyme Complex

The fungal cultures showing positive results for extracellular cellulase enzyme activity on CMC agar plates during qualitative testing, were evaluated for quantitative estimation of cellulase enzyme complex using CMC containing cellulase production liquid medium.

Composition of Cellulase Production Medium

Components	Concentration (gL^{-1})
CMC	10.0
$(NH_4)_2SO_4$	1.0
K_2HPO_4	0.5
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.5
$FeCl_3$	0.004
$CaCl_2.2H_2O$	0.002
pH	7.0

The cell free crude enzyme was used for measurement of cellulase enzyme activities. Filter paper activity (FPase) and carboxymethyl-cellulase (CMCase) activity were determined using the IUPAC standard procedures. One unit of enzyme activity was defined as "the amount of enzyme releasing one micromole of reducing sugar per minute" and determined using glucose as a standard. The beta-glucosidase enzyme activity was measured by the hydrolysis of *p*-nitrophenyl-D-glucopyranoside (pNPG) as the substrate. In this case "one unit of the β-glucosidase activity was defined as the "micromoles of the *p*-nitrophenol released per minute" upon hydrolysis of pNPG under standard enzyme assay conditions" (**Ghose, 1987)**.

Stocks and Reagents

(i)	Stock solution of Glucose	10 mg ml^{-1}
(ii)	3', 5' Dinitrosalisylic acid (DNS) Reagent	1g DNS, 0.2g crystalline phenol, 0.05g sodium sulphite, and Rochelle Salt (40%) were dissolved in 100 ml of 1% NaOH. Finally volume was made up to 1000 ml.

(I) Standard Curve Preparation

(a) Standard Curve of Glucose

Aliquots of 25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 µl (0.25-2.5 mg of glucose with an interval of 0.25) from stock solution of glucose, were taken in test

tubes and the volume in each tube was made up to 1.5 ml with acetate buffer (pH 5.5), a blank of 1.5 ml of acetate buffer (pH 5.5) was also prepared. Then, 3', 5' dinitrosalicylic acid (DNS) reagent (3 ml) was added to each test tube. The samples were boiled for 5 min in a boiling water bath and cooled using cold ice water bath for stabilization of colour. The tube content was diluted using 20 ml distilled water and mixed properly by inverting the tubes several times. The absorbance (O.D.) was measured at 540 nm and the linear standard curve was constructed using the amount of glucose plotted against absorbance (**Appendix I**).

(b) Standard Curve of p-Nitrophenol

The standard curve of p-nitrophenol was prepared by taking the aliquots of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 nano mol) from stock solution of p-nitrophenol (1.0 mM) into a series of test tubes. The volume was made up to 1.0 ml using acetate buffer (pH 5.5). Then 2.0 ml of chilled sodium carbonate (1M) was added. The colour development was read at 405nm against reagent blank (**Appendix 1**).

(II) Measurement of Enzyme Activities

(a) Filter Paper (FPase) Assay

The appropriately diluted supernatant (0.5 ml) was equilibrated to 50^0C, for 5 min in a water bath, and then substrate (Whatman filter paper No.1 strip, 1.0X6.0 cm, 0.05g) was added. This mixture was incubated at 50^0C for 1h and reaction was stopped by adding DNS reagent (3.0 ml). The samples were boiled for 5 min in a boiling water bath, and then cooled in ice water bath for colour stabilization. The tube content was diluted using distilled water (20 ml) and mixed properly by inverting the tubes several times. The absorbance (O.D.) was measured at 540 nm and the values of released glucose were quantified using the standard curve. Calibration was done using reagent blank (1.5 ml of acetate buffer, 0.05M, pH 5.5) and values were quantified after substraction of enzyme and substrate blank. FPase activity was calculated as Filter Paper Unit (FPU) using following formula:

$$\text{FPU} \quad \text{mg glucose released X} \frac{1.0 \quad \text{mol per min per ml}}{0.18 * 0.5 * 60}$$

(b) Endoglucanase (CMCase) Assay

Appropriately diluted crude enzyme solution (0.5ml) was equilibrated to 50^0C for 5 min and then 2% of CMC dissolved in 0.05M acetate buffer, pH5.5 (0.5ml) was added to it. The mixture was incubated at 50^0C for 30 min and then the reaction was stopped by adding 3.0 ml of DNS reagent. The samples were then boiled for 5 min followed by cooling in ice cold water bath for colour stabilization. The tube content was then diluted using 20 ml of distilled water, mixed properly and OD was read at 540nm. CMCase activity was calculated using following formula:

$$\text{CMCase units} \quad \text{mg glucose released X} \frac{1.0 \quad \text{mol per min per ml}}{0.18 * 0.5 * 30}$$

(c) Beta-Glucosidase (BGL) Assay

β-glucosidase enzyme assay was performed by taking appropriately diluted supernatant (0.5ml) into a test tube and equilibrated for 5 min to 50^0C. Then, 0.5 ml of the substrate (10mM pNPG in 0.05M acetate buffer of pH5.5) was added. The tubes were then incubated at 50^0C in a boiling water bath for 30 min and the reaction was stopped by adding 2.0 ml of chilled sodium carbonate (1M). The colour development was read at 405nm against reagent blank. Calibration was done using reagent blank (1.5 ml of acetate buffer, 0.05M, pH 5.5) and values were quantified using the standard curve of pNP after subtraction of absorption of enzyme and substrate blank.

UNIT II: LACCASE ENZYME PRODUCTION AND CHARACTERIZATION

3.6 Enzyme Production Optimization

After quantification of laccase enzyme in synthetic broth medium, the highest laccase producing wood rot fungal isolate WDP2 showing maximum laccase activity in culture filtrate was selected for delignification studies. The culture conditions were optimised using classical method of optimization. The classical method involved optimization of culture conditions for high enzyme production by optimizing 'one variable at a time'. Various process parameters that affect laccase enzyme production were optimized over a wide range. The approach implemented for standardization of bioprocess parameters was to evaluate the effect of an individual parameter and to incorporate it at standardized level before standardizing the next parameter. The process parameters thus standardized included substrate/carbon source (Rice bran, bamboo powder, rice husk, wheat bran, and poplar powder as a carbon source each at the concentration of 1%, w/v), substrate or carbon source concentration (0.5-3.0% w/v, with an interval of 0.5), type of nitrogen source (Chickpea flour as a natural source of nitrogen, tryptone, sodium nitrate, yeast extract, ammonium sulphate, urea, and peptone, each at the concentration 0.1% w/v), concentration of nitrogen source (0.1-0.5 %, with an interval of 0.1%), pH of the culture medium (3.0-8.0), incubation temperature (24, 28, 32, 36, and 40^0C), type and concentrations of various surfactants such as Triton X100, tween 20, and the natural surfactant soap-nut (*Sapindus*) extract, each at the rate of 0.01-0.05% (v/v) with an interval of 0.01; and 0.1-0.5%, v/v with an interval of 0.1 were evaluated for maximal laccase enzyme production.

3.7 Purification and Characterization of Laccase Enzyme

3.7.1 Partial Purification of Enzyme

The selected fungal isolate WDP2 was inoculated in optimised laccase production medium and incubated at 30°C on static condition. After 12 d of growth, the mycelium was removed using cheese cloth and culture filtrate was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and an equal volume of chilled acetone was slowly added with constant stirring under ice chilled conditions and stored at 4^0C. The precipitated proteins were separated using centrifugation 10,000 rpm for 10 min at 4 °C.

After centrifugation, the pellet was dissolved in 5 ml of 50 mM citrate buffer (pH 3.0), and dialyzed against same buffer. The partially purified laccase enzyme used for further studies.

3.7.2 Characterization of Laccase Enzyme

Each specific enzyme has specific characteristic properties, at which the enzyme is maximally active. Thus, laccase enzyme activity was characterised by studying the temperature profile, pH profile, thermal stability, pH stability and storage stabilities of partially purified enzyme. These parameters are very important for industrial use of the enzyme. The pH profile of the laccase enzyme was determined by measuring the laccase activity at different pH (2.0-10.0) values under the optimum fermentation conditions at room temperature using following buffers: 0.1M citrate phosphate buffer (pH 3.0-7.0), Tris (hydroxymethyl) aminomethane buffer (pH 7.5-9.0) and Glycine-NaOH buffer (pH 9.5-10.5). Temperature profile of enzyme was determined by measuring the laccase activity at the optimum pH after 12d of fermentation period at different reaction temperatures ranging from 20-100^{0}C with an interval of 10^{0}C. Thermal (40, 50, 60, 70, 80, 90^{0}C and 100^{0}C) stability of the enzyme was determined at respective temperatures with the pre-incubation of the enzyme for 30 and 60 min and stability of enzyme at different pH values (pH 4.0-10.0) was determined at respective pH with pre-incubation of enzyme for 60 min under the optimized conditions and then assaying the residual activities. The storage stability of the enzyme was also determined by keeping the enzyme at 4^{0}C in refrigerator for 28 d and then assaying the residual activity at an interval of 7 d. In all the cases initial laccase activity was assumed to be 100% and used to calculate the residual enzyme activities as percentage of the initial activity (relative activities) during the incubation period.

3.8 Molecular Characterization of Enzyme

Partially purified laccase enzyme produced under optimised culture conditions was used for extracellular protein profiling. The extracellular proteins were visualized through native polyacrylaide gel electrophoresis (PAGE) and sodium dodecyl sulphate polyacrylaide gel electrophoresis (SDS-PAGE) following the protocols described by **Sambrook *et al*. (2000)**. Loading was based upon equal protein quantities for each sample.

3.8.1 Stock Solutions

(i) Acrylamide & bis-acrylamide Stock Solutions (30%): Acrylamide (29.2g) and N,N'-methylene bis-acrylamide (0.8g) were dissolved in 100 ml of triple distilled water using a magnetic stirrer, filtered and stored at 4^0C in dark coloured bottle.

(ii) Sodium Dodecyl Sulphate (SDS), 10% (w/v): 10.0g SDS was dissolved in triple distilled water (100 ml).

(iii) Stacking Gel Buffer: 0.5M Tris-HCl (pH6.8).

(iv) Separating Gel Buffer: 1.5 M Tris-HCl (pH 8.8).

(v) Electrophoresis Buffer: Tris glycine buffer (pH 8.3).

Components	SDS-PAGE	Native-PAGE
Tris base	25.0 mM	25.0 mM
Glycine	192.0 mM	192.0 mM
SDS	0.1%	-

(vi) Sample Buffer (2X)

Components	SDS-PAGE	Native-PAGE
Tris-HCl (pH 6.8)	62.0 mM	62.0 mM
SDS	2.0%	-
Glycerol	10.0%	10.0%
B- mercapto-ethanol (BME)	5.0%	-
Ethylenediamine-tetra-acetic acid (EDTA)	0.1%	0.1%
Bromophenol-blue (BPB)	0.1%	0.1%

(vii) Fixing Solution: Methanol (50%) and glacial acetic acid (10%).

(viii) Staining Solution: Coomassie Brilliant Blue-R250 dye (0.25%) dissolved in methanol (50%) and glacial acetic acid (10%).

(ix) Destaining Solution: Methanol (10%) and glacial acetic acid (7%).

(x) Ammonium-per-sulphate (APS, 10%, w/v) solution: APS (0.1 g) dissolved in triple distilled water (1.0 ml).

(xi) Tetramethylethylenediamine (TEMED): Commercially available TEMED (HiMedia) was used.

3.8.2 Preparation of gel

Proteins were analysed using 5% stacking gel in both Native- and SDS-PAGE, while 12% and 8% resolving gels were used for SDS- and Native-PAGE respectively. The following composition of resolving and stacking gels were used for the SDS- and Native-PAGE analysis of laccase enzyme.

I. Composition of Resolving Gel

Components	**Quantity (ml)**	
	SDS-PAGE (12%)	**Native-PAGE (8%)**
Triple distilled water	4.90	7.05
Acrylamide stock (30%)	6.00	4.00
Resolving gel buffer (1.5 M Tris, pH 8.8)	3.80	3.80
SDS (10%)	0.15	-
APS (10%, w/v)	0.15	0.15
TEMED	0.006	0.009
Total volume	15.006	15.009

II. Composition of Stacking Gel (5%)

Components	Quantity (ml)	
	SDS-PAGE	**Native-PAGE**
Double distilled water	5.50	5.58
Acrylamide stock (30%)	1.30	1.30
Stacking gel buffer (1.0 M Tris, pH 6.8)	1.00	1.00
SDS (10%)	0.08	-
APS (10%, w/v)	0.08	0.08
TEMED	0.008	0.008
Total volume	7.968	7.968

3.8.3 Gel Casting

Cleaned dried gel plates were assembled with the spacers and clamps, separating gel solution was poured between the glass plates, overlaid with double distilled water and allowed to polymerize at room temperature. When the gel was fully set, overlay solution was removed; the gel washed 2-3 times with triple distilled water. Then, the stacking gel was poured and the comb was inserted 1.5 cm above the resolving gel & the gel was allowed to polymerize. The comb was then removed and the wells were cleaned. The gel plates were assembled in electrophoresis chamber and filled with electrophoresis buffer. Air bubbles were carefully removed through a syringe. The gel was pre run at 80V for 15 min.

3.8.4 Loading of Sample

For SDS-PAGE analysis, the protein samples were denatured prior to loading on gel. Each protein sample (20 to 50 µg) was mixed with 2X SDS-PAGE sample buffer (1:1) and boiled in water bath for 5 min. For native PAGE, protein samples were not heat denatured prior to loading. The samples were mixed with 2X Native-PAGE sample buffer (1:1), centrifuged at 5,000 rpm for 2-3 min and loaded onto wells with the help of micropipette using gel loading tips. The gel was first run at 80V till the sample was in

stacking gel, and then the voltage was raised to 100V; the gel was allowed to run till the dye reached 0.5 cm from the lower edge of the gel. After completion of electrophoresis, gel was kept in fixing solution for 30 min.

3.8.5 Staining and Destaining of Gel

The gel was stained for a minimum of 1 to 2 h or maximum overnight in staining solution. Destaining was done till the background was fairly reduced to make the protein bands visible.

3.9 Laccase Isoform Analysis

Different isoforms (isozymes) of the laccase enzyme were identified and visualised as activity bands by activity staining through native PAGE. Activity staining for laccase was performed with ABTS as the substrate. Samples were run through Native PAGE on 7% of resolving gel in non-denaturating conditions. Protein samples were not heat denatured prior to loading. The samples were mixed with 2X Native-PAGE sample buffer (1:1), centrifuged at 5,000 rpm for 2-3 min and loaded onto wells with the help of micropipette using gel loading tips. The gel was first run at 80V till the sample was in stacking gel, and then the voltage was raised to 100V; the gel was allowed to run till the dye reached 0.5 cm from the lower edge of the gel.

Following the electrophoresis, the gel was washed with triple distilled water and allowed to stand in 50 ml of sodium acetate buffer (100 mM, pH 4.5) with 1 ml of 10 mM ABTS for 20–30 min for visualization of laccase isoform activity bands (**Patel *et al.*, 2014**). Laccase activity bands were indicated by the development of a low to dark green coloured bands.

UNIT III: LIGNOCELLULOSIC BIOMASS: SELECTION & PRETREATMENT

3.10 Bamboo Biomass: Screening & Chemical Analysis

3.10.1 Bamboo Biomass as Substrate

Four different bamboo wood samples (*Bambusa balcooa*, *Dendrocalamus hemiltonii*, *Bambusa nutans*, & *Dandrocalamus asper*) were taken from Agroforestry research centre, G.B. Pant University of Agriculture and Technology Pantnagar, Uttarakhand, India. The freshly collected samples were cut into small pieces and were cut into small pieces and weighed to get the fresh weight of the biomass.

3.10.2 Moisture Content Determination

The samples were oven dried at 60-70^{0}C and periodically checked for the decrease in weight by loss of moisture, until constant weight was achieved. The following formula was used for the calculation of the moisture content of wood:

$$\text{Moisture Content} \quad \frac{\text{Fresh Weight of Wood} - \text{Dry Weight of Wood}}{\text{Fresh Weight of wood}} \quad 100$$

3.10.3 Physical Processing of the Substrates

The dried wood samples were then converted into fine wood powder (~1-2 mm diameter) using a local electronic wheal saw, and then stored 4^{0}C in zip-lock bags until further use.

3.10.4 Chemical Analysis

In order to select potential bamboo sp., as a source for fermentable sugars, all four powdered biomasses were analysed for their cellulose and lignin contents following the methods given by **Sadasivam and Manickam, 1991** with slight modifications**.**

(I) Cellulose Content

(a) Stocks and Reagents

(i) Acetic/ nitric reagent: 150 ml of 80% acetic acid+5 ml of conc. nitric acid

(ii) Anthrone reagent: 200mg anthrone+100 ml conc. sulphuric acid

(iii) 67% sulphuric acid

(b) Standard Curve of Cellulose

For preparation of standard curve of cellulose, cellulose (100 mg) was taken in a test tube and 10 ml of 67% sulphuric acid was added to it. The tube was allowed to stand for 1h. A series of volumes from this test tube (0.4, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8 ml corresponding to 40, 80, 120, 160, 200, 240 and 280 µg of cellulose) were taken in separate test tubes and these were diluted to 100 ml. To 1.0 ml of diluted solution, 10 ml of anthrone reagent was added and mixed thoroughly. The samples were then heated in a boiling water bath for 10 min, cooled and the absorbance was recorded at 630 nm. A blank was set with anthrone reagent and triple distilled water and the standard curve was plotted by taking cellulose concentration on x-axis and absorbance on y-axis (**Appendix I**).

(c) Cellulose Estimation

The oven dried bamboo wood powder (0.5g) was mixed with acetic/ nitric reagent (3.0 ml) and incubated in a water bath at 100^0C for 30 min. The mixture was cooled, centrifuged at 5000 rpm for 10 min. The residue was washed with distilled water and 10 ml of 67% sulphuric acid was added to it. The mixture was allowed to stand for 1h and then 1 ml of this solution was diluted to 100 ml. Then 1.0 ml of this diluted solution was taken in a test tube and mixed with 10 ml of anthrone reagent. A tube containing 1 ml of distilled water and 10 ml of anthrone reagent served as control. The tubes were heated in a boiling water bath for 10 min. The absorbance was recorded at 630 nm after cooling. The values were quantified using the standard curve of cellulose.

(II) Lignin Estimation

The amount of lignin was calculated as the difference between acid detergent lignin (ADL) and ash. The ADL and ash were determined according to procedure given below.

(a) Reagents

(i) Acid Detergent Solution

Cetyl trimethyl ammonium bromide (CTAB, 20.0 g) was dissolved in 1.0 N sulphuric acid (1.0 litre).

(ii) 72% H_2SO_4

(b) Determination of Acid Detergent Fibre (ADF)

Oven dried bamboo wood powder (1.0 g) was mixed with acid detergent solution (100 ml). The mixture was heated to boil for 10 min and refluxed for 1 h. The heat was reduced to avoid the foaming as boiling begins. The resulting contents were filtered and washed with hot water twice and then by acetone for breaking up the lumps. The acetone washing was repeated until the filtrate was colourless. The residue was transferred to crucible, dried overnight at 100^0C and then weighed after cooling in desiccator. The ADF content was expressed in percentage and calculated using following formula:

$$\text{ADF } \% \quad \frac{\text{Weight of acid detergent fibre}}{\text{Weight of wood}} \quad 100$$

(c) Determination of Acid Detergent Lignin (ADL)

Acid detergent fibre (ADF) was mixed with 72% H_2SO_4 (20-50 ml) and allowed to stand for 3 h with intermittent stirring with a glass rod. After that the mixture was diluted and filtered with pre-weighted What-man No.1 filter paper. For preparation of pre-weighted What-man no.1 filter paper, the filter paper was dipped in hot water, dried in oven at 120^0C for 2h and then used. The glass rod and residue was washed with water several times until acid-free. The residue was dried to a constant weight at 100^0C in an oven and ADL (%) was calculated using following formula:

$$\text{ADL } \% \quad \frac{\text{Weight of acid detergent lignin}}{\text{Weight of wood}} \quad 100$$

(d) Determination of Ash

The residue left after acid treatment (ADL) was transferred to muffle furnace at 575^0C ad heated for 3 h. The ash content was calculated using following formula:

$$\text{Ash } \% \quad \frac{\text{Weight of ash}}{\text{Weight of wood}} \quad 100$$

(e) Calculation of Lignin Content

The lignin (%) in the wood was calculated using following formula:

$$\text{Lignin } \% \quad \frac{\text{Acid detergent lignin} - \text{Ash}}{\text{Initial weight of wood powder}} \quad 100$$

3.11 Pretreatment of Selected Bamboo: *Bambusa balcooa*

Various physical, physicochemical, chemical and biological pretreatments were compared in order to design the process/bioprocess for delignification of substrate.

3.11.1. Physical Pretreatments

(a) Liquid hot water Pretreatment

Fine wood powder (2.0 g) was mixed with 20 ml of the distilled water (1:10 solid:liquid) in a test tube and kept in a boiling water bath for 30 minutes. After the pretreatment, the sample was washed with distilled water, overnight oven dried at 50 ± 1^0C, and analysed for its cellulose and lignin content.

(b) Autoclaving Pretreatment

Fine wood powder (2.0 g) was mixed with 20 ml of the distilled water (1:10 solid:liquid) and in a test tube and autoclaved for 60 minutes. After the pretreatment, the sample was washed with distilled water, overnight oven dried at 65 ± 2^0C, and analysed for its cellulose and lignin content.

(c) Microwave Heating Pretreatment

Fine wood powder (2.0 g) was mixed with 20 ml of the distilled water (1:10 solid: liquid) kept in a microwave for 10 minutes at 150^0C with intermittent heating for avoiding the over boiling. After the pretreatment, the sample was washed with distilled water, overnight oven dried at 65 ± 2^0C, and analysed for its cellulose and lignin content.

(d) Ultraviolet Light Pretreatment

Fine wood powder (2.0 g) was kept under UV light (UV-C, 253.7 nm) in a Laminar air flow for 1h. After exposure, the sample was washed with distilled water, oven dried at 65 ± 1^0C, and analysed for its cellulose and lignin content.

3.11.2 Chemical Pretreatments

(a) Liquid Hot Water with Dilute Acid Pretreatment

Fine wood powder (2.0 g) was mixed with 20 ml of the distilled water (1:10 solid:liquid) and sulphuric acid (2%) and kept in a boiling water bath at 100^0C for 30 minutes. The sample was washed with distilled water, overnight oven dried at 65 ± 2^0C, and analysed for its cellulose and lignin content.

(b) Acid Catalysed Autoclaving Pretreatment

Fine wood powder (2.0 g) was mixed with 20 ml of the distilled water (1:10 solid:liquid) and sulphuric acid @2% in a test tube. Then the test tube was kept for autoclaving for 60 minutes. The sample was then washed with distilled water, overnight oven dried at 65 ± 2^0C, and analysed for its cellulose and lignin content.

(c) Alkali {NaOH/ $Ca(OH)_2$} Pretreatment

Fine wood powder (2.0 g) was mixed with 20 ml of the distilled water (1:10 solid:liquid) containing NaOH/ $Ca(OH)_2$ @ for 120min. The sample was washed with distilled water, overnight oven dried at 65 ± 2^0C, and analysed for its cellulose and lignin content.

(d) Sulphite Pretreatment of Recalcitrant Lignin (SPORL)

Fine wood powder (2.0 g) was mixed with 20 ml of the distilled water (1:10 solid:liquid), sulphuric acid (0.2%) and sodium bisulphate (2%). The sample was washed with distilled water, overnight oven dried at 65 ± 2^0C, and analysed for its cellulose and lignin content.

(e) Ammonia Fibre Explosion (AFEX) Pretreatment (Dry and Wet)

The ammonia fibre explosion was carried out in two different ways. (I): two grams of the fine wood powder was directly treated with ammonia solution (1:1). (II): the wood powder (2.0 g) was mixed with water in 1:1 ratio and then treated with ammonia solution in 1:1 ratio. Both pretreatments were conducted for at 90^0C for 30 min. The samples were then washed with distilled water, overnight oven dried at 50 ± 1^0C, and analysed for its cellulose and lignin content.

(f) Microwave Assisted Alkali Pretreatment

Two grams of the fine wood powder was mixed with 20 ml of the distilled water (1:10 solid:liquid) and NaOH (0.5%) in a test tube, and kept in a microwave for 10 minutes with intermittent heating for avoiding the over boiling. After the pretreatment, the sample was washed with distilled water, overnight oven dried at 50 ± 1^0C, and analysed for its cellulose and lignin content.

(g) Titanium Dioxide Photocatalyst Nanopowder Pretreatment

Titanium dioxide (TiO_2) nanopowder is considered as a photocatalyst that can degrade lignin or related compounds. The following combinations (using solid:liquid ratio 1:10) were developed for the evaluation of the effective TiO_2 pretreatment.

S.No.	Pretreatment Method	Procedure
1.	TiO_2+ *hv*	Wood powder (2.0g) was treated with 0.1% of titanium dioxide for 1h in sunlight
2.	TiO_2+H_2O_2+ *hv*	Wood powder (2.0g) was treated with 0.1% of titanium dioxide with 1% $H_2O_2$for 1h in sunlight
3.	TiO_2+NaOH+*hv*	Wood powder (2.0g)was treated with 0.1% of titanium dioxide with 0.5% NaOH for 1h in sunlight
4.	TiO_2+NaOH+H_2O_2+ *hv*	Wood powder (2.0g) was treated with 0.1% of titanium dioxide with 0.5% NaOH and 1% H_2O_2 for 1h in sunlight

(h) Hydrogen Peroxide Pretreatment (Oxidative Delignification)

Hydrogen peroxide is widely used for oxidative delignification of wood. The oxidative delignification can be coupled by various other conditions to yield the better results. Generally high temperature is required for the process. During present study, energy expensive high temperature requirement was replaced by use of natural source of energy *i.e.*, sunlight of a particular summer season with the temperature between 37-42^0C (May and June). The temperature at that time varied from 37^0C to 42^0C (**http://www.accuweather.com)**. The following seven different combinations were designed during present study. The solid: liquid ratio in all combinations was put constant as per requirement to wet the complete wood powder (1:10).

S.No.	Pretreatment Method	Procedure
1	H_2O_2+*hv*	Wood powder (2.0g) was treated with 1% of Hydrogen peroxide for 4h in sunlight
2	H_2O_2+NaOH+*hv*	Wood powder (2.0g) was treated using 2% NaOH with 1% H_2O_2 for 4h in sunlight
3	H_2O_2+Iron shavings+ *hv*	Wood powder (2.0g) was treated with 1% H_2O_2 in presence of iron shavings (1g) for 4h in sunlight
4	H_2O_2+Aluminium shavings+ *hv*	Wood powder (2.0g) was treated with 1% H_2O_2 in presence of Aluminium shavings (1g) for 4h in sunlight
5	H_2O_2+Zink metal+ *hv*	Wood powder (2.0g) was treated with 1% H_2O_2 in presence of zinc metal (1g) for 4h in sunlight
6	H_2O_2+Copper shavings+ *hv*	Wood powder (2.0g) was treated with 1% H_2O_2 in presence of copper shavings (1g) for 4h in sunlight
7	H_2O_2+Al+Fe+Zn+Cu+ *hv*	Wood powder (2.0g) was treated with 1% H_2O_2 in presence of iron shavings, Aluminium shavings, zinc metal, copper shavings (each 0.25g) for 4h in sunlight

* *hv*= Sunlight of summer season (May and June)

3.11.3 Biological Pretreatments

Biological pretreatments of bamboo biomass were given using either by sour fruit extract or by lignolytic wood rot fungal isolate WDP2.

(I) Using Crude Fruit Extracts

Pretreatments of wood powder was done using three different types of sour (acidic) fruits viz. cranberry (water extract), lemon and citrus (absolute juice). After removing seeds , the aqueous fruit extracts were prepared and used to treat wood powder with (pH 2-2.5) and solid liquid ratio of 1:10 for 1 hour in each case.

(II) Fungal Pretreatment

The whole cell biological pretreatment of wood sample was done using the selected lignolytic fungal culture WDP2 following the method described by **Nazarpour *et al.*,(2013)** with slight modifications, wood powder (7.0 gm) was taken in 250 ml of erlenmayer flask and supplemented with 12 ml of distilled water to maintain 75%

moisture content. The experiments was carried out in batch cultures to establish moisture content.Three treatments were taken(T1) wood sample were subjected to only H2O(with 75% moisture content), In T2 wood suspended in half concentration of synthetic laccase production medium adjusted with 75% moisture content. In T3 the wood sample was suspended in half concentration of optimized laccase production medium adjusted with 75% moisture content. The flasks were inoculated with the active culture of WDP2 and incubated upto 48d. 0.5gm treated wood samples was suspended in 0.5 ml of autoclaved triple distilled water (pH 7.0), and used for the determination of laccase activity as described in the section 3.5.1, II (for delignification), cellulase activities as described in section 3.5.2 (for simultaneous Saccharification), reducing sugar yield as described in section 3.13 (to check the sugar loss) and simultaneous ethanol production as described in section 3.14. The treated wood samples were then dried and checked for cellulose (as described in section 3.10.4, I) and lignin content (as described in section 3.10.4, II).

3.11.4 Optimization of Alkaline Oxidative Pretreatment

Among different oxidative pretreatments using H_2O_2 with or without metal shavings, the alkaline oxidative ($NaOH+H_2O_2$) pretreatment showed most significant results and hence were further optimised using Box Behnken Design using Response Surface Methodology (RSM). But, the model was found non significant and no conclusion could be drawn. Therefore, in order to enhance delignification of biomass by this method, further experiments were carried out using different combinations of alkali and H_2O_2. Thus to find maximum delignification using minimum alkali concentration (%, w/v), a simple manual optimization experiment was designed by using three levels of solid:liquid (1:6, 1:10, 1:14) were chosen. For each experiment of these three levels, two levels of NaOH concentration (0.5%, and 2.0%) and two levels of H_2O_2 concentration (3.0%, and 1.0%) were chosen. As the hydrogen peroxide having very minute or no toxicity to the environment or it is easily degradable to water and oxygen, we selected a very high concentration of hydrogen peroxide (3%). The high concentration of hydrogen peroxide was used with low concentration of alkali and *vice versa*. The six experiments were done (**Table 3.2**) using 2 g of wood powder for each experiment. The pretreatment experiments were carried out under intense sunlight of a particular summer season (May or June) for 4h. After completion of experiment, samples were washed using triple distilled water,

dried in an oven at 60±2^{0}C and checked for delignification (lignin content) using the protocol described in section **3.10.4, II**.

Table 3.2: Manual optimization of alkali concentration for delignification

S. No.	Solid:Liquid	NaOH Concentration (%)	H_2O_2 Concentration (%)
1.	1:6	0.5	3.0
2.	1:6	2.0	1.0
3.	1:10	0.5	3.0
4.	1:10	2.0	1.0
5.	1:14	0.5	3.0
6.	1:14	2.0	1.0

UNIT IV: ENZYMATIC SACCHARIFICATION OF PRETREATED BIOMASS & MONITORING WOOD HYDROLYSIS

3.12 Optimization of Enzymatic Saccharification of Fungal Pretreated Substrate

Fungal pretreated *Bambusa balcooa* powder was dried in oven at 65 ± 2^0C, and then used for optimization of enzymatic saccharification, based on maximum reducing sugar yield (% saccharification). For optimization, a second order Box-Behnken design (BBD) was used (**Box *et al.*, 1978**). The crude cellulase enzyme complex from the fungal isolate PCF1 was used for saccharification studies.

3.12.1 Experimental Design

The enzymatic saccharification of biologically (lignolytic fungal) pretreated *Bambusa balcooa* wood powder was performed and analyzed using 17 experiments in Design-Expert® Software Version 10. Second order model was fitted in each response using multiple regression analysis. The significance of saccharification efficiency or percent saccharification (Y) was studied by considering three variables: enzyme loading (FPU g^{-1} substrate) X1, substrate concentration (% w/v) X2, and temperature (^{0}C) X3. Thus, the experiments were comprised of three independent variables at three levels. The coded and uncoded (actual) levels of the variables are given in **Table 3.3**, and the design of experiments of Box-Behnken is described in **Table 3.4**, and the experimental runs and combinations are given in **Table 3.5**.

Table 3.3: Coded and actual (uncoded) levels of the independent variables for design of experiments for enzymatic saccharification

Independent variable		**Coded levels**		
Name	**Code**	**-1**	**0**	**1**
		Actual levels		
Enzyme concentration (FPUg^{-1} substrate)	A	15	20	25
Substrate concentration (%)	B	1.0	2.5	4.0
Temperature (^{0}C)	C	30	40	50

Table 3.4: Box-Behanken design for three variables at three levels each for enzymatic saccharification

Coded variables			No. of combinations	No. of experiments
A	B	C		
0	0	0	1	8
±1	±1	±1	3	3
±1	0	0	2	2
0	±1	0	2	2
0	0	±1	2	2

Table 3.5: Box-Behnken design for enzymatic saccharification

Exp. No.	Coded variables			Uncoded Variables		
	A	B	C	Enzyme concentration ($FPUg^{-1}$ substrate)	Substrate concentration (%)	Temperature (^0C)
1	-1	-1	0	15	1	40
2	1	-1	0	25	1	40
3	-1	1	0	15	4	40
4	1	1	0	25	4	40
5	-1	0	-1	15	2.5	30
6	1	0	-1	25	2.5	30
7	-1	0	1	15	2.5	50
8	1	0	1	25	2.5	50
9	0	-1	-1	20	1	30
10	0	1	-1	20	4	30
11	0	-1	1	20	1	50
12	0	1	1	20	4	50
13	0	0	0	20	2.5	40
14	0	0	0	20	2.5	40
15	0	0	0	20	2.5	40
16	0	0	0	20	2.5	40
17	0	0	0	20	2.5	40

3.12.2 Statistical Analysis

The FINV function was used to calculate the F_{tab} values. The F distribution can be used in an F-test that compares the degree of variability in two data sets. p is a probability with $0 < p < 1$ and df1 >= 1 and df2 >= 1 are the numbers of degrees of freedom for the numerator and denominator of the ratio that is assumed to have an F-distribution. Because df1 and df2 are integers, Excel truncates (or rounds down) the value to an integer value if a non-integer value is used for either df1 or df2. The FINV (p, df1, df2) function is the inverse function for FDIST (x, df1, df2). For any particular x, FDIST(x, df1, df2) returns the probability that an F-distributed random variable with df1 and df2 degrees of freedom is greater than or equal to x. The FINV (p, df1, df2) function returns the value of x where FDIST(x, df1, df2) returns p. Therefore, FINV is evaluated by a search process that returns the appropriate value of x by evaluating FDIST for various candidate values of x until it finds a value of x where FDIST(x, df1, df2) is "acceptably close" to p.

3.12.3 Calculation of Optimum Values of Parameters for Saccharification Efficiency

The coded independent variable were A, B, C for cellulase enzyme (FPU/g of substrate), substrate concentration (%, w/v), and temperature (^{0}C). The actual values after optimization experiments were calculated using following formulas:

$$\text{For A: } -0.332 \quad \frac{\text{Ao} - 20}{5}$$

$$\text{For B: } -1 \quad \frac{\text{Ao} - 2.5}{1.5}$$

$$\text{For C: } -0.051 \quad \frac{\text{Ao} - 40}{10}$$

3.13 Determination of Reducing Sugar Yield and Saccharification Efficiency

The mount of reducing sugar liberated in the reaction mixture was measured by Dinitrosalicylic (DNS) acid method (**Miller, 1959**). The hydrolysate samples were appropriately diluted and final volume made up to 1.5 ml in test tube. The DNS reagent (3.0 ml) was added to each sample and test-tubes were boiled in water bath for 5 min. After cooling the tubes to room temperature, the absorbance was measured at 540 nm and released sugar was estimated by using standard curve (**Appendix 1**). After calculating the

amount of reducing sugar in hydrolysate, saccharification efficiency was determined by using the following formula:

$$\text{Saccharification Efficiency } \% \quad \frac{\text{reducing sugar mg/ml} * 0.9 * 100}{\text{initial substrate mg/ml}}$$

3.14 Estimation of Alcohol

Alcohol was estimated colorometrically according to **Pinyou *et al.*** (**2011**), with slight modifications. A standard curve was prepared by using 1.0 to 10.0 mg/ml of absolute alcohol to which 2 ml of standardized 0.36 N cerric ammonium nitrate reagent was added. After proper mixing for 5 min, the extinction was read at 486nm on an UV Vis spectrophotometer against the distilled water blank. The preparation of standard curve of ethanol described in the given table:

Test Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Blank**
Alcohol stock solution (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	-
Distilled water (ml)	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	-	0.5
Reagent (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Concentration (mg/ml)	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	-

5 ml of the supernatant sample was appropriately diluted in order to get colour in range of standard curve. Five ml of diluted sample was taken and 2 ml of cerric ammonium nitrate was added, and after 5 min extinction was read at 486 nm. The amount of ethanol (mg/ml) was determined by computing the absorbance against a standard curve of the absolute ethanol.

3.15 Biophysical Techniques Used for Monitoring Wood Hydrolysis

3.15.1 Scanning Electron Microscopic (SEM) Studies

Untreated, pretreated by best physical, best chemical and biological method, and pretreated saccharified biomass of *B. balcooa* were analysed by scanning electron microscope for structural changes. The samples washed with deionized water, and

dehydrated in a grade series of ethanol solutions (20%, 50%, 70%, 95%, and 100%). The dried samples were then mounted on stubs and sputter coated with gold and examined under 100KV JEOL 6600 LV electron microscope at different magnifications.

3.15.2 Spectral Analysis Using UV-Vis Spectrophotometry

For UV-Vis spectral analysis, the untreated, and pretreated wood samples were scanned at 200-700nm using UV-Vis spectrophotometer (Thermo) and the degradation product study was observed as the disturbance in peaks as compared by control.

3.15.3 Fourier Transmission Infrared (FTIR) Spectroscopic Studies

For FTIR spectra analysis, the samples (untreated and pretreated) and potassium bromide (KBr) were dried overnight at 60^0C in an oven to remove the moisture. The chemical structural changes in the pretreated samples were analysed using FTIR spectrometer at the wavelength range of 400-4000 cm^{-1}.

UNIT V: IDENTIFICATION AND CHARACTERIZATION OF LIGNOLYTIC FUNGAL CULTURES

3.16 Polyphasic Approach

A polyphasic approach was used to identify selected lignocellulolytic fungal isolates. Four potential lignolytic fungal isolates were characterized and identified using phenotypic (morphological and biochemical) and molecular characteristics.

3.16.1 Morphological Characterization

The fungal cultures were evaluated for various cultural (colonial) characters such as colour, margin, surface, and texture of colony. The wet (live) mycellial preparations were prepared using cover slip, and were examined using a phase contrast microscope under 10x, 20x, 40x, and 100x objective lenses for various mycellial characteristics and arrangement of the spores if present.

3.16.2 Biochemical Characterization

The selected fungal isolates were tested for sugar utilization using Hi Carbohydrate test kits (HiMedia). The test was based on the principle of the colour change of medium on substrate utilization. The fungal mycelial suspension in sterile distilled water was prepared and used as inoculum. The sugar wells of the kit were inoculated by this mycellial suspension (100µl) of test organism and incubated at 28 ± 1^0C, for 3-5 d. The kits were observed for the colour changes. Red colour of the medium was changes to yellow, if organism produced the acid during carbon assimilation. Thus using this experiment, the cultures were tested for their efficiency to utilize various monosaccharides, disaccharides, oligosaccharides and sugar alcohols, and organic carbon compounds. The selected cultures were further evaluated for extracellular protein profiling using SDS and Native PAGE as mentioned in section 3.9.

3.16.3 Molecular Characterization

Only two potential fungal isolates (WDP2 and WFPn1) were characterised on the basis of 5.8S ITS rRNA gene sequence analysis. The pure cultures of potential lignin degraders were sent for identification based on sequence analysis of the PCR product of 5.8S ITS rRNA gene sequence to Yaazh Xenomics Chenni, India. Fungal genomic DNA for molecular identification was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit. The PCR amplification of 5.8S ITS r-RNA gene was carried out using universal forward primer ITS1 (5' TCCGTAGGTGAACCTGCGG3'), and ITS4 (5' TCCTCCGCTTATTGATATGC3'). The PCR amplification was carried out under following conditions:

PCR Conditions

1.	Initial denaturation	**:**	94^0C for 2 min
2.	Denaturation	**:**	94^0C for 45 sec
3.	Anneling	**:**	55^0C for 60 sec
4.	Extension	**:**	72^0C for 60 sec
5.	Final Extension	**:**	72^0C for 10 min
	Total Cycles		35

Unincorporated PCR primers and dNTPs from PCR products were removed using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the ITS1/ITS4 primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using below 18s rRNA ITS universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The

samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

3.16.4 *In silico* Studies

The 5.8S ITS r-RNA gene sequence was analysed using nucleotide Basic Local Alignment Search Tool (BLAST N) using National Centre for Boitechnology Information (NCBI) database (**http://blast.ncbi.nlm.nih.gov/**) was used for homology search with other 5.8S ITS r-RNA gene sequences available in the database. Program MUSCLE 3.7 was used for multiple alignments of sequences (**Edgar, 2004**). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) **(Talavera and Castresana 2007)**. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. The program Tree Dyn 198.3 was used for tree rendering (**Dereeper *et al*., 2008**).

3.17 Statistical Analysis

All the experiments were conducted in triplicates and data were scored in Microsoft Excel. Data were analysed by two way ANOVA (Analysis of Variance) using the software programme stpr. The statistical significance level (p-value) was set at 0.05 and the results were reported in terms of critical difference (CD).

3.18 Materials Used

All the chemicals and reagents used in the study were of analytical grade and purchased from standard manufacturers as HiMedia Mumbai, Sisco Research Lab. Pvt. Ltd., Mumbai, Merck India Ltd., Mumbai, and Banglore Genei, Banglore, etc. All the glassware and plasticware were supplied from Borosil, Tarsons, Schoot Duran, Himedia, Greiner and Axygen etc.

Table 3.6: Instrument Used in the Present Study

S.No.	Instrument		Manufacturer
1	-20^0C/-80^0C deep freezers	:	Vest frost
2	Autoclave	:	Sanco
3	Electronic balance	:	Adair Dutt Instruments Pvt. Ltd.
4	Hot air oven	:	Sanco
5	Gel Documentation system	:	BioRed
6	Electrophoresis Units	:	Tarsons, Technoconcept
7	Incubator	:	Sanco
8	Incubator Shaker	:	Remi, Orbitek
9	Leminar Air Flow Bench	:	Sanco, Adair Dutt Instruments Pvt. Ltd.
10	Ice flaker mechine	:	Harrison Scientific Instruments
11	Microscopes	:	Olympus
12	Microwave oven	:	Bajaj
13	pH meter	:	Genei
14	Centrifuge, Refrigerated	:	Remi/Sigma
15	Refrigerator	:	LG, Samsung
16	UV-Visible Spectrophotometer	:	Thermo, Biomate
17	Water Bath	:	Sanco
18	Spin win	:	Remi
19	Distillation Unit	:	Borosil
20	Vortex	:	Merck

Results and Discussion

Chapter 4 RESULTS AND DISCUSSION

Increasing demand and rapid depletion of fossil fuel are forcing the global scientific community to focus an intensive research on alternative renewable energy sources. Bio-ethanol, the clean and eco-friendly green fuel, can be accepted either as such or with blending for different energy purposes worldwide. Production of bio-ethanol from renewable lignocellulosic plant biomass using microbial enzyme technology is one of the greatest challenges for bio-fuel industries (**Zhao *et al.*, 2007**). However, recalcitrance of the structural components of the lignocellulosic plant biomass makes its hydrolysis more difficult. A wide range of physical, physicochemical, and chemical and biological pretreatments have been developed and studied to remove or reduce the recalcitrant component (lignin) and crystalinity of the crystalline cellulose (**Sun and Cheng, 2008; da Costa Sousa *et al.*, 2009; Chundawat *et al.*, 2011**). Although, all the processes are having various challenges to complete such as proper technology and cost effectiveness during the pretreatment of feedstock (**Sun and Cheng, 2002**). Different combinations of the alkaline pretreatment, and the biological pretreatment methods are now being in focus of many workers and are generally more effective (**Chang and Holtzapple, 2000; Dashtban *et al.*, 2010**). Among various pretreatments, biological pretreatment seems to be more promising and eco-friendly process, and moreover no inhibitor generation occurs during the process. In the current scenario there, are few limitations in using this strategy for pilot scale process (**Sindhu *et al.*, 2016**).

Lignin forms an integral part of secondary walls in plants and is thus tough to degrade. In biological pretreatment process, mainly the white rot fungus helps in delignification of the substrate with the help of lignin peroxidase and laccase enzymes, which in turn improve the enzymatic saccharification rate (**Sindhu *et al.*, 2016**). The proper utilization of these enzyme systems for enzymatic process development requires a complete identification and characterization of a highly stable enzyme, that can be able to perform at extreme pH values and wide temperature ranges. Therefore, present study was carried out to isolate, identify and characterize the potential lignolytic microorganism capable of producing high concentration of stable, extracellular laccase enzyme system, that can be utilize for sustainable biological pretreatment of the potential lignocellulosic biomass.

UNIT I: SELECTION OF LIGNOCELLULOLYTIC CULTURES

4.1 Qualitative Selection of Potential Lignocellulolytic Microbial Gene Pool Based on Relative Enzyme Activity Indices

Production of extracellular enzyme using a potential microbial strain is the first step of any industrial process that requires the enzyme. Therefore identification of new indigenous microbial strains and characterization of their lignolytic enzymes is of immense importance for their improved biotechnological applications (**Eggert *et al.*, 1996**). In view of above, various rotten wood samples and fruiting bodies growing on various forest trees were collected from different sites during rainy season and used for the isolation and *in vitro* cultivation of ligninases enzymes producing microorganisms during present investigation. Detailed description of the bacterial and fungal cultures isolated from different sources is given in **Table 3.1**. A total of forty one bacterial cultures and seventy three fungal cultures including both mushrooms and non mushroom fungi were isolated on nutrient agar and potato dextrose agar (PDA) respectively. The isolated cultures were preserved on nutrient agar and potato dextrose agar plates/slants at 4^0C at refrigerator till further use. Mushrooms are well characterised by type of fruiting bodies. Some of the selected fruiting bodies used for the isolation of wood rotting fungi are shown in **Plate 4.1**. The colonial growth pattern on potato dextrose agar plate of some of the fungal isolates and photomicrographs depicting mycellial structures of some of the fungal isolates are shown in **Plate 4.2**. Many previous studies have reported the involvement of several lignin degrading enzyme systems in lignin biodegradation and mushrooms have been well characterised for this purpose. A clear-cut correlation between the activities of different types of peroxidases and laccases in lignin biodegradation has also been well documented, that has been well studied in case of various mushrooms such as *Agaricus bisporous*, *Strobilurus ohshimae* and others (**Kalyani *et al.*, 2013**).

A. Selection for Lignolytic Potential:

Basically three types of lignin degrading enzyme activities such as lignin peroxidase (LiP), laccase (lac), manganese dependent peroxidase (MnP), are involved in lignin degradation; besides these some of the other versatile peroxidases and other polyphenol oxidases are also associated with lignin biodegradation. But all these types of lignolytic enzymes may or may not be produced by an individual fungal culture. Wood

rotting fungi, among various microorganisms have been reported as most potential lignin degraders. While bacteria are known to have very little or no lignolytic activity. Considering these facts all the 114 microbial (fungal and bacterial) cultures during the present study were first screened qualitatively using agar plate assay for differentiating lignolytic and non-lignolytic strains. The cultures were first evaluated for their overall lignin modifying ability using mineral salt medium (MSS) containing glucose as the carbon source and tannic acid as the indicator compound. None of the bacterial culture showed any type of lignolytic enzyme activity during the plate assay. In previous studies also, several bacteria isolated from soil decaying wood samples were also not found to have lignin degrading potential. While a very few bacterial species are currently known to synthesize extracellular lignin degrading laccase and peroxidases (**Woo *et al*., 2014**). Most of the previous reports have been studied the lignin degrading enzyme systems from mushroom fungus. **Homma *et al.* (2007)** have reported *Strobilurus ohshimae*, (an edible mushroom), with high lignin degrading activity. They checked the enzyme activity directly using degradation of the native lignin in 18 strains. The correlation between the activities of peroxidase and laccase and the degradation of lignin in *A. bisporus* was studied by **Bonnen *et al*. (1994)**, & they suggested significant roles for these two enzymes in lignin degradation by this fungus. Thus mushrooms have been well characterised for their high lignolytic activities (**D'souza *et al*., 1999; Fen *et al*., 2014**), while only few bacterial species (*e.g. Pseudomonas*, *Cellulomonas*, and few Actinomycetales) are known to produce extracellular lignin degrading enzymes (**Woo *et al*., 2014**). There is a rare property of the bacterial system as observed by us during present investigation.

Among 73 test fungal cultures, only nine wood rotting fungal and the standard culture of *Phanerochaete chrysosporium* showed brown zone formation around the colony indicating their lignin degrading/ modifying potential (**Plate 4.3 a, b**). Tannic acid was found a very good indicator for ligninases enzymes production and it also act as selective agent to check the organisms' overall lignin modifying ability (**Mahalingam and Maruthamalai, 2014**). Moreover, tannic acid that is used as an indicator for lignin degrading enzyme activities, is hydrolyzed tannin; and the tannins are the recalcitrant polyphenolic compounds which are found abundantly in vascular plants, hence it acts as good substrate for detecting the desired activity. The reason for the brown zone formation around the colony may be formation of some oxidised brown coloured compound from

tannic acid through ligninase activity (**Sliva *et al*., 2010**). Thus among all the test microbial cultures, only nine fungal isolates and standard culture of *Phanerochaete chrysosporium* showed positive results for overall lignin modifying activity, laccase activity shown by only six fungal isolates but not by the standard culture of *Phanerochaete chrysosporium* (**Plate 4.3 a, b, c, d**). The standard culture of *Phanerochaete chrysosporium* showed lignin peroxidase activity but not the laccase activity. Among the lignolytic isolates, only two isolates (WDP2 and WFPn1) showed lignin peroxidase activity (**Plate 4.3 e and f**). Moreover, same fungal isolates (WDP2 and WFPn1) along with standard culture of *Phanerochaete chrysosporium* were found positive for lignin peroxidase production when qualitatively screened using azure B agar assay (**Plate 4.3 g and h**). The fungal isolates WFPn1 and WDP2 were found positive for lignin peroxidase enzyme production. Thus it is evidence from our results that lignin degrading/modifying activity is not very prominent even among wood rot fungi. Out of 73 fungal isolates, only 10 were capable of showing overall lignin modifying activity (**Table 4.1**). Rest of the 63 fungal cultures along with all the bacterial cultures were characterised as non-lignolytic and were not used for further studies.

The relative enzyme activity indices for all the three lignolytic enzymes (Overall lignin modifying, laccase and lignin peroxidase) were calculated based on the diameter of colony and coloured zone formed around the colony. In each case the values of relative enzyme activity indices of overall lignin modifying enzymes for various cultures (I_{lig}) varied from 1.06-2.0 for various cultures (**Table 4.2**).The minimum I_{lig} value was recorded for the standard culture of *Phanerochaete chrysosporium*, while all the isolates except for WDP2 (1.07), DWFP1 (1.08), and TAFP41 (1.10) showed significantly higher values for same. Maximum I_{lig} value (2.0) was recorded for the isolate CAFP7, the value being significantly higher than that recorded for all the other cultures. Selection of lignolytic fungal isolate for the laccase activity was done using guaiacol as a substrate. The three fungal cultures (WDP1, DWFP1, and BAFP1) did not show any coloured zone formation around their colonial growth indicating that these strains were laccase negative. The values of laccase enzyme activity indices (I_{lac}) for various cultures varied from 1.0 to 3.0 (**Table 4.2**). It is apparent from the data that significantly lower value for I_{lac} (1.0) was recorded for the standard culture of *Phanerochaete chrysosporium* as compared to rest of laccase positive isolates. Maximum I_{lac} value (3.0) was recorded for the isolate AAFP5, the value being significantly higher than rest of the cultures. It is apparent from these results that all

the selected (six) fungal isolates showed a very high laccase enzyme activity when qualitatively compared to the standard culture of *P. chrysosporium*. Different workers have used different substrates for detecting lignolytic activity but in most of the cases guaiacol and tannic acid are used as preferable chromogenic substrate as observed by us during present investigation (**Desai *et al*., 2011; Ueda *et al*., 2012**).

Guaiacol is used as a sole carbon source for selective laccase production by the microbial system and metabolic oxidation of this chromogenic substrate during growth may be corrylated with dark brick red brown colour formation around the colony throughout laccase activity (**Gao *et al*., 2011**). For qualitative detection of peroxidase enzyme, ABTS plus H_2O_2 were used as substrate-indicator; formation of green zone around the colony on glucose malt extract salt agar medium was considered as positive test (**Thiyagarajan *et al*., 2008**). During present investigation, the lignin peroxidase activity was found very rare among the various cultures as only two fungal isolates (WFPn1 and WDP2) and the standard culture of *Phanerochaete chrysosporium* could showed lignin peroxidase activity while remaining seven cultures did not showed any green zone formation indicating no enzyme production. Maximum value for relative lignin peroxidase enzyme activity index (I_{per}), was recorded for the isolate WFPn1 (2.5) followed by the isolate WDP2 (1.6), the values being significantly higher than that recorded for standard culture of *Phanerochaete chrysosporium* (1.2). Decolorization of the dye azure-B dye also given the same results by fungi. This decolourization of azure B positively correlated with production of lignin peroxidase and Mn dependent peroxidase by **Pointing, 1999**. Thus, based on relative enzyme activity indices of all the three lignin degrading enzymes (I_{lig}, I_{lac}, I_{per}), only five fungal isolates (WFPn1, WDP2, AAFP5, CAFP7, TAFP41) were selected for quantification of lignin degrading laccase enzyme and compared for their activities with the standard culture of *Phanerochaete chrysosporium* (**Figure 4.1**). Thus it appears from the results that qualitative tests, that were used as preliminary screening of the cultures for selection of lignolytic isolates, are having useful and rapid methods for identifying potential isolates. Thus selection, handling and development of active wood rot fungal cultures for pretreatment of biomass, development of cost effective fermentation media, and control over the entire process are the basic factors that contribute significantly to achieve the high level of extracellular accumulation or secretion of such enzymes for a proper delignification process development.

Table 4.1: Selection and diversification of indigenous wood rotting fungal isolates for lignolytic potential

Site/source of isolates	Total no. of isolates	No. of non lignolytic isolates	No. of lignolytic isolates	No. of isolates showing diverse lignin degrading enzyme activities			Selected lignolytic isolates
				Overall lignin modifying activity*	Laccase activity**	Lignin peroxidase activity***	
Pithoragarh/fruiting bodies from decaying tree wood samples	09	09	-	-	-	-	-
AFRC, Pantnagar/ fruiting bodies from decaying tree wood samples	39	31	08	08	07	02	Eight isolates: WFPn1, WDP1, WDP2, BAFP1, BAFP3, AAFP5, CAFP7, TAFP41
Pantnagar/ decaying door wood sample	12	11	01	01	-	-	One isolate: DWFP1
Departmental Culture Collection	12	12	-	-	-	-	-
MTCC, IM Tech, Chandigarh,Standard Culture	01	-	01	01		01	*Phanerochaete chrysosporium*
Pantnagar/Bacterial Cultures from Decaying wood sample	41	41	0	0	0	0	-
Total no. of isolates	**114**	**104**	**10**	**10**	**07**	**03**	**10**

* For overall lignin modifying activity, the fungal cultures were grown in LME basal agar media containing 1% (w/v) aqueous tannic acid solution at 28±1^{0}C for 10d.

**For laccase activity determination, the fungal cultures were grown in PDA containing 0.01% (v/v) guaiacol at 28±1^{0}C for 10d.

***For lignin peroxidase activity determination, the fungal cultures were grown in glucose malt extract agar medium at 28±1^{0}C for 10d.



(a)



(b)



(c)



(d)

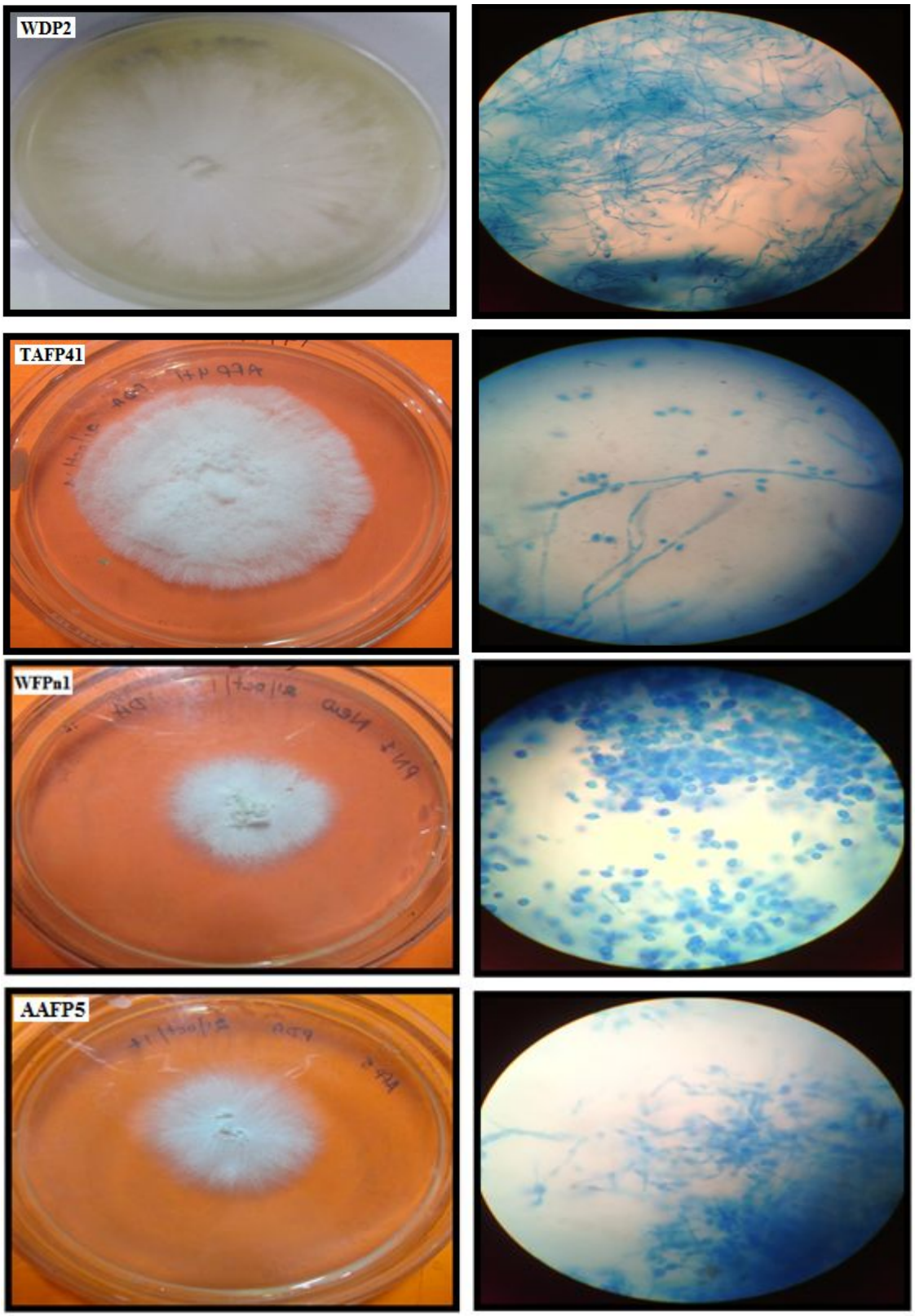


(e)



(f)

Plate 4.1: Some selected mushroom fruiting bodies used for isolation of wood rotting fungi (a-f).



(a) **(b)**

Plate 4.2: Colonial growth pattern (*In vitro*) on PDA (a), and photomicrographs depicting mycellial structure (b) of selected wood rotting fungal isolates.

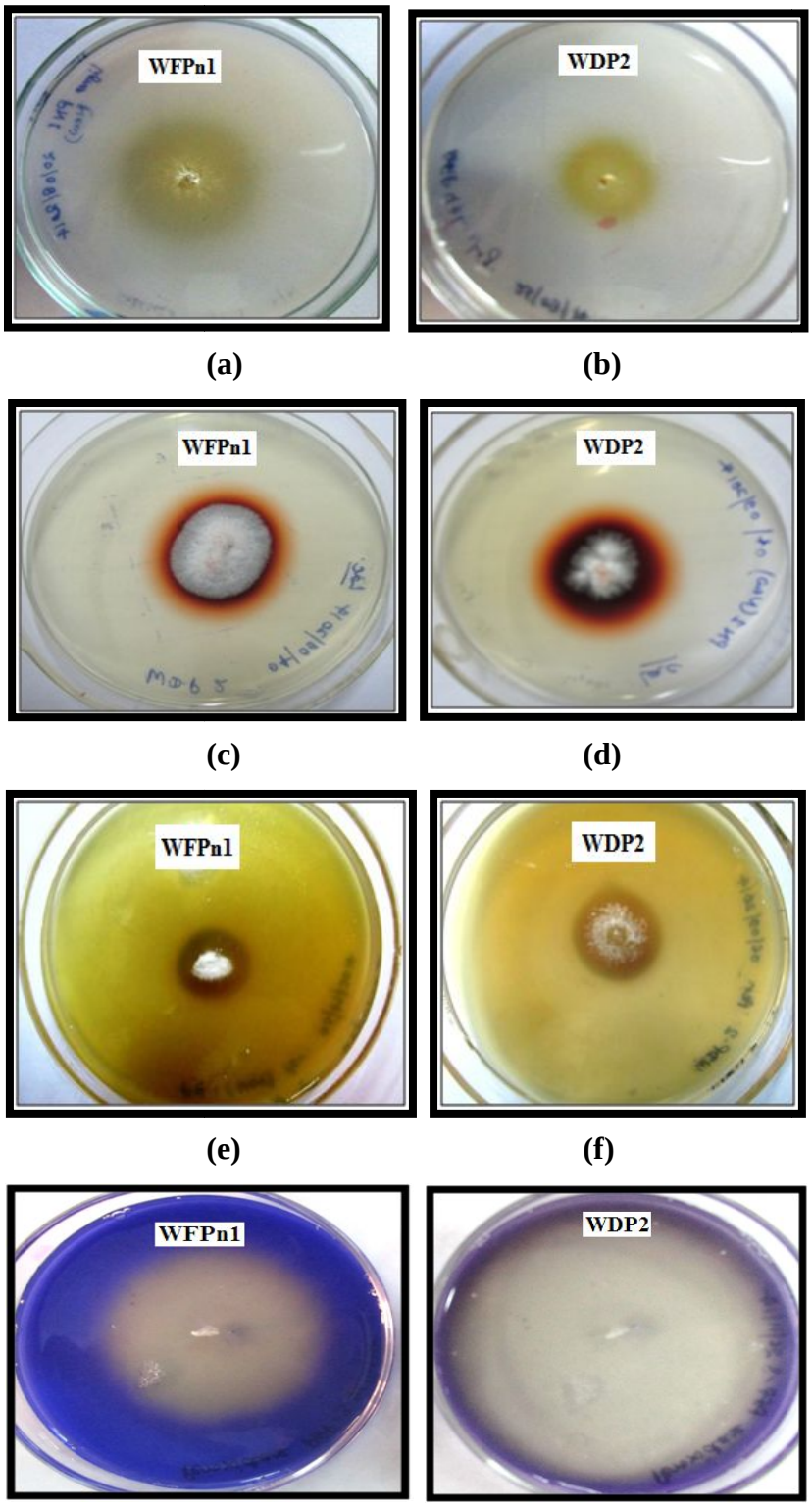


Plate 4.3: Qualitative detection for overall lignin modifying enzyme activity (a, b); laccase enzyme activity (c, d); and lignin peroxidase enzyme activity using ABTS+H_2O_2 (e, f) and using azure B assay (g, h) of two wood rotting fungal isolates WFPn1 & WDP2 respectively.

Table 4.2: Relative extracellular overall lignin modifying enzyme activity (I_{lig}); laccase enzyme activity (I_{lac}); and lignin peroxidase enzyme activity (I_{per}) indices of selected wood rot fungal cultures* as a qualitative measure of lignin degrading enzyme production

Cultures	Relative enzyme activity indices (I) for		
	Overall lignin modifying enzymes (I_{lig})*	Laccase enzyme (I_{lac})*	Lignin Peroxidase activity (I_{per})**
WFPn1	1.59	2.44	2.5
WDP1	1.16	-	-
WDP2	1.07	2.3	1.6
DWFP1	1.08	-	-
BAFP1	1.46	-	-
BAFP3	1.40	1.36	-
AAFP5	1.81	3.00	-
CAFP7	2.00	1.66	-
TAFP41	1.10	2.7	-
P. chrysosporium	1.06	1.0	1.2
S.Em ±	0.019	0.041	0.018
CD (5%)	0.056	0.12	0.073

* The cultures were grown on respective media containing tannic acid (for overall lignin modifying enzyme activity); and guaiacol (for laccase activity) as substrate at 28±2^{0}C.

**For detecting peroxidase activity, ABTS plus H_2O_2 solution was overlapped on 3d fungal growth at 28±2^{0}C on respective medium.

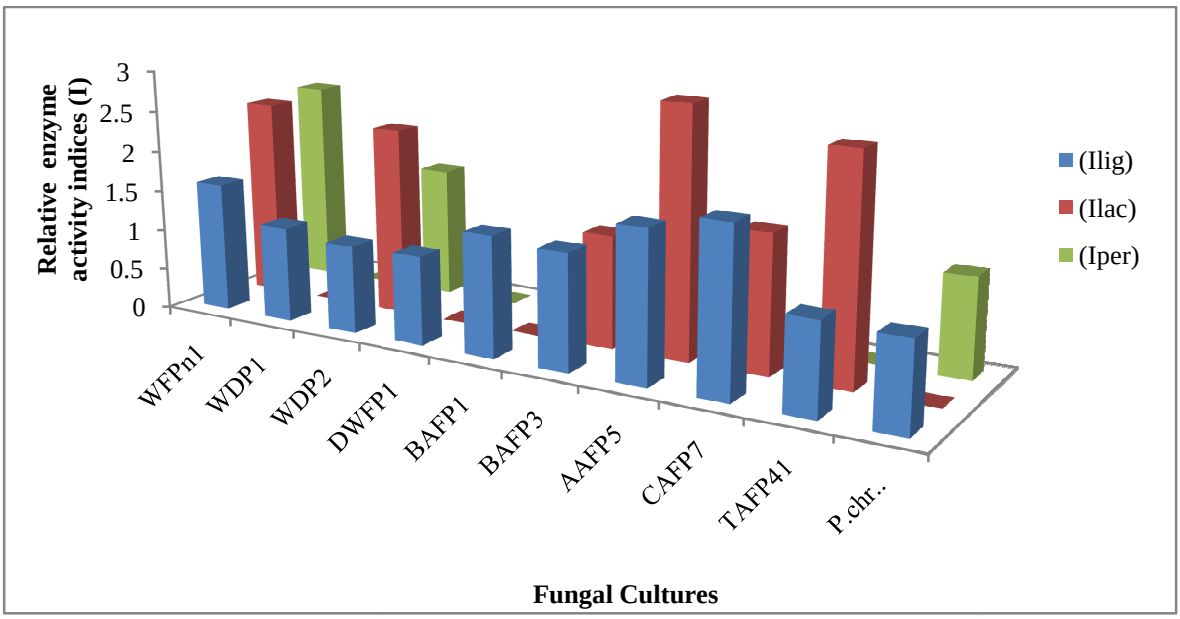


Fig 4.1: Relative enzyme activity indices of the selected lignolytic fungal cultures for the three lignin degrading enzymes: overall lignin modifying enzyme (I_{lig}), laccase enzyme (I_{lac}) and lignin peroxidase enzyme (I_{per}).

B: Selection for Cellulolytic Potential

After proper pretreatment of potential lignocellulosic biomaterial, saccharification of exposed polysaccharides to fermentable sugars by microbial cellulase enzyme has attracted an attention to researchers, as this is the second most important step in bioconversion of organic material into valuable products such as reducing sugars and then finally to biofuels (**El-Shishtawy *et al*., 2015**). Thus for performing the saccharification part, all the selected lignolytic and other remaining fungal/ bacterial strains were tested qualitatively for cellulase enzyme production. It is very important to have a minimum or no cellulase activity in the strain that is used only for delignification, or otherwise the strain should have both ligninase and cellulase activities to be used for simultaneous delignification and saccharification process, or, if the strain used only for delignification experiment, there is a need of a potential cellulase producer with the high cellulase enzyme activity for separate saccharification experiment. Keeping these facts in mind, all fungal and bacterial cultures were tested qualitatively by plate assay using CMC agar medium. No bacterial culture was observed to produce the halo-zone around their colony. A halo zone appeared only around the colonies of seventeen wood rot and non wood rot fungal isolates (**Plate 4.4; Fig 4.2**). Since carboxymethyl cellulose was only the carbon source in the medium, the results strongly evident the cellulase production in order to degrade cellulose. CMC is used for qualitative screening of cellulase enzyme production, but it is mainly reported to used as substrate for determining CMCase or endoglucanase and β-glucosidase activity of particular microbial strain (**Atri and Sharma, 2012**). The plate assay use CMC as substrate is a good indicator especially for cellulolytic ability of various fungi since CMCase or endoglucanase is generally produced in larger amounts than the cellobiohydrolase enzyme by most of the fungi (**Cai *et al*., 1994; Atri and Sharma, 2011**). The complex formation between the dye (congo red) and cellulose or cellulose derivative (CMC) is a very common approach for the detection of cellulose degradation ability of microbial cultures. The degradation of cellulose by cellulase enzyme led to the removal of unbound dye during the washing with NaCl solution on CMC agar plate showing colourless zone around the colony (**Rautela and Cowling, 1966; Pointing, 1999)**. The cellulase production was calculated as the ratio of diameter of CMC degradation halo-zones and their respective colony diameter. Most of the fungal isolates did not show any significant difference in their I_{CMC} index. The relative cellulase enzyme activity index

(I_{CMC}) values varied from 1.11 to 2.9 for various fungal cultures (**Fig 4.2**). The lowest I_{CMC} value (1.111) was observed for the fungal isolate CAFP7 while, maximum I_{CMC} value (2.9) was recorded for the non wood rot fungal isolate PCF1 that was significantly higher than the I_{CMC} values obtained for other fungal isolates. It was followed by the potential wood rot fungal isolate AAFP5 (2.4) and the standard culture of *Aspergillus niger* 282 (1.63). Thus two fungal isolates *viz*. PCF1, and AAFP5 showed significantly higher relative cellulase enzyme activity indices then that of the standard culture of *A. niger*. The I_{CMC} values of 1.511 and 1.2 were obtained for other potential wood rot fungal isolates WDP2 and TAFP41 respectively, followed by the minimum I_{CMC} value (1.13) was recorded for wood rot fungal isolate WFPn1 (**Fig 4.2**). Out of seventeen cellulase producers, six wood rot and other fungal isolates with standard culture of *A. niger* were selected for further quantification of the enzyme.

Many previous workers have considered and reported the relative enzyme activity index for selection of cellulolytic bacteria based on halo zone formation around the colony for selection (**Cavaco-Paulo and Gübitz, 2003; Rawat and Tewari, 1012; Saini and Tewari, 2012**). Moreover, various other workers have also used the same congo red method for qualitative selection of fungal and bacterial strains for cellulase enzyme production (**Pečiulytė, 2007; Khokhar *et al*., 2012**). Thus, congo red dye clearly be used for screening of cellulase producing microorganisms. Mostly, the cellulase detection procedure using congo red looks qualitative, but the procedure gives the relative extracellular cellulase enzyme activity indices (estimate of zone diameter of hydrolysis of CMC) that truly correspond to the findings obtained from the quantitative estimation of the cellulase enzyme complex from microbial cultures (**Sazci *et al*., 1986**). The fungi belonging to the genus *Trichoderma* sp. and *Penicillium* sp. followed by *Aspergillus* sp. have also have also been reported as potential cellulase producer (**Cavaco-Paulo and Gübitz, 2003** and **Khokhar *et al*., 2012**).

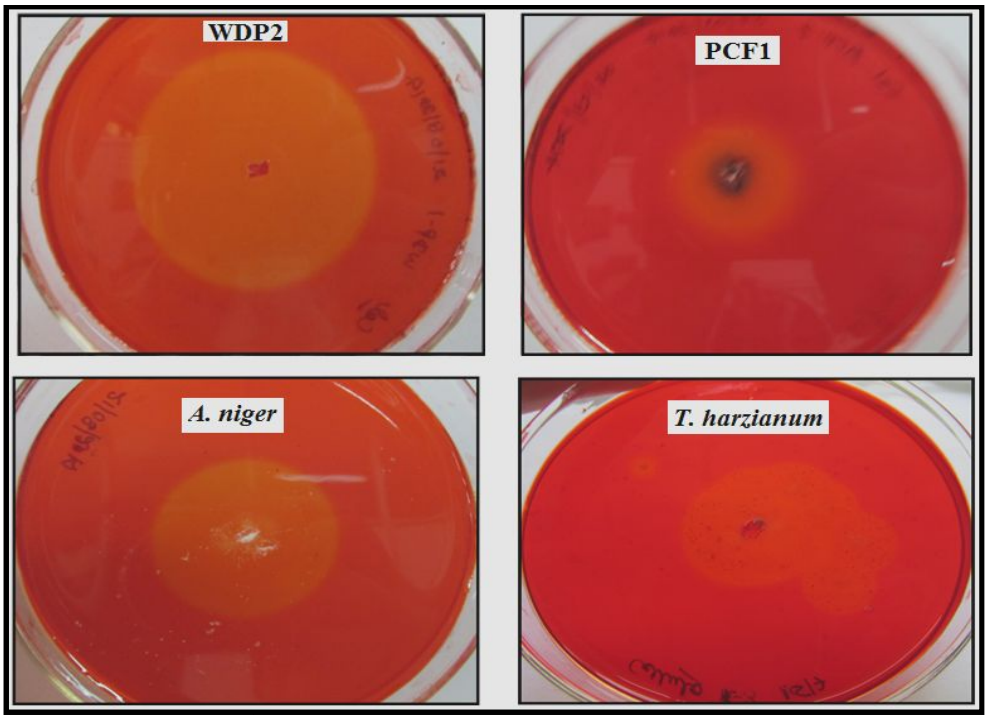


Plate 4.4: Qualitative detection of cellulase enzyme production by some fungal isolates using CMC as sole carbon source

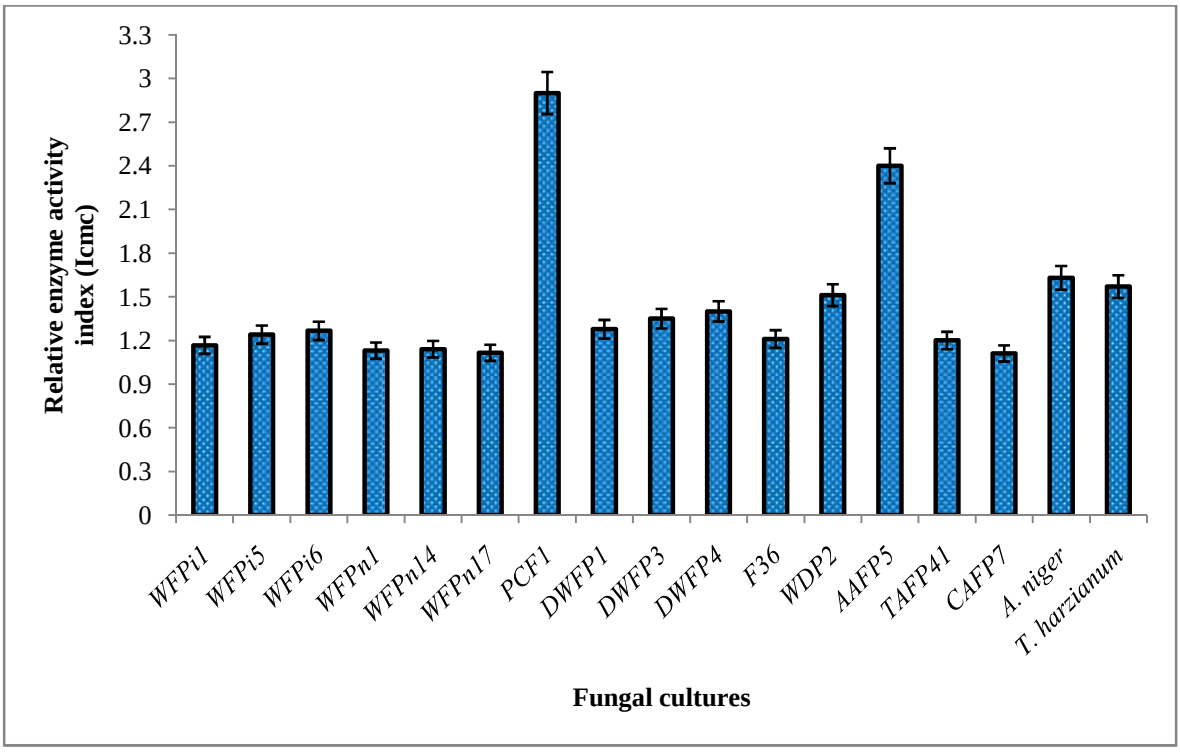


Fig 4.2: Relative cellulase enzyme activity indices (I_{cel}) of the selected lignolytic and non lignolytic fungal cultures

4.2 Efficacy of Laccase Producing Cultures for Synthetic Dye Removal/ Decolourization

The lignin-degrading enzymes have been reported to be not only directly involved in the degradation of lignin from their natural lignocellulosic substrates but also in the degradation of various synthetic xenobiotic compounds, including synthetic dyes (**Okino *et al*., 2000**). Although these dyes or coloured cyclic organic compounds generally impart only a minor fraction in wastewater, but their colour and carcinogenic properties renders them aesthetically unacceptable.

A. Preliminary Selection

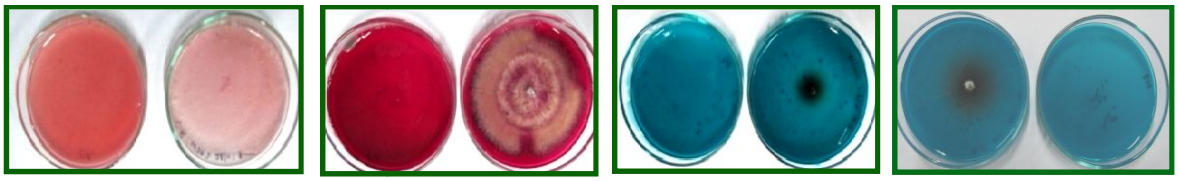
The preliminary study was conducted to check the efficacy of selected lignolytic fungal cultures for dye decolourisation capacity by agar plate assay using low nitrogen basal medium amended with synthetic dye (congo red, crystal violet, fuchsin basic, malachite green and brilliant green, @100 mg/l). All the test cultures were unable to grow on crystal violet, but they showed growth and slight decolourisation of the three test dyes congo red, malachite green, and brilliant green dyes while only one isolate WFPn1 was observed to grow on the agar plate containing the dye fuchsin basic during the plate assay (**Plate 4.5 I; a, b, c, d**). The similar agar plate screening method for determining dye decolourizing potential of wood rot fungi *Ganoderma* sp. has also been performed in earlier studies (**Arulmani *et al*., 2005**). The dye decolourising capability might be correlated with the production of lignolytic enzymes and it can be used as a confirmatory test for dye decolourization. All the five cultures were further checked for their *in vitro* dye removing potential in dye containing liquid media. It is evident from the results that during the broth culture experiments, the dye containing liquid media turned from an initial deep coloration to a lighter colour, eventually becoming colourless in most of the cases (**Plate 4.5 II; e, f, g**). In some cases the bio-sorbed dye was accumulated within the fungal structures as is evident from the photomicrographs shown in **Plate 4.5 III; h, i**. The accumulation of coloured dye (congo red and brilliant green) in the live fungal cultures could also be visualised during phase contrast microscopy (**Plate 4.5 IV; j, k**). It is also confirmed from the results that the isolate TAFP41 did not show bio-sorption of the dye rather it removed the dye through some enzymatic/other mechanism.

B. Quantification of Dye Decolourization

The results of preliminary experiments on dye decolourisation revealed clearly the potential of fungal cultures for bioremediation of synthetic dyes. The extent of removal of three selected dyes (brilliant green, malachite green and congo red) in broth cultures was further monitored spectrophotometrically as percent decolourization (%) at the λ_{max} of each dye. The results revealed clearly the high decolourization potential of fungal cultures towards brilliant green, malachite green, and congo red dyes (**Table 4.3**). The degree of maximum percent decolourization of the liquid containing brilliant green dye by the test cultures varied from 50.21 (CAFP7) to 97.68 (AAFP5). Maximum removal (%) of malachite green dye by various fungal cultures achieved from 14.18 to 90.32 with WFPn1 and TAFP41 respectively. Congo red dye was removed more efficiently using bio-sorption mechanism by all the test cultures and percent decolourization of the broth varied from 92.63 (TAFP41) in 15d to 97.89 (WFPn1, and AAFP5) within 9d. In case of the remaining two test dyes (crystal violet and fuchsin basic) there was no apparent change in the colour of liquid media even up to 50d in any case, therefore there removal was not quantified spectrophotometrically. The enzymatic decolorization of potential fungal isolate AAFP5 was also confirmed by checking laccase enzyme activities in completely decolorized (30 d old) culture filtrates of congo red, malachite green and brilliant green dye containing media (**Fig 4.3**) because due to the instance colour of media one could not calculate the activity in dye containing broth. Although the laccase activity is very less in culture filtrate of congo red (10 Uml^{-1}), it may be due to the maximum part of the dye was bio-absorbed by fungus and moreover aging of the culture filtrate. Maximum laccase activity of 68.5 Uml^{-1}, followed by 55.9 Uml^{-1} were observed in completely decolourized culture filtrates of brilliant green and malachite green dyes respectively. This induction of enzymes was correlated with their involvement in dye decolourization. Thus the results revealed that laccase enzyme play vital roles in the bioremediation of used dyes during present investigation.

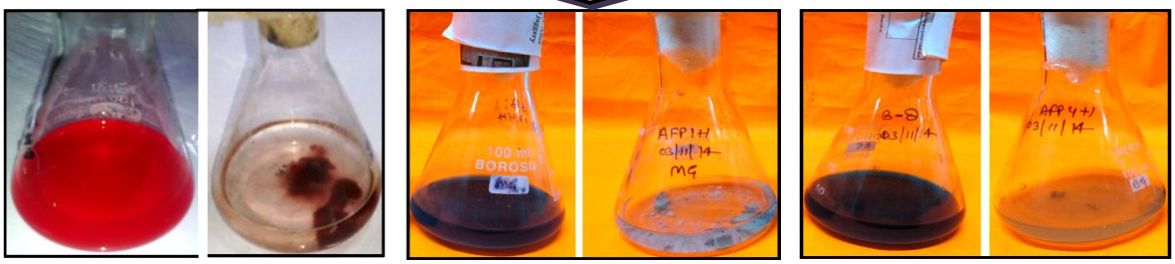
Earlier studies have also reported differential dye removing capabilities of diverse microbial cultures but wood rot fungi specifically have been reported to be more efficient for dye removal through its enzymatic/ non enzymatic mechanisms. Previously only 40% decolourization of anthraquinone dye, Remazol Brilliant Blue R (RBBR) by *Ganoderma* sp. was reported which could be increased up to 92.4% upon addition of HBT as redox

mediator (**Murugesan *et al*., 2006**). However, contrary to previous findings, much higher decolourization rate for the two dyes congo red, and brilliant green by our indigenous fungal isolates could be achieved during present study indicating higher bioremedial potential of the cultures. All the five test fungal isolates decolouried all three dyes efficiently in comparision to the standard culture of *Phanerochaete chrysosporium*. It is evident from the results that the cultures showed better biosorption efficacy towards the two dyes brilliant green and congo red but only two wood rot fungal isolates, AAFP5 and TAFP41, could show better efficacy for removal of the dye malachite green. As per many earlier reports, the bioremediation of dye by the ligninolytic fungi and their ligninases enzymes (lignin peroxidase, manganese dependent peroxidase and laccase) is more environmentally friendly strategy to degrade synthetic textile dyes (**Raghukumar *et al*., 2008; Plácido *et al*., 2016**). The decolourization of dyes with the help of extracellular lignin degrading/modifying enzymes is also supportable because of the broad spectrum substrate range that is very low substrate specificities of these enzymes from the fungal system. The low substrate specificities of ligninases enzymes from wood rot fungi make them more capable to degrade a wide range of xenobiotic compounds including the synthetic industrial dyes (**Spadaro *et al*., 1992; Chander and Arora, 2007; Kaushik and Malik, 2009**).



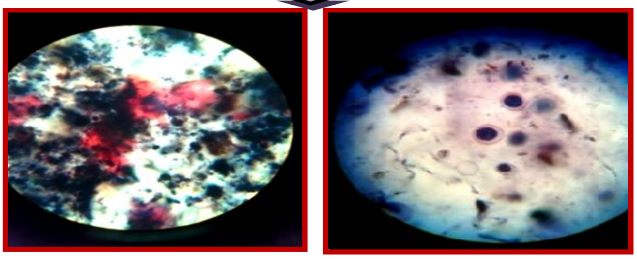
(a) **(b)** **(c)** **(d)**

(I) Decolourization of Congo red by the isolate WDP2 (a); fuchsin basic (b), Brilliant Green (c) and Malachite Green (d) by the Isolate WFPn1



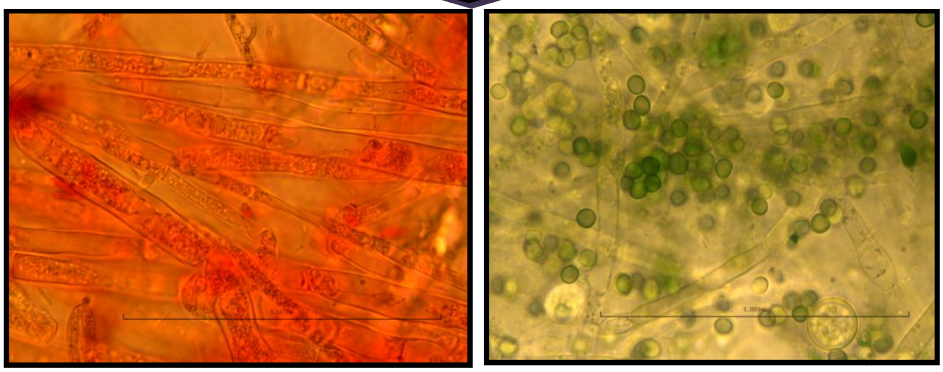
(e) **(f)** **(g)**

(II) Bio-sorption of congo red by fungal isolate WDP2 (e), decolorization of malachite green by WFPn1 (f), and decolorization of brilliant green (g) by the fungal isolate TAFP41 in broth cultures.



(h) **(i)**

(III) Bright field Photomicrograph of stained preparations showing accumulation of congo red dye into the fungal mycellia at 400X (h) and in spores at 1000 X (i).



(j) **(k)**

(IV) Phase contrast photo-micrographs of live fungal preparations showing accumulation of congo red and malachite green dyes in fungal mycelia of the isolate WDP2 (j) and in spores of WFPn1 (k) at 1000X.

Plate 4.5: *In Vitro* Decolourization of Synthetic Industrial Dyes by Potential Lignolytic Fungal Isolates (I-IV)

Table 4.3: Time Period required for maximum percent removal of different synthetic dyes from liquid media by the selected lignolytic fungal cultures

S. No.	Fungal Culture*	Time Period required for maximum removal (%) of dyes from liquid media					
		Brilliant Green		Malachite Green		Congo Red	
		Dye decolourization (%)	Period Required (d)	Dye decolourization (%)	Period Required (d)	Dye decolourization (%)	Period Required (d)
1	WDP2	92.67	18	58.61	24	95.89	09
2	WFPn1	96.59	21	14.18	09	97.89	09
3	CAFP7	50.21	12	14.91	09	94.74	09
4	AAFP5	97.68	21	88.86	21	97.89	09
5	TAFP41	95.20	12	90.32	12	92.63	15
6	*P. chrysosporium*	58.63	12	33.00	12	94.74	09
SEm± (% decolourization)		0.11		0.085		0.071	
CD at 5% (% decolourization)		0.36		0.28		0.23	

*The cultures were grown in mineral salt medium supplemented with dye (100 mg/L) at 30±1^{0}C for 27 d.

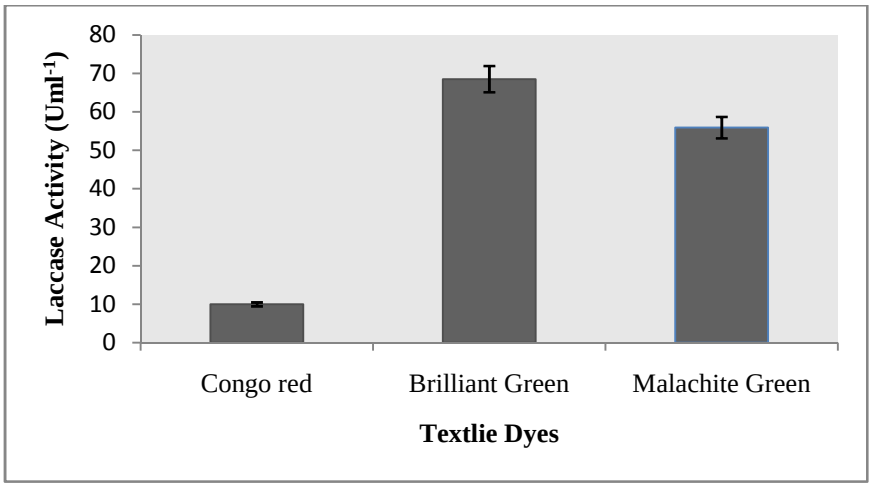


Fig 4.3: Laccase enzyme activity in dye containing decolourized broth by potential fungal isolate AAFP5.

4.3 Quantification of Lignolytic Enzymes

The five selected fungal isolates (WFPn1, WDP2, AAFP5, CAFP7, TAFP41) and the standard culture of *P. chrysosporium*, showing extracellular lignolytic enzyme production qualitatively, were further evaluated for quantification of the three lignin degrading enzymes (lignin peroxidase, manganese dependent peroxidase and laccase) in liquid minimal salt medium supplemented with veratryl alcohol (as inducer) and $MnSO_4$ salt in case of manganese dependent peroxidase (MnP) enzyme production.

4.3.1 Lignin Peroxidase (LiP) Activity

Extracellular lignin peroxidase activity of the test cultures was measured up to 24d & very low initial enzyme activity for all the selected fungal cultures was observed in culture filtrate (**Table 4.4, a**). The LiP enzyme activities for various cultures varied from 0.000 to 0.0349 Uml^{-1} at different incubation periods. Maximum enzyme activity was shown by the fungal isolate CAFP7 (0.0349 Uml^{-1}), followed by the standard culture of *P. chrysosporium* (0.032 Uml^{-1}) and the isolate WDP2 (0.02 Uml^{-1}). Most of the cultures showed maximum enzyme activity within 12-18d of incubation period except TAFP41, which showed the maximum LiP activity (0.005 Uml^{-1}) at 6d of incubation. Our results are with the earlier findings where workers have reported the production of lignin peroxidase enzyme by various wood rot fungal cultures but in very low amounts (**Tortella *et al.*, 2008**). They have observed the production of lignin peroxidase by *Trametes hirsuta* (0.0667 Uml^{-1}), *P. chrysosporium* (0.0581 Uml^{-1}), *Lengites betulina* (0.00 Uml^{-1}), *Trametes versicolor* (0.017 Uml^{-1}), *Anthracophyllum discolour* (0.097 Uml^{-1}).

4.3.2 Mn Dependent Peroxidase (MnP) Activity

Very low amount of initial extracellular Mn dependent peroxidase enzyme activities were observed for the test fungal cultures. Majority of the fungal isolates showed maximum MnP enzyme production within 12-21d of incubation period (**Table 4.4, b**). Maximum MnP activity (0.00224 Uml^{-1}) was observed for the fungal culture TAFP41, at 21d of incubation period, followed by the fungal isolate WDP2 (0.0015 Uml^{-1}), at 18d of incubation, and the isolate AAFP5 (0.0013Uml^{-1}) at 12 d of incubation period. Maximum MnP activity (0.007 Uml^{-1}) at 6d of incubation was recorded for the standard fungal culture *P. chrysosporium*. Similar results with low MnP activity during initial growth period were also reported by many workers (**Xu *et al.*, 2013**). Further work on optimization of culture conditions is required for enhancing the enzyme yield.

4.3.3 Laccase (Lac) Activity

All of the five isolates showed very high laccase enzyme activities when quantified using ABTS, as substrate. The extracellular laccase enzyme activities were varied from 0.082 Uml^{-1} to 156.793 Uml^{-1} for various cultures, maximum during 12-18d incubation period. Maximum laccase enzyme activity was shown by the fungal isolate WDP2 (156.793Uml^{-1}) at 15 d of incubation period, followed by the fungal isolate WFPn1 (156.65 Uml^{-1}) at 18d of incubation period and TAFP41 (155.53 Uml^{-1}) with significant difference in the values among three cultures. The standard culture (*P. chrysosporium*) showed the less laccase enzyme activity (0.25Uml^{-1}) within 12 d of incubation period as compared to other isolates except for the isolate CAFP7 (**Table 4.4, c**). **Srinivasan *et al.*, 1995**, reported that the white rot basidiomycete *P. chrysosporium*, unlike most other white rot fungi, does not produce laccase but again they have suggested the use of cellulose in the medium for the induction of laccase enzyme system in this fungi. however, *P. chrysosporium* is well reported for its high peroxidase enzyme (both LiP and MnP)activities associated with its lignin biodegradation. Moreover it has been extensively studied as a model organism for fungal lignin degradation because of the presence of these two peroxidase enzymes (**Buswell and Odier, 1987; Gold and Alic 1993**). **Heinzkill *et al.* (1998)** reported the maximum laccase enzyme production of 70-80 Uml^{-1} by white rot fungi *Panaeolus sphinctrinus*, using ABTS as substrate. Our findings showed relatively higher laccase production during the fermentation time in minimal salt medium contacting glucose and veratryl alcohol. Thus, on the basis of maximum extracellular laccase enzyme activity the fungal isolate WDP2 was considered as the potential isolate for lignin degrading extracellular laccase enzyme, and thus selected for the further studies.

Table 4.4: Initial extracellular lignin peroxidase enzyme (a), Mn dependent peroxidase enzyme (b), and laccase enzyme (c) activities in the filtrates of the selected fungal cultures*

4.4 (a) Lignin peroxidase enzyme activity

Fungal Cultures	Enzyme activity (Uml^{-1}) at different time intervals (d)							
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**
WFPn1	0.0000	0.0050	0.0050	0.0050	0.0050	0.0200	0.0000	0.0000
WDP2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0200	0.0000	0.0000
AAFP5	0.0000	0.0000	0.0000	0.0000	0.0050	0.0050	0.0050	0.0000
CAFP7	0.0050	0.0199	0.0150	0.0248	0.0247	0.0248	0.0349	0.0198
TAFP41	0.0000	0.0050	0.0050	0.0050	0.0050	0.0000	0.0000	0.0000
P.chrysosporium	0.00	0.005	0.032	0.032	0.005	0.005	0.00	0.00
Factors	Time (a)	Culture (b)	Interaction (a*b)					
S.Em.±	0.001	0.001	0.004					
CD (5%)	0.005	0.004	0.115					

4.4 (b) Mn-dependent peroxidase activity

Fungal Cultures	Enzyme activity (Uml^{-1}) at different time intervals (d)							
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**
WFPn1	0.0005	0.0008	0.0008	0.0012	0.0002	0.0002	0.0000	0.0002
WDP2	0.0000	0.0000	0.0000	0.0013	0.0013	0.0015	0.0013	0.0011
AAFP5	0.0000	0.0000	0.0012	0.0013	0.0012	0.0012	0.0012	0.0012
CAFP7	0.0000	0.0000	0.0000	0.0002	0.0002	0.0002	0.0003	0.0002
TAFP41	0.0000	0.0000	0.0000	0.0005	0.0015	0.0021	0.0022	0.0022
P.chrysosporium	0.005	0.007	0.002	0.002	0.002	0.00	0.00	0.00
Factors	Time (a)	Culture (b)	Interaction (a*b)					
S.Em.±	0.00001	0.00001	0.00004					
CD (5%)	0.00005	0.00004	0.00012					

4.4 (c) Laccase activity

Fungal Cultures	Enzyme activity (Uml^{-1}) at different time intervals (d)							
	3	**6**	**9**	**12**	**15**	**18**	**21**	**24**
WFPn1	1.341	3.426	6.251	6.750	100.350	156.650	122.243	119.574
WDP2	0.083	0.330	37.589	109.009	156.793	117.842	114.350	14.669
AAFP5	2.670	3.933	7.669	4.538	19.572	148.430	117.370	79.169
CAFP7	0.084	0.083	0.083	0.252	0.167	0.166	0.082	0.084
TAFP41	18.499	18.499	28.348	61.662	115.530	87.756	85.612	16.138
P.chrysosporium	0.083	0.083	0.083	0.25	0.16	0.16	0.083	0.083
Factors	Time (a)	Culture (b)	Interaction (a*b)					
S.Em.±	0.0478	0.037	0.106					
CD (5%)	0.134	0.106	0.301					

*The cultures were grown in modified minimal salt medium containing 0.3% glucose and veratryl alcohol for lignin peroxidase and laccase enzymes, and with 0.05% $MnSO_4$+0.3% glucose+veratryl alcohol for Mn dependent peroxidase production.

4.4 Quantification of Cellulolytic Enzymes

Based on relative cellulase activity indices, six fungal isolates (WDP2, WFPn1, TAFP41, AAFP5, PCF1, and CAFP7) along with the standard culture (*A. Niger*) were selected for quantification of overall (filter paper) cellulase (FPase), carboxymethyl cellulase (CMCase) and β-glucosidase (BGL) enzyme activities.

4.4.1 Filter Paper (FPase) Activity

The initial FPase activity for various test fungal cultures varied from 0.00 to 0.73 Uml^{-1}. The FPase activity increased gradually with incubation period and maximum activity was recorded between 9-12h of incubation period (**Table 4.5**). Among various cultures under test, the isolate CAFP7 showed minimum FPase activity (0.026 Uml^{-1}) at 6d, thereafter it showed no enzyme activity in the culture filtrate even up to 15d (**Fig 4.4**). The isolate PCF1 showed maximum FPase activity (0.52 Uml^{-1} and 0.732 Uml^{-1}), the values being significantly higher than those recorded for all the other isolates as well as standard culture of *A. niger* (0.064 Uml^{-1} and 0.21 Uml^{-1}) at 9d at 12d respectively. For most of the fungal isolates including *A. niger*, the enzyme activity decreased after 12d of incubation period. Several previous studies have also reported variable cellulase activities for lignolytic microbial cultures. A gradual increase in enzyme activity up to certain optimum period followed by a decline with time as observed by us during present investigation has also been reported by several other workers (**Romero *et al*., 1999; Saini *et al*., 2013**).The decrease in cellulase production after a particular time of incubation period may be due to the catabolic repression of enzyme by accumulated glucose moieties that are generated through β-glucosidase enzyme activity of the cellulase complex. Although many studies have reported the production of cellulase enzyme complex within the fermentation period of 7-10d on incubation by *A. niger* and other fungi (**Hussain *et* al., 2012; Das *et al*., 2013**), but the standard culture (*A. niger*) used in the present study gave the highest activity in somewhat longer time (12d). This delayed enzyme production might be correlated with the culture conditions and the use of synthetic complex cellulose substrate for enzyme production. However, using the natural substrates such as wheat bran, rice bran, and husks, etc., maximum amount of cellulase enzyme production can be achieved at lesser time. **Oberoi *et al*. (2010)** got highest cellulase production (FPase, CMCase, BGL) by *Trichoderma* sp. during solid state fermentation

using kinnow pulp with different combinations of wheat bran and rice bran within 3-5d of incubation period. It is thus clear that the efficacy of enzyme production by microbial culture can be enhanced by optimising the substrate as well as other culture conditions.

4.4.2 CMCase and β-Glucosidase Enzyme Activities

The results indicated clearly that the carboxymethyl cellulase enzyme activity was not prominent in selected wood rot and non wood rot fungal isolates. The fungal isolate CAFP7 showed no CMCase enzyme activity even up to 15d of incubation period (**Table 4.5**), while rest of the fungal cultures also showed very little CMCase activity very little or no increase in enzyme activity with increase in incubation period. The maximum CMCase activity was observed at ninth d of incubation period and varied from 0.023 Uml^{-1} for wood rot fungal isolate WFPn1 to 0.047 Uml^{-1} for standard culture (*A. Niger*). Only fungal isolate PCF1 showed highest CMCase activity (0.03 Uml^{-1}) at 12d of incubation. For rest of the cultures, CMCase activity got decreased after the 9d of incubation period and then reached 0.00 up to 15d of incubation.

Majority of the isolates showed a higher β-Glucosidase enzyme activity as compared to FPase and CMCase activities (**Table 4.5**). The fungal isolate CAFP7 showed no β-Glucosidase activity even up to 15d incubation period. The β-Glucosidase enzyme activity for rest of the test fungal isolates varied from 0.00 Uml^{-1} (CAFP7) at 3d to 0.398 Uml^{-1} (PCF1) at 9d. The fungal isolate PCF1 showed a significantly higher β-Glucosidase enzyme activity (0.398 Uml^{-1}) as compared to rest of fungal isolates and the standard culture (*A. niger*). For all the fungal isolates including standard culture the enzyme activity gradually increased up to 9d and therefore it decreased in further incubation.

Some of the earlier workers have reported mthe maximum CMCase and BGL activity at 6d followed by a decline during submerged fermentation medium supplemented with soy-bran and wheat bran (**Todero-Ritter *et al.*, 2013**). The higher activity observed in lesser time by previous workers might be correlated with the use of natural optimum carbon source. Therefore it is observed that by optimizing the substrate and other fermentation conditions, the enzyme yield can be further enhanced in the present case also. The fungal isolate PCF1 showing maximum cellulase activity was selected as an efficient fungal culture for the cellulase enzyme complex production, and thus used for further saccharification study.

Table 4.5: Initial (crude) extracellular crude cellulase enzyme activities (Uml^{-1}) in the filtrates of selected fungal* cultures

	Cellulase enzyme activities (Uml^{-1}) at different time intervals (d)														
	3			6			9			12			15		
	FPase	CMCase	BGL**	FPase	CMCase	BGL	FPase	CMCase	BGL	FPase	CMCase	BGL	FPase	CMCase	BGL
WDP2	0.023	0.034	0.04	0.055	0.035	0.137	0.074	0.04	0.176	0.081	0.00	0.202	0.021	0.00	0.32
WFPn1	0.015	0.018	0.056	0.033	0.01	0.135	0.04	0.023	0.132	0.07	0.003	0.181	0.054	0.00	0.091
TAFP41	0.023	0.018	0.034	0.138	0.01	0.127	0.064	0.027	0.123	0.018	0.002	0.05	0.018	0.00	0.005
AAFP5	0.020	0.019	0.062	0.101	0.019	0.147	0.064	0.025	0.148	0.037	0.00	0.102	0.029	0.00	0.032
PCF1	0.129	0.011	0.043	0.393	0.012	0.25	0.52	0.034	0.398	0.732	0.03	0.295	0.32	0.00	0.15
CAFP7	0.02	0.00	0.00	0.026	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A. niger	0.02	0.034	0.012	0.11	0.034	0.15	0.064	0.047	0.18	0.21	0.00	0.2	0.121	0.00	0.185

Factors	**For FPase**			**For CMCase**			**For β-glucosidase**		
	Culture (a)	Time (b)	Interaction(a*b)	Culture(a)	Time (b)	Interaction (a*b)	Culture(a)	Time (b)	Interaction (a*b)
SEm±	0.0044	0.0037	0.01	0.0004	0.0003	0.0008	0.0026	0.0022	0.022
CD (5%)	0.012	0.01	0.028	0.0011	0.001	0.0024	0.0075	0.0063	0.0168

*Cultures were grown in cellulase production broth medium containing CMC as sole carbon source at 28 ± 1^0C.

**β- glucosidase

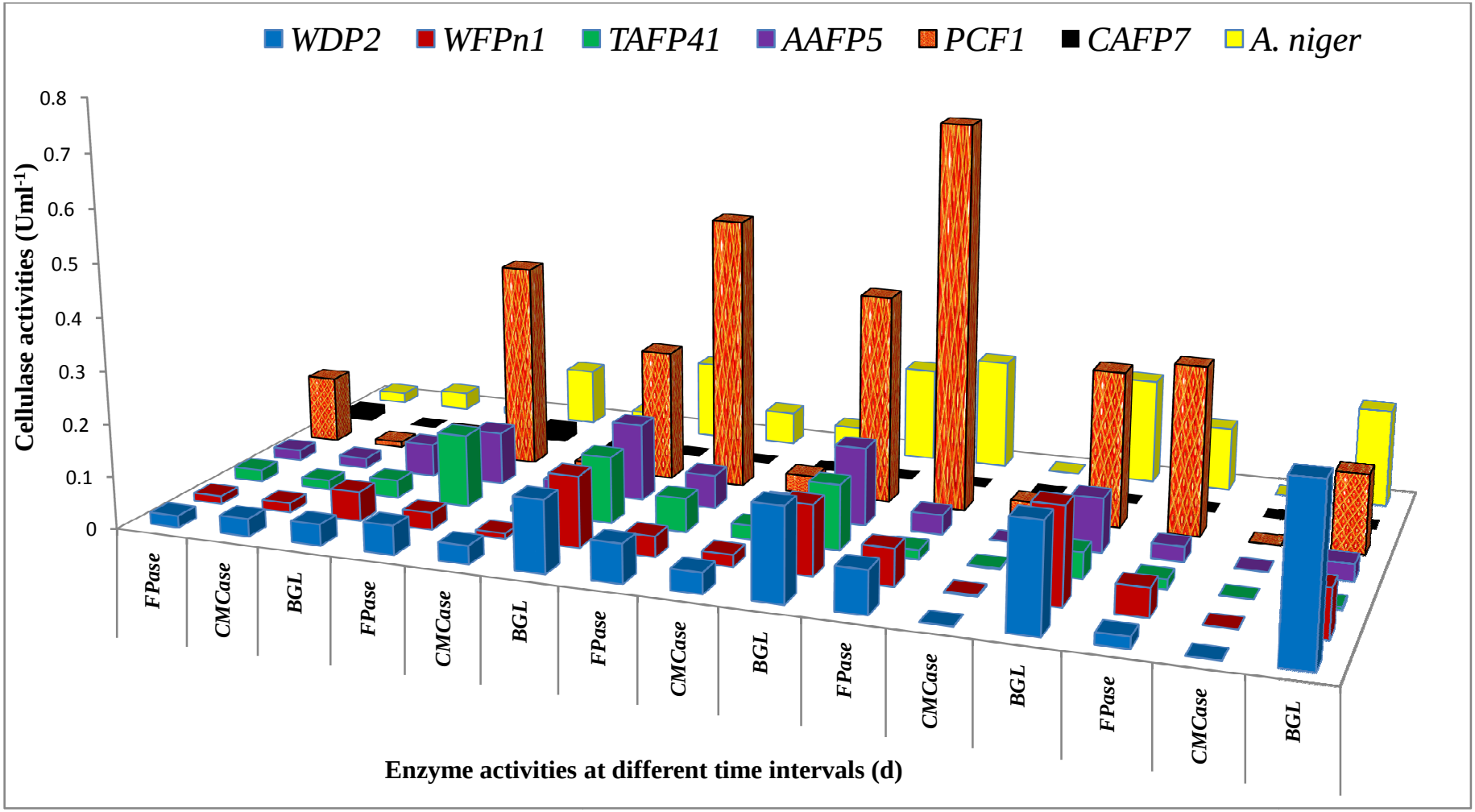


Fig 4.4: Differential extracellular cellulase enzyme activities (Uml^{-1}) in the filtrates of selected fungal cultures

UNIT II: LACCASE ENZYME PRODUCTION & CHARACTERIZATION

4.5 Optimization of Enzyme Production

Culture conditions for maximum production of laccase were optimized by studying different parameters such as carbon source and its concentration, nitrogen source and its concentration, incubation temperature and period, pH of fermentation medium, and presence of different surfactants, that affect the enzyme production. The study was undertaken by taking one parameter at a time while keeping the other variables constant.

4.5.1 Effect of Carbon Source

Carbon is one of the major nutrients required for microbial growth, hence a non limiting supply of carbon is required to assure the proper growth and production of large quantities of microbial enzymes for industrial purposes. Synthesis of laccase can be induced by the presence of some inducer molecules such as some lignocellulosic substrates (**Leonowicz *et* al., 1978; Leonowicz and Malinowska, 1982; Koroljova-Skorobogat'ko *et al*., 1998**). The cost of enzyme production at commercial level mostly depends upon the choice of the substrate as carbon source. One strategy for reducing the enzyme production cost might be lowering the raw material cost. In view of above, several abundantly available, cheap, natural substrates such as rice bran, wheat bran, bamboo wood powder, rice husk, and poplar powder along with the synthetic substrate (glucose) were evaluated as sole carbon source for maximal production of the extracellular lignin degrading laccase enzyme.

The extracellular laccase enzyme production was significantly influenced by the nature of carbon source and concentration of the carbon source available in medium (**Table 4.6, a**). The enzyme activity with the different substrates varied from 112.34 to 162.49 Uml^{-1}. Similarly a significant influence of the cheap organic substrates on enzyme productivity was observed as it varied from 390.07 to 564.21$UL^{-1}h^{-1}$. The laccase enzyme yield varied from 11234.00 to 16249.33 Ug^{-1}, maximum yield (16249.33 Ug^{-1}) recorded with wheat bran as the carbon source followed by the Bamboo powder (16044.57) (**Fig 4.5).** Wheat bran has also been reported to enhance the laccase enzyme production under solid state fermentation by earlier workers. Moreover, significantly higher enzyme activity, productivity, and yield obtained with wheat bran as compared to other substrates, indicated

it as a most suitable substrate for laccase production. Addition of wheat bran and peptone together were found to increase laccase enzyme production from 9300 UL^{-1} to 17390 UL^{-1} during the earlier study (**Bakkiyaraj *et al*., 2013**). Several other studies also reported an enhanced the production of the laccase enzyme from various fungal cultures up on addition wheat bran (**Hou *et al*., 2004**; **Nandal *et al*., 2013; Levin *et al*., 2010**). Our results indicated clearly the suitability of using wheat bran as a cheap and abundantly available substrate for production of laccase enzyme, thereby making the process cost effctive.

After selection of best suited substrate (wheat bran), for laccase enzyme production, further experiments were carried out to optimize the concentration of wheat bran for enzyme production by selected fungus WDP2. The laccase enzyme production was significantly influenced by different concentrations of the carbon source in the basal medium. It is evident from the data that enzyme activity varied from 87.80 Uml^{-1} to 182.89 Uml^{-1} at different concentrations of wheat bran. Maximum laccase activity (182.89 Uml^{-1}) recorded with 2% wheat bran concentration, thus values being significantly higher than that recorded with other concentrations of wheat bran (**Table 4.6, b**). Maximum laccase yield (25585.73 U g^{-1}), was found with the 0.5% of wheat bran (**Fig 4.6**). During earlier studies also, maximum laccase production was obtained with 2% substrate concentration from white rot fungus *Trametes hirsuta*, the potent laccase producer (**Periasamy *et al*., 2001; Bakkiyaraj *et al*., 2013; Nandal *et al*., 2013**). Although we have observed the laccase activity inhibition while concentration of the wheat bran has been increased.

4.5.2 Effect of Nitrogen Source

Nitrogen is one of the most essential elements required for growth and metabolic activities of all organisms; hence, nitrogen source in the production medium is a very crucial factor limiting enzyme production. Since the laccase enzyme production recognized to be very sensitive to nitrogen source present in the production medium, therefore effect of the various organic (Chick pea flour, tryptone, peptone, and yeast extract), and inorganic (sodium nitrate, ammonium sulphate, and urea) nitrogen sources on enzyme production was studied.

During present study, maximum enzyme activity (184.42 Uml^{-1}), productivity (640.34 $UL^{-1}h^{-1}$), and yield (9220.87 Ug^{-1}) were observed with ammonium sulphate, the values being significantly higher than those recorded with other nitrogen sources (**Table**

4.7 a). Urea as a nitrogen source was found completely inhibits the production of the laccase enzyme during present study. The effectiveness of the laccase enzyme production significantly decreased in the following order: Ammonium sulphate > peptone > chick pea flour > sodium nitrate > tryptone > yeast extract > urea (**Fig 4.7)**. Most of studies have reported the organic substrate as best nitrogen source for laccase production, however, only few workers have reported inorganic nitrogen source $(NH_4)_2SO_4$ as the optimum nitrogen source for enzyme production (**Niladevi and Prema, 2008)**. Although the suitability of the nitrogen source varies with the microbial strain is being used. Although the organic nitrogen source considered to be the best for production, surprisingly, it was noticed that the inorganic nitrogen source, ammonium sulphate, was stimulated the highest laccase production in the broth medium. But, ammonium sulphate has the advantage that it is relatively very cheap nitrogen source than that of the other inorganic sources.

Since each enzyme has an optimum nitrogen concentration requirement, therefore different levels of ammonium sulphate ranging from 0.1 to 0.5% with an interval of 0.1 were used during present study to optimize laccase production. Maximum laccase enzyme activity (184.42 Uml^{-1}), productivity (640.34 $UL^{-1}h^{-1}$), and yield (9220.87 Ug^{-1}) were achieved in the presence of 0.1% of the ammonium sulphate concentration; the values being significantly than those regarded at other concentrations of ammonium sulphate (**Table 4.7 b**). However, the values of enzyme activity, productivity & yield gradually decreased with increasing concentration of nitrogen source (**Fig 4.8**). Thus it is apparent from the results that laccase production occurs at low nitrogen concentration *i.e.*, nitrogen stress conditions are required for expression of laccase enzyme. It may be concluded that being a secondary metabolite, it requires longer time to achieve significant enzyme production as compared to other extracellular enzymes such as cellulase & others. Moreover, the study also confirms that laccase is a nitrogen stress induced enzyme. In this way to maintain the concentration of the nitrogen source is very critical for laccase production. The findings are in agreement with those of earlier findings, who have reported the inhibition of the laccase and Mn dependent peroxidase enzymes at high nitrogen concentration (**Buswell *et al.*, 1995**; **Gianfreda *et al.*, 1999**). However, this is a controversial subject and many other authors as authors have found that laccase activity increases under N limiting conditions (**Eggert *et al.*, 1996; Pointing *et al.*, 2000; Galhaup *et al.*, 2002**). Thus our findings support that nitrogen starvation conditions favour laccase production by fungi.

Table 4.6 (a): Influence of exogenous carbon source on production and yield of extracellular laccase enzyme from the white rot fungal isolate WDP2*

Carbon Source	Enzyme Activity ($U\ ml^{-1}$)	Productivity ($UL^{-1}h^{-1}$)	Yield ($U\ g^{-1}$)
Rice Bran	112.34	390.07	11234.00
Wheat Bran	162.49	564.21	16249.33
Bamboo Powder	160.45	557.10	16044.57
Rice Husk	146.16	507.51	14616.33
Poplar Powder	157.37	546.43	15737.13
Glucose	155.97	541.57	15597.20
SEm±	0.248	0.866	25.014
CD (5%)	0.783	2.728	78.799

*The culture was grown in modified minimal salt medium containing different carbon sources (@1%, w/v) at 28±1^{0}C for 12d.

Table 4.6 (b): Influence of wheat bran concentration on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2*

Carbon Source	Enzyme Activity ($U\ ml^{-1}$)	Productivity ($UL^{-1}h^{-1}$)	Yield ($U\ g^{-1}$)
0.5	110.0	475.31	27377.73
1.0	117.71	408.70	11770.67
1.5	129.37	449.20	8624.67
2.0	182.89	635.02	9144.25
2.5	101.32	351.82	4052.93
3.0	87.80	304.86	2926.67
SEm±	0.355	1.233	18.950
CD (5%)	1.095	3.799	58.381

*The culture was grown in modified minimal salt medium containing wheat bran as carbon source at 28±1^{0}C for 12d.

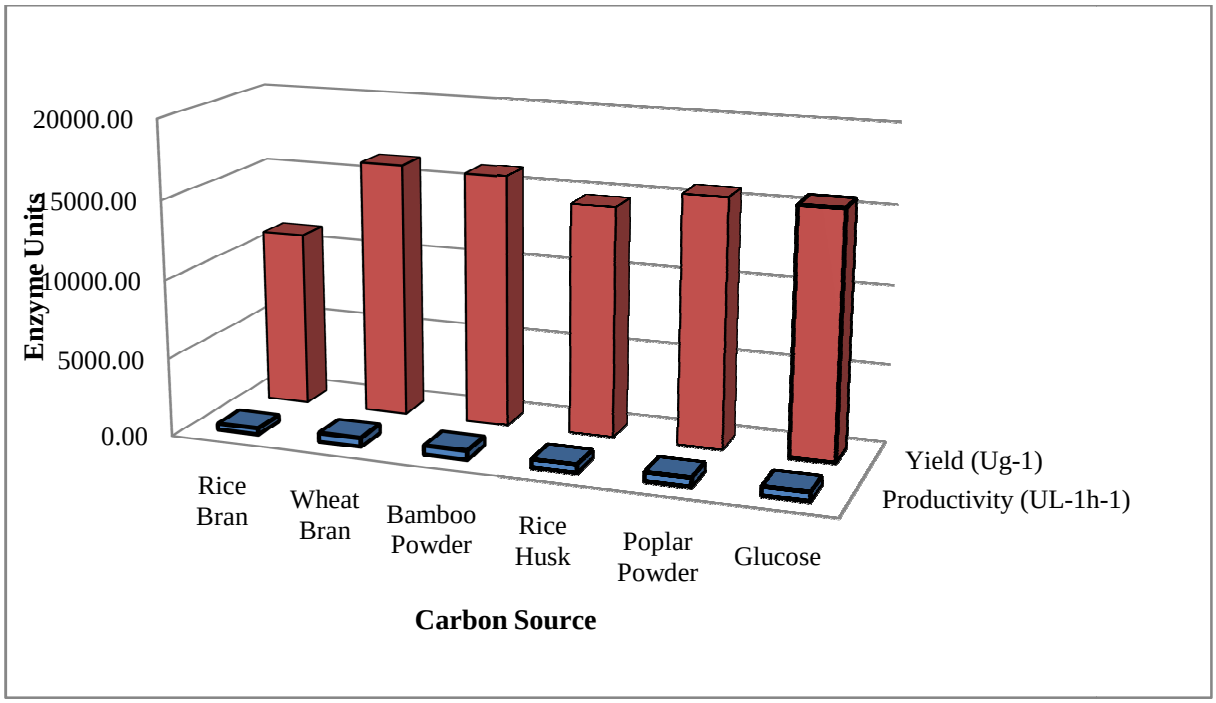


Fig 4.5: Effect of carbon source on production of extracellular laccase enzyme by wood rot fungal isolate WDP2

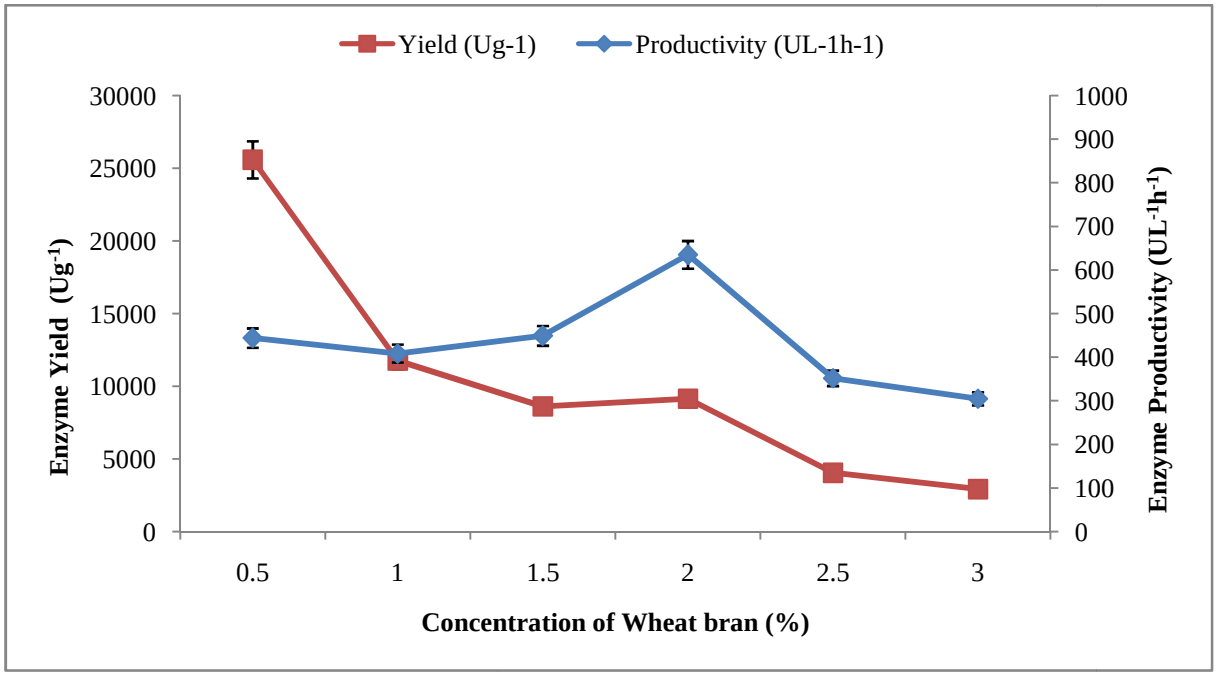


Fig 4.6: Effect of wheat bran concentration on extracellular laccase production by wood rot fungal isolate WDP2

Table 4.7 (a): Influence of exogenous nitrogen source on production and yield of extracellular laccase enzyme from the white rot fungal isolate WDP2*

Nitrogen Source	Enzyme Activity ($U\ ml^{-1}$)	Productivity ($UL^{-1}h^{-1}$)	Yield ($U\ g^{-1}$)
CPF	126.14	437.99	6307.05
Tryptone	122.67	425.93	6133.37
Sodium Nitrate	122.80	426.38	6139.88
Yeast Extract	117.66	408.55	5883.10
$(NH_4)_2SO_4$	184.42	640.34	9220.87
Urea	0.00	0.00	0.00
Peptone	183.42	636.87	9170.98
SEm±	0.224	0.794	11.380
CD (5%)	0.682	2.409	34.516

*The culture was grown in modified minimal salt medium containing wheat bran (@2%, w/v) supplemented with different nitrogen sources (@ 0.1%) at 28±1^{0}C for 12d.

Table 4.7 (b): Influence of concentration of nitrogen source (ammonium sulphate) on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2*

Concentration of $(NH_4)_2SO_4$ (%)	Enzyme Activity ($U\ ml^{-1}$)	Productivity ($UL^{-1}h^{-1}$)	Yield ($U\ g^{-1}$)
0.1	184.42	640.34	9220.87
0.2	114.99	396.45	5708.83
0.3	83.06	292.18	4207.35
0.4	21.21	71.60	1031.10
0.5	13.59	49.81	717.27
SEm±	0.453	1.573	22.662
CD (5%)	1.429	4.957	71.390

*The culture was grown in modified minimal salt medium containing wheat bran (@2%, w/v) supplemented with different concentrations of ammonium sulphate, at 28±1^{0}C for 12d.

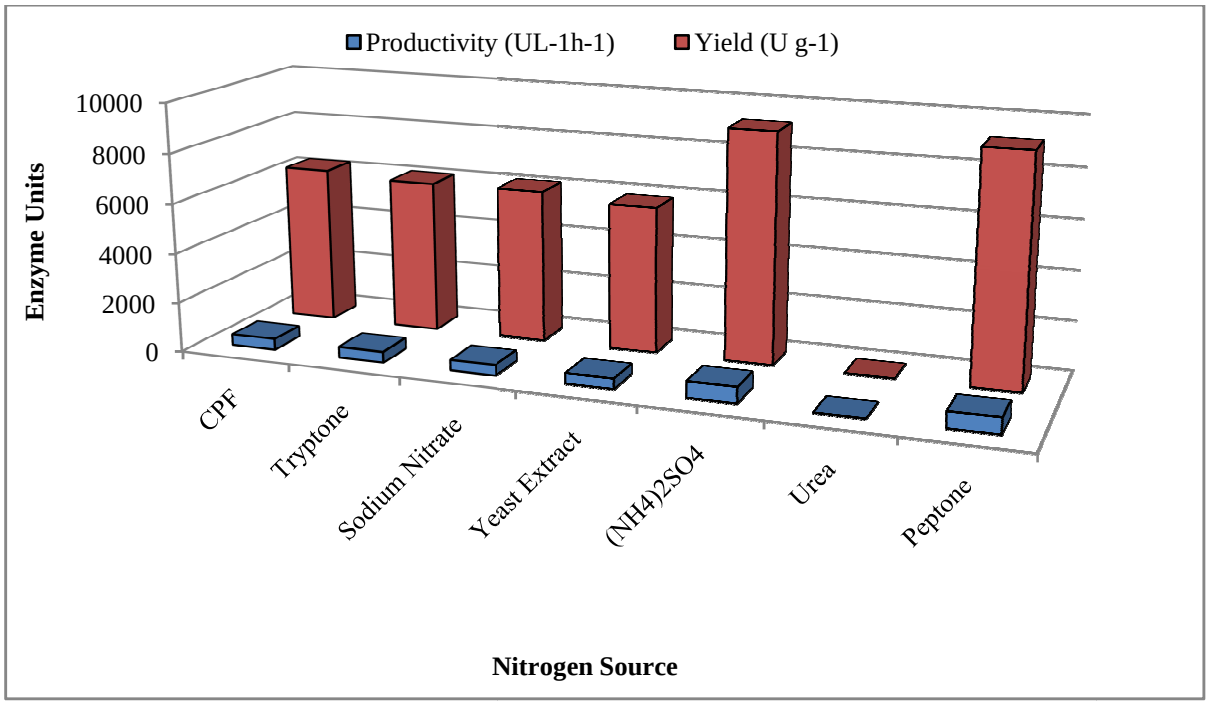


Fig 4.7: Effect of nitrogen source on extracellular laccase production by wood rot fungal isolate WDP2

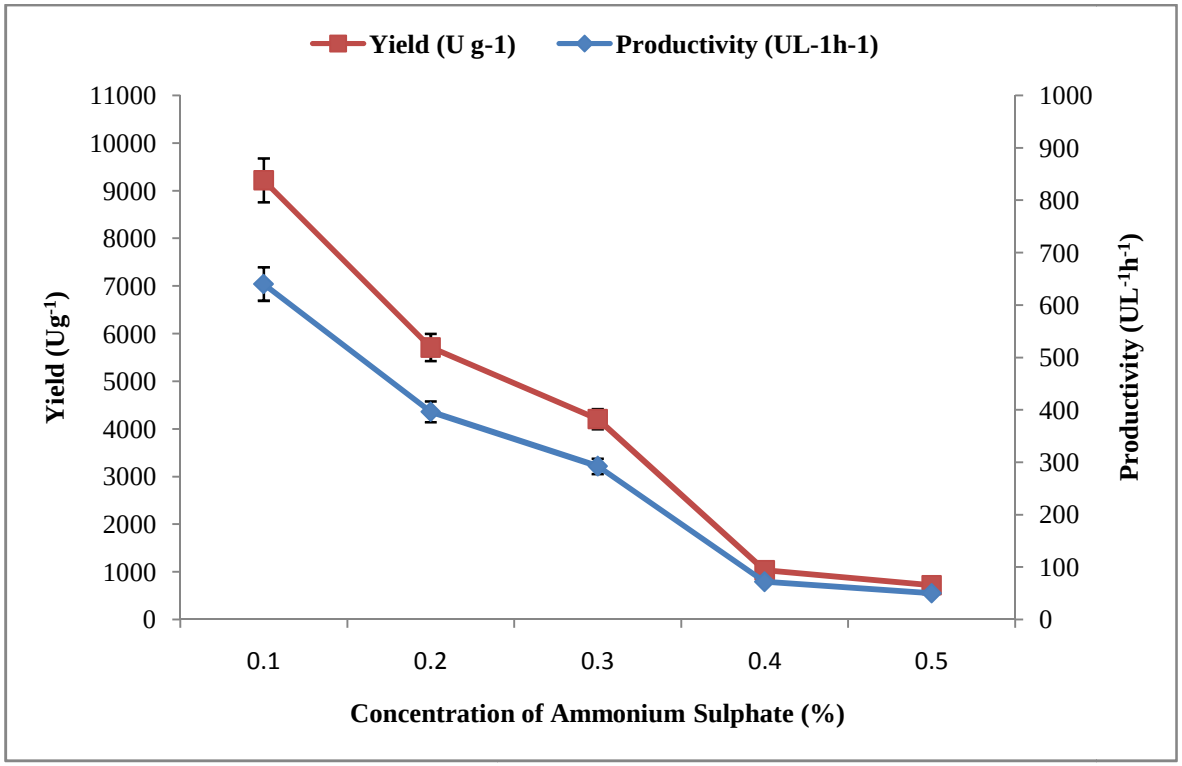


Fig 4.8: Effect of different concentrations of nitrogen source on extracellular laccase production by wood rot fungal isolate WDP2

4.5.3 Effect of pH

Each organism has an optimum pH requirement for its growth, multiplication and synthesis of various macromolecules constituents including enzyme protein. Not only synthesis but activity of an enzyme is also highly influenced by the pH of surroundings. Therefore influence of pH ranging from 3.0 to 8.0 on laccase production was evaluated. In the present study, optimal laccase enzyme production ($185.77 Uml^{-1}$) was achieved at pH 7.0, that was significantly higher than the values recorded at other pH levels; while the values of productivity and yield were 645.03 $UL^{-1}h^{-1}$, and 9288.38 Ug^{-1}, respectively at the same pH 7.0 (**Fig 4.9**). Enzyme activity was minimum at highly acidic conditions (pH 3-4), it increased gradually with increasing pH values, attaining maximum at neutral pH value and thereafter, the activity decreased again gradually with increasing the pH (**Table 4.8**).

Results indicated that the laccase yield gradually increased as pH range increased from 4.0 to 7.0 (3991.05 Ug^{-1} to 9288.38 Ug^{-1}, respectively), and then it reduced to 3929.83 Ug^{-1}) at pH 8.0. As observed by us, an initial pH of 7.0 was also reported as the most favourable pH for optimal growth and laccase production by a newly isolated strain of *T. modesta* (**Nyanhongo *et al.*, 2002**). Although pH of culture medium has been optimised for several microbial enzymes, but very little information is available on its influence of laccase production. Most of the reports have given optimum pH value (in the range of pH 4.5-7.0) for enzyme production. However, reports are also indicating acidic pH (3-5) as the optimum pH for laccase production by different fungal cultures (**Kunamneni *et al.*, 2007**; **Moreira Neto *et al.*, 2009**). It is thus apparent that different fungi vary in their pH optima for laccase production. Although, the optimum pH for laccase activity falls in acidic pH range (3.0-5.0), but the production of the enzyme depends on various other factors (**Palmieri *et al.*, 1997**).

4.5.4 Effect of Temperature

The temperature is another factor regulating the growth of fungi and thereby affecting the synthesis of enzyme. During present study, effect of temperature on laccase enzyme production using fungal isolate WDP2 was evaluated at different temperatures ranging from 24^0C to 40^0C, with an interval of 4^0C. It was observed in the study that

optimum temperature for the growth and maximum laccase enzyme production was 28^0C, indicating the mesophilic nature of the organism. At this temperature, the fungal isolate showed the maximum laccase enzyme activity (187.37 Uml^{-1}), that was significantly higher than the enzyme activities observed at temperatures. The values of the productivity and yield were 650.60 $UL^{-1}h^{-1}$, and 9368.67 Ug^{-1}, respectively (**Table 4.9**). Initially the activity was increased from 88.42 Uml^{-1} at 24^0C to 187.37 Uml^{-1} at 28^0C, thereafter, with increasing the incubation temperature from 28^0C to 40^0C, the enzyme activity, productivity and yield were gradually decreased from 187.37 Uml^{-1} to 53.36 Uml^{-1}, 650.60 $UL^{-1}h^{-1}$ to 195.71 $UL^{-1}h^{-1}$, and 9368.67 Ug^{-1} to 2818.18U g^{-1}, respectively (**Fig 4.10**). The values for enzyme productivity and yield at 28^0C were also significantly higher from those obtained at other temperatures. This could be attributed to thermal denaturation of the enzyme protein that occurred at higher temperatures (>28^0C). The results are close to the findings of **Park & Park, (2008)** and **Murugesan *et al*. (2006)**. They have also reported optimum laccase production under mesophilic temperature range ***i.e.*** from 25 to 30^0C for the maximum laccase recovery from white rot fungus. Some other workers have **also** the same temperature as observed by us for maximum laccase production by white rot fungus *Ganoderma lucidum* during submerged fermentation using effluents of paper and pulp industry as substrate (**Aftab and Ahmad, 2015**).

Present investigation thus reveals the mesophilic nature of the organism therefore at very high or very low temperatures the physiological/ metabolic activities of organism are ceased thereby affecting the enzyme activities. Many other studies have also reported the mesophilic nature of laccase producing fungi (**Gochev & Krastanov, 2007; Eugenio *et al*., 2010; He *et al*., 2014; El-Batal *et al*., 2015; El Monssef *et al*., 2016**). Thus, in accordance with the earlier reports, the mesophilic temperature of 28 ± 1^0C was found as the optimum temperature for laccase production during present study also, and therefore subsequent experiments on enzyme production were carried out at this temperature.

Table 4.8: Influence of pH on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2*

pH	Enzyme Activity ($U\ ml^{-1}$)	Productivity ($UL^{-1}h^{-1}$)	Yield ($U\ g^{-1}$)
3.0	0.00	0.00	0.00
4.0	79.82	277.16	3991.05
5.0	135.43	470.24	6771.50
6.0	163.14	566.45	8156.93
7.0	185.77	645.03	9288.38
8.0	78.60	272.91	3929.83
SEm±	0.164	0.569	8.326
CD (5%)	0.505	1.755	25.652

*The culture was grown in modified minimal salt medium containing wheat bran (@2%, w/v), and ammonium sulphate (@ 0.1%), at 28±1^{0}C for 12d.

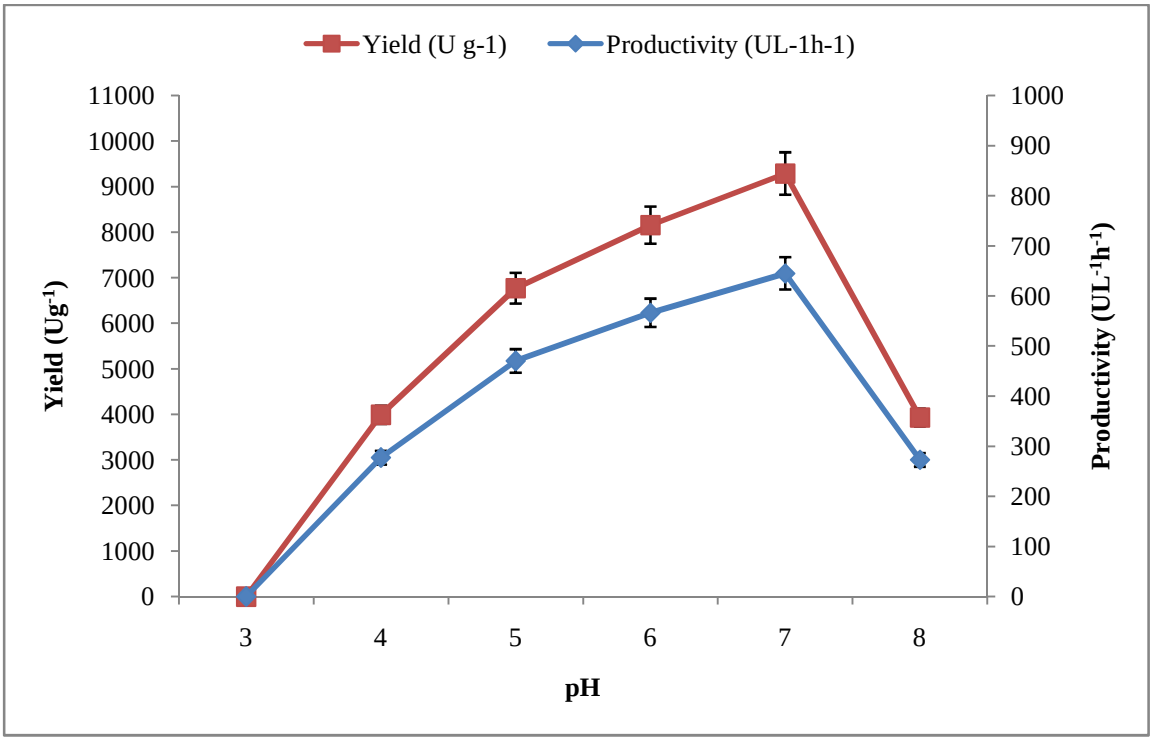


Fig 4.9: Effect of pH on extracellular laccase production by wood rot fungal isolate WDP2

Table 4.9: Influence of temperature on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2*

Temperature (^{0}C)	Enzyme Activity (U ml^{-1})	Productivity (UL^{-1}h^{-1})	Yield (U g^{-1})
24	88.42	307.01	4420.90
28	187.37	650.60	9368.67
32	109.00	378.46	5449.83
36	70.40	244.44	3519.92
40	56.36	195.71	2818.18
SEm±	0.245	0.848	12.252
CD (5%)	0.772	2.672	38.598

*The culture was grown in modified minimal salt medium containing wheat bran (@2%, w/v), ammonium sulphate (@ 0.1%), at pH 7.0 for 12d.

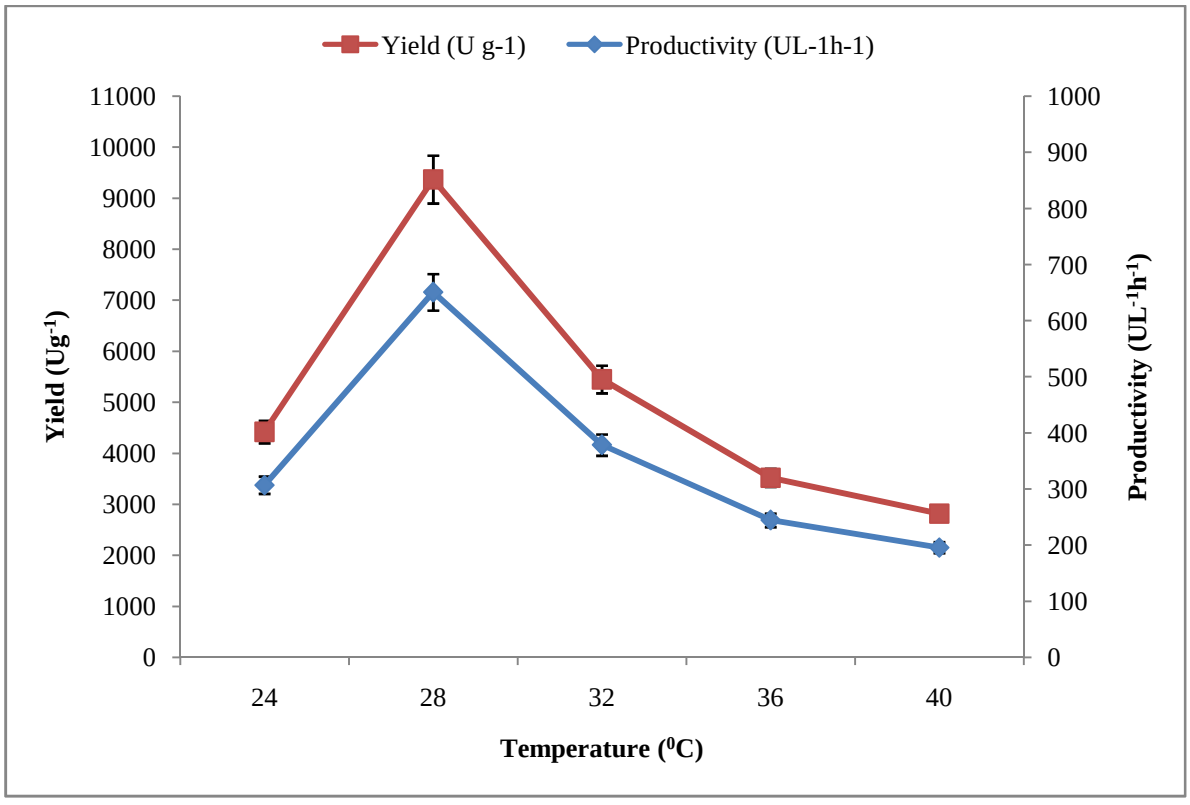


Fig 4.10: Effect of incubation temperature on extracellular laccase production by wood rot fungal isolate

4.5.5 Effect of Surfactants

Influence of supplementation of the pre optimised medium with different surfactants on laccase enzyme production was studied using two non-ionic *viz.* Triton-X-100, and Tween 80; and one natural surfactant soap-nut extract; different concentrations of each of the surfactant ranging from 0.01% to 0.5 % were evaluated. In case of both the synthetic surfactants the maximum enzyme activity (165.04 Uml^{-1} and 73.32 Uml^{-1} with tween 80 and triton X100 respectively) was observed at 0.02% concentration; the values were significantly higher than those recorded at other concentrations of the two surfactants (**Table 4.10**). Contrary to it, in case of the natural surfactant (soapnut extract), maximum and significantly higher enzyme activity (189.26 Uml^{-1}) was observed at 0.03% concentration as compared to those at other concentrations followed by a gradual decline in the activities with increasing surfactant concentrations. Triton X 100 had shown more inhibitory effects on enzyme activity, productivity and yield as was indicated by their complete inhibition at the concentration of ≥0.2 %. Similarly, maximum enzyme productivity and yield with tween 80 (573.06 $UL^{-1}h^{-1}$ and 8252.07 Ug^{-1} respectively) and with triton X100 (253.44 $UL^{-1}h^{-1}$ and 3666.17 Ug^{-1} respectively) were recorded at 0.02% concentration, while in case of soapnut extract the enzyme productivity and yield were maximum (657.15 $UL^{-1}h^{-1}$ and 9463 Ug^{-1} respectively) at 0.03% concentration. The results revealed clearly that all the three surfactants were effective only at very low concentration (0.02-0.03%) while with increasing concentration of the surfactant, rapid decline in enzyme activity, productivity and yield was recorded. Among the three surfactants tested, the natural surfactant soapnut extract was found better as compared to triton X100 and tween 80. The increase in laccase enzyme yield upon addition of soap-nut extract in comparison to Tween 80 and Triton X 100 has been given in **Fig 4.11**. The results revealed that the relative laccase enzyme yield increased with soapnut extract by 33.327%, and 63.58% in comparison to Tween 80 and Triton X 100, respectively. It is noteworthy that none of the three surfactants (tween80, triton X100 and soapnut extract) could enhance the enzyme activity.

It is noteworthy that out of the three surfactants (Tween 80, Triton X100, and Soapnut extract) the two synthetic surfactants (tween 80 and triton X100) did not influence the enzyme production significantly while the soapnut extract enhanced the enzyme

activity from 187.37 Uml^{-1} to 189.26 Uml^{-1} at 0.03% concentration. It may thus be concluded from the data that the natural surfactant (soapnut extract) was better in comparison to the synthetic surfactants. Previous workers have reported an enhancement of laccase or other lignolytic enzymes production by various fungi upon addition of surfactants (**Leštan *et al*., 1994; Pardo, 1996; D'Souza-Ticlo *et al*., 2009**). Enhancement of enzyme activity might be correlated with the higher oxygen permeability and extracellular enzyme transport through the cell membrane caused by the surfactant (**Eggert *et al*., 1996**). Laccase activity has also been reported to be influenced greatly by the concentration of the surfactant used (**Zhang *et* al., 2012**). The stability of laccase has also been reported to be improved in the presence of triton X100 (**Guanglei, *et al*., 2009**). However in our studies, results showed inhibition of laccase enzyme by triton X 100 (**Table 4.10**). Many studies reported that the saponin (extract) from *Sapindus* sp. is a natural non-ionic surfactant (**Hong & Tokunaga, 2000; Rao & Sang, 2006**). It has not only good emulsifying, separating and dispersing capabilities but is also a good foam stabilizer with a great cleaning capacity (**Lin, 1977; Zhang *et al*., 1993; Du *et al*., 2014**). In spite of having good emulsifying and surfactant properties, soapnut extract has not yet been used as to check the enzyme production.

Table 4.10: Influence of exogenous surfactants on production and yield of extracellular laccase enzyme from white rot fungal isolate WDP2*

Surfactant conc. (%)	Enzyme Activity ($U\ ml^{-1}$)			Productivity ($UL^{-1}h^{-1}$)			Yield ($U\ g^{-1}$)		
	Tween 80	Triton X 100	Soap-nut extract	Tween 80	Triton X 100	Soapnut extract	Tween 80	Triton X 100	Soapnut extract
0.01	135.22	3.74	158.41	469.52	13.00	550.02	6761.07	187.23	7920.33
0.02	165.04	73.32	160.22	573.06	253.44	556.31	8252.07	3666.17	8010.83
0.03	126.18	68.93	189.26	438.14	239.33	657.15	6309.15	3446.42	9463.00
0.04	54.72	66.22	126.92	190.01	229.94	440.71	2736.17	3311.07	6346.17
0.05	52.74	26.42	99.35	183.13	91.74	344.97	2637.00	1321.07	4967.60
0.1	53.5	13.25	76.08	185.76	46.00	264.16	2675.00	662.50	3804.00
0.2	47.8	0	69.8	165.97	0.00	242.36	2390.00	0	3490.00
0.3	19.25	0	66.41	66.84	0.00	230.59	962.50	0	3320.5
0.4	16.05	0	42.5	55.72	0.00	147.56	802.50	0	2125.00
0.5	1.5	0	12.0	5.20	0.00	41.66	75.00	0	600.00
SEm±	0.473	0.427	0.222	1.645	1.483	0.785	23.703	21.366	11.153
CD (5%)	1.491	1.345	0.699	5.184	4.674	2.473	74.669	67.307	35.134

**The culture was grown in modified minimal salt medium (pH 7.0) containing wheat bran (@2%, w/v) and ammonium sulphate (@ 0.1%) at 28 ± 2^0C for 12d.

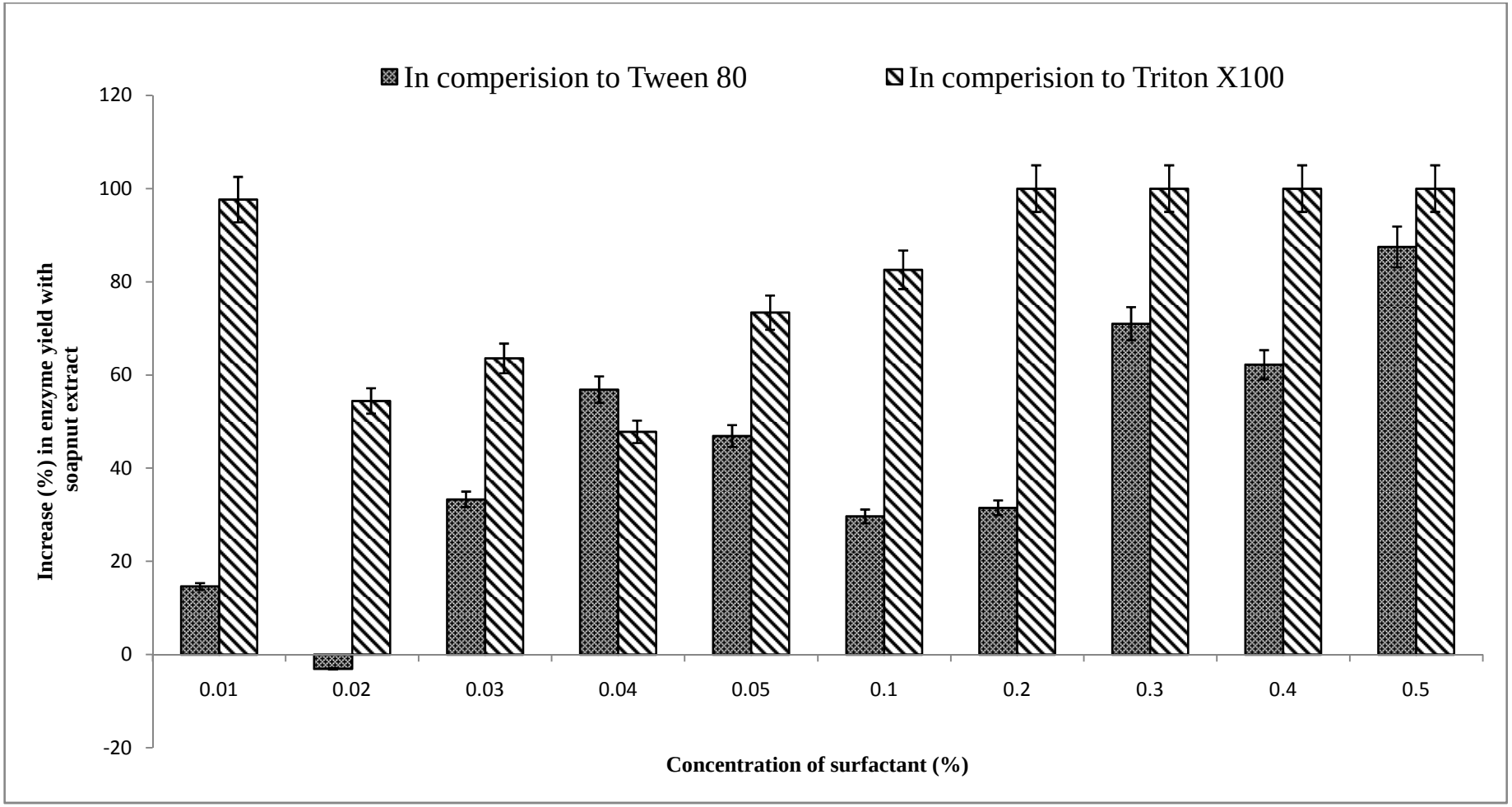


Fig 4.11: Percent increase in laccase yield upon addition of soap-nut extract in comparison to Tween 80 and Triton X 100, from the fungal isolate WDP2.

4.6.6 Effect of Agitation and Inducer (Veratryl Alcohol)

Ligninolytic systems of white-rot fungi were mainly activated during the secondary metabolic phase, when conditions for the growth of fungi are not good. The effect of agitation (aeration) and the veratryl alcohol on laccase enzyme production were checked using two types of media: (i) the initial modified carbon limited basal liquid medium (CLM) and (ii) optimised laccase production medium; with or without agitation and with or without veratryl alcohol. Modified Carbon Limited Basal Liquid Medium (CLM) was taken for the comparison. The results clearly indicated adverse effect of agitation and absence of inducer on the laccase enzyme production. Data indicated clearly that the maximum laccase activity (189.25 Uml^{-1}) was obtained in the optimised medium containing varatryl alcohol under static conditions at 12d incubation period, while in the same medium without inducer (veratryl alcohol), even under static conditions, the enzyme activity was significantly reduced to 10.908 Uml^{-1}; while the same optimised medium with inducer under shake conditions also showed significantly reduced enzyme production (100.66 Uml^{-1}) at the same time interval (**Table 4.11**). The enzyme activity was significantly reduced when CLM medium was used. In CLM medium maximum enzyme production (156.793 Uml^{-1}) was recorded in the presence of varatryl alcohol (VA) under static conditions while in the same medium even in the presence of VA under shake conditions, the enzyme activity was significantly reduced to 56.259 Uml^{-1}; while absence of inducer in the same medium under static conditions further reduced the activity to 9.67 Uml^{-1} (**Fig 4.12**). It may be concluded from the results that the media optimised during the present investigation was better in comparison to the initial synthetic medium (CLM) for enzyme production. The results further confirmed enhancement of laccase activity by VA indicating it as an inducer for the expression of laccase enzyme. Moreover, it is also apparent from the data that the enzyme is induced under oxygen stress conditions and that too at the later phase of the growth (12-15d). Significantly lower enzyme production in the absence of inducer (VA) in both the cases indicated the inducible nature of laccase enzyme secreted by the fungal isolate WDP2. It also appears that the enzyme synthesis is repressed by agitation even in the presence of inducer indicating oxygen stress induced synthesis of the enzyme (**Fig 4.12**).

Veratryl alcohol is secondary metabolite firstly described in lignin degradation by the wood rot fungi *Phanerochaete chrysosporium*. The addition of veratryl alcohol in the production media has also been reported to exert a positive effect on synthesis of laccase enzyme by several lignolytic fungi (**Vasconcelos *et al.*, 2000**; **Patel *et al.*, 2009**). Enhancement of laccase activity from various fungi upon addition of veratryl alcohol in low amount has also been well documented (**Dekker and Barbosa, 2011**). Considering the fact that veratryl alcohol acts as an inducer only at low concentrations, we had also used it at low concentrations and achieved induction of the enzyme. Ability of varatryl alcohol to act as an enzyme inducer at low concentration might be correlated with the retardation of the fungal growth, lower biomass production and inhibition of laccase enzyme as at high concentration it acts as potential inhibitor for laccase enzyme. Although **Flores *et al.* (2009)**, reported enhancement of the laccase production in shake cultures with better aeration, but our results we could observed an adverse effect of agitation on laccase production by the fungal isolate WDP2, our results indicated that laccase from WDP2 isolate was an inducible enzyme that is sensitive to aeration and is also induced better under oxygen stress conditions. Laccases have been reported by several workers to be produced as secondary metabolites during the secondary metabolic phase of different fungi (**Gayazov and Rodakiewicz-Nowak, 1996; Gochev and Akrastanov, 2007**). Our results are also in agreement with these findings as the laccase production was observed during the later growth phase (12-15d). Since agitation of the fermentation medium may lead to the normal growth phase, hence decreased laccase production, while during static phase due to limited oxygen supply, the secondary growth phase was achieved in which the enzyme is better expressed.

4.7 Extracellular Laccase Enzyme Activity under Optimised Culture Conditions

After optimization of culture conditions, all the four selected wood rot fungal isolate along with the standard culture of (*P. chrysosporium*) were tested again for their abilities to produce the laccase enzyme under the optimised conditions at 12 d of incubation period. A significant difference in the laccase enzyme activities among various fungal isolates was recorded. Data indicated the superiority of the wood rot fungal isolate WDP2 over other fungal cultures (**Table 4.12**). The results indicated that maximum

laccase enzyme activity (189.272 Uml^{-1}), productivity (657.195 $UL^{-1}h^{-1}$), and yield (9463.610Ug^{-1}) were obtained from the fungal isolate WDP2; followed by the isolate AAFP5 showing 176.040 Uml^{-1} enzyme activity, 611.250 $UL^{-1}h^{-1}$ productivity, 8802.00 Ug^{-1} enzyme yield. Minimum enzyme activity (0.2984 Uml^{-1}) was recorded for the standard culture (*P. chrysosporium*), that was significantly lower than that observed for other cultures. In comparison to synthetic medium a significant increase in enzyme production under optimum conditions was observed. The results indicated that the laccase enzyme activity increased by 8.11 %, 17.16 %, 15.68 %, and 19.62% for the wood rot fungal isolates WFPn1, WDP2, AAFP5, and TAFP41, respectively, in optimised conditions while a significant relative enzyme activity increase of 44.52% was observed using standard fungal culture of *P. chrysosporium* (**Fig 4.13**).

During present study maximum laccase production was obtained after 12d incubation period. Although, different incubation periods have been reported for laccase production by different workers, but most of the studies have reported longer optimum period (12-15d) for enzyme production as observed by us during present study (**Saito *et al.*, 2003; Zouari-Mechichi *et al.*, 2006; Forootanfar *et al.*, 2011**). Although bacterial cultures have been reported to produce laccase within very less incubation period, butfungi are considered as potential organisms for laccase enzyme production.

Table 4.11: Influence of the exogenous inducer (veratryl alcohol) on laccase production by the fungal isolate WDP2 under static/shake culture conditions

S. No.	Culture Condition	Enzyme activities at different time intervals (d)				
		3	6	9	12	15
1	CLM*+VA**+Agitation	0.21	0.41	0.125	25.833	56.259
2	CLM +VA+NoAgitation	0.083	0.330	37.589	109.009	156.793
3	CLM -VA+No Agitation	0.002	0.004	3.32	8.92	9.67
4	Opt***+ VA+Agitation	0.666	3.083	83.833	100.66	80.66
5	Opt+VA+No Agitation	0.144	5.75	65.25	189.25	160.35
6	Opt-VA+ No Agitation	0.003	0.0059	2.385	10.908	8.82
Factors		Time (a)	Culture (b)	Interaction (a*b)		
S.Em.±		0.037	0.038	0.102		
CD (5%)		0.125	0.149	0.401		

* Modified Carbon Limited Basal Liquid Medium (used for the laccase enzyme production)

** Veratryl alcohol (Inducer for laccase production)

*** Optimised medium (used for laccase enzyme production)

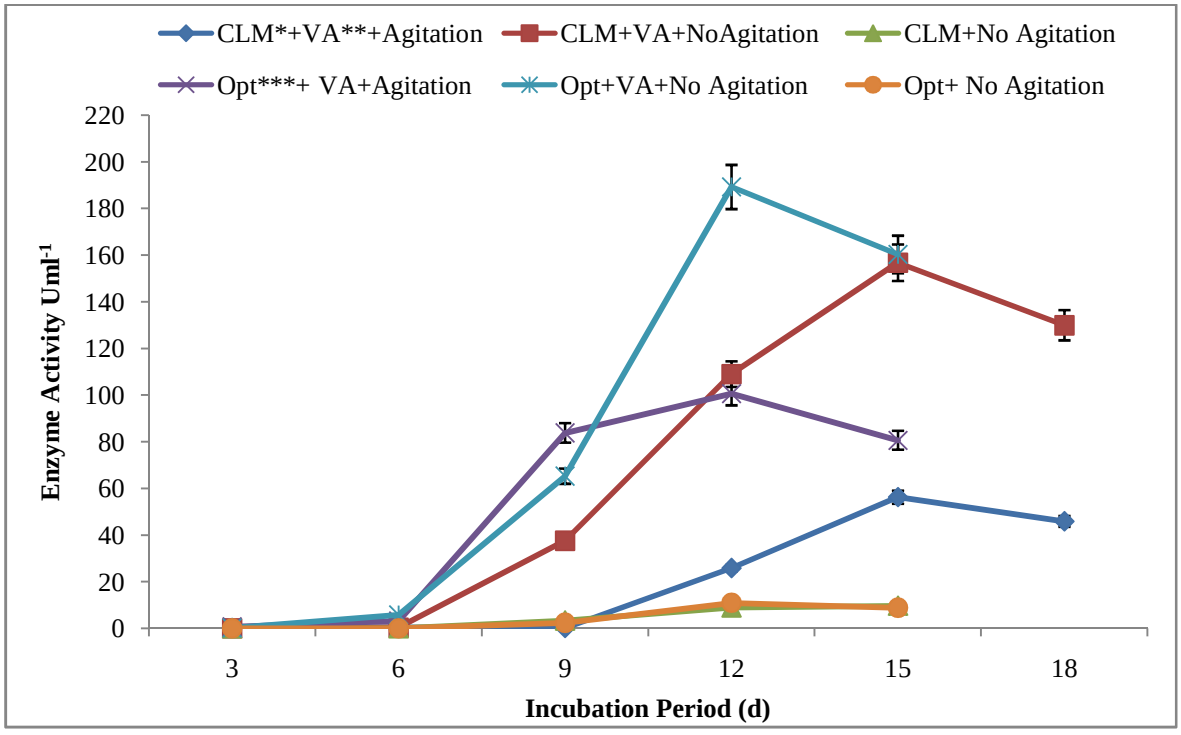


Fig 4.12: Effect of agitation and inducer (veratryl alcohol) on laccase production using wood rot fungal isolate WDP2

Table 4.12: Comparative evaluation of lignocellulolytic fungal cultures for laccase enzyme activity under optimised culture conditions

Cultures	Enzyme Activity ($U\ ml^{-1}$)	Productivity ($UL^{-1}h^{-1}$)	Yield ($U\ g^{-1}$)
WFPn1	170.490	591.978	8524.483
WDP2	189.272	657.195	9463.610
AAFP5	176.040	611.250	8802.000
TAFP41	143.739	499.095	7186.967
P. chrysosporium	0.298	1.036	14.917
SEm±	0.347	1.204	17.404
CD (5%)	1.093	3.793	54.82

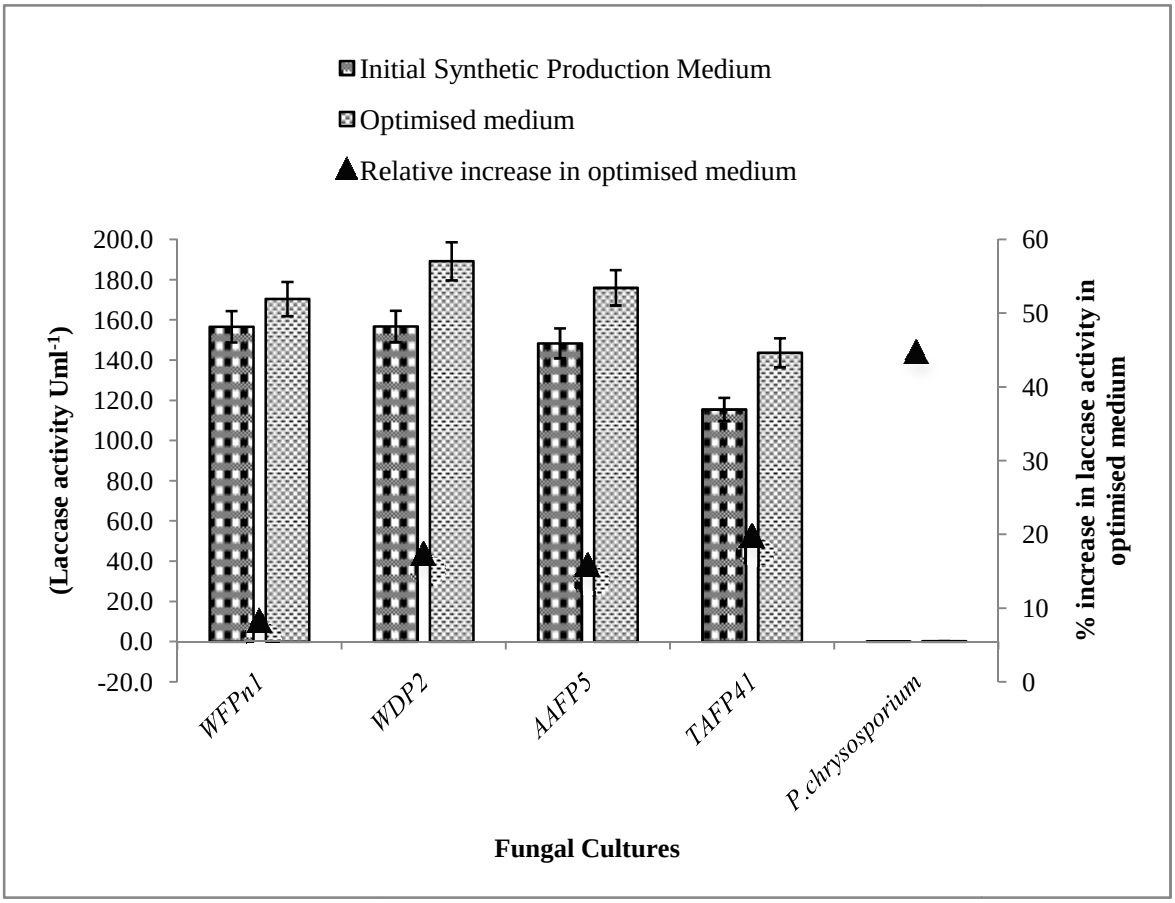


Fig 4.13: Percent increase in laccase activity by the fungal isolate WDP2 in optimised medium as compared to that in initial synthetic medium.

4.8 Characteristics of Laccase Enzyme

The fungal isolate WDP2 showing maximum enzyme producing efficiency was selected for further studies. The partially purified laccase enzyme was characterised for enzyme activity and stability at different pH and temperature conditions.

4.8.1 pH Optima

The effect of the pH on the laccase enzyme activity was examined at various pH values ranging from pH 2.0 to pH 10.0. The enzyme from the fungal isolate WDP2 exhibited the maximum activity profile at highly acidic pH values (**Table 4.13 a**). The enzyme was optimally active at pH 3.0 with the highest enzyme activity of 189.25 Uml^{-1}, followed by subsequent decrease in enzyme activity with increasing pH (**Fig 4.14**). The enzyme activity continuously decreased up to pH 6.5 (1.644 Uml^{-1}), thereafter it became 0.0. The results are close to those of various other findings on the laccase characterization. The optimum pH of enzyme has been reported to be highly influenced by the type of substrate used. With phenolic substrate, the optimum pH can range from 3.0 to 7.0 for fungal laccases and up to 9.0 for the laccases produced by plants (**Heinzkill *et* al., 1998**; **Madhavi and Lele, 2009**). It has been also reported that with ABTS as substrate, the optimum pH for laccase activity is more acidic (pH 3.0-5.0). **Atalla *et al.* (2013)** reported that the laccase enzyme purified from *Trematosphaeria mangrovei* showed maximum activity at acidic pH (pH 4.0), above or below this pH, the enzyme activity decreased. Similarly, during present study also, the laccase from the fungal isolate WDP2 was found to have acidic pH as optima of pH 3 and after pH 6.0, the enzyme showed complete inactivity. The maximum ability attained at acidic pH might be due to the higher stability of the enzyme in acidic conditions as repeated earlier also for good stability of *T. atroviride* laccase in acidic pH values (**Chakroun *et al.*, 2010**). The higher stability of enzyme in acidic condition is a rare property among fungal laccaseas laccase from the most of the fungi have high solubility at neutral or slighi alkaline pH values (**Kiiskinen *et al.*, 2002; Palmieri *et al.*, 2003; Ben Younes *et al.*, 2007**).

4.8.2 Temperature Optima

Each enzyme has an optimum temperature requirement to show the maximum enzyme activity. The impact of temperature on the laccase enzyme activity was determined at different temperatures ranging from 20^0C to 100^0C. Among various temperatures tested, 50^0C was found to be the optimum showing maximum laccase enzyme activity (**Table**

4.13 a). On either side of the 50^0C, there was decline in enzyme activity. The optimal temperature requirement for laccase activity varies from organism to organism. Results indicated clearly that the extracellular laccase enzyme from the fungal isolate WDP2 has a wide temperature range between 40^0C to 90^0C, during which a fairly high activity was retained by the enzyme (**Fig 4.15**). The enzyme retained its more than 70% activity even after increasing the temperature from 70^0C to 100^0C. Thus results clearly revealed the thermophilic nature of the laccase enzyme secreted extracellularly from fungal isolate WDP2. Previous workers have also reported that pre-incubation of enzymes at 40°C and 50°C greatly increased laccase activity (**Farnet *et al*., 2000 and Kunamneni *et al*., 2007**). However, the optimal temperature of laccase can differ greatly from one strain to another. The high temperature optima (40^0C to 90^0C) as observed by us for the laccase from from the isolate WDP2, the laccase from *P. ostreatus* is almost fully active in the temperature range between 40°C-60°C, with a maximum activity at 50°C (**Palmeiri *et* al., 1993)** Furthermore they checked the stability and found that the activity remains unaltered after prolonged incubation at 40°C for more than 4 h.

4.8.3 pH Stability

To determine pH stability of laccase enzyme from the fungal isolate WDP2, the enzyme activities were evaluated at different pH values (4.0 to 10.0). The laccase enzyme from fungal culture WDP2 revealed good stability towards acidic pH conditions (pH 4.0 and 5.0) (**Table 4.13, b**). It is appeared from the figure (**Fig 4.16**), that the laccase enzyme from WDP2 retained more than 90% of its activity at the pH values of 4.0 to 5.0, while a gradual decline in stability of the enzyme was observed up to pH 7.0, and becoming 0.00 at pH 8.0 up to pH 10.0. In alkaline environment, the enzyme was totally degraded or deactivated as shown by no (0.00) residual enzyme activity. Maximum laccase enzyme activity observed at the pH 3.0, thus the higher activity/stability of the enzyme at acidic pH values showed acidophilic nature of enzyme. Our results are agreement with previous reports showing acidiphilic nature of laccase enzyme (**Saito *et al*., 2003; Zouari-Mechichi *et al*., 2006; Stoilova *et al*., 2010**). The same pH stability pattern as observed by us during present investigation has also been reported previousy for laccase enzyme from marine fungus fungus *Trematosphaeria mangrovei* that was adversally affected at pH 5.5-6.0, (**Atalla *et al*., 2013**). However, broad pH range stability of laccase enzyme has also been reported from the thermophilic ascomycetus fungus *Chaetomium thermophilium*, but most of the studies reveal the acidic stable nature of the laccase enzyme (**Chefetz *et al*., 1998**).

4.8.4 Thermal Stability

Most of the enzymatic industrial processes such as enzymatic delignification and enzymatic saccharification, are generally carried out at the high temperatures (more than 50^0C), and, therefore, require preferentially thermostable enzymes. Although most of the enzymes are protein in nature, thus are highly sensitive to high temperatures as get denatured losing their catalytic activities. Therefore, the extracellular laccase enzyme from the isolate WDP2 was further evaluated for its thermal stability at the temperature range of 40-100^0C. During present study, the enzyme was incubated at different temperature ranges and checked for the relative laccase enzyme activity within half an hour and within one hour of incubation period. It is appeared from the results that the enzyme retained 100% laccase activity at 40^0C and >95% activity at 50^0C for one hour (**Fig 4.17**). Further at the temperature of 60^0C the laccase enzyme showed 76% relative laccase activity after half an hour; thereafter declined to 72.6% when incubation time was increased to one hour (**Table 4.13, b**). The findings showed the thermostable nature of the laccase enzyme from fungal isolate WDP2 indicating its possible application in industrial fermentation processes that require high temperature (up to 60^0C). However, at and above 70^0C, the laccase enzyme completely lost its activity only after half an hour of incubation period. The results thus, indicated clearly that above 60^0C, the enzyme protein completely gets denatured, hence completely inactivated at these high temperatures temperatures. It may be concluded from the results that laccase enzyme production by the fungal isolate WDp2 is a thermostable acidophilic enzyme.

Thermostable enzyme are classified mainly as moderately thermostable (45-60^0C) thermostable (60-80^0C), and extremely thermostable (>80^0C). It was observed that depending upon the source microorganism, the thermal stability varied at different temperatures. Currently, laccases from most wood rot fungi show a high thermal stability at acidic pH range of 2.0-4.5 (**Sadhasivam *et al.*, 2008**). The higher stability of the enzyme at acidic pH values may also be correlated as the acidic pH optima of the enzyme showing higher activity under acidic conditions. Many other reports have shown the laccase enzyme from *Trichoderma harzianum* retained 70% of its initial activity after 1 h incubation at 55^0 C; moreover, the laccase from *Daedalea quercina* (wood rot mushroom in the Polyporales order) was very stable at or below 45^0C (**Sadhasivam *et al.*, 2008**).

4.8.5 Storage Stability: Shelf Life of Enzyme

For industrial application of the enzyme, longer shelf life is highly desirable, so that the enzyme can be used cost effectively for carrying out delignification of lignocellulosic biomass. Therefore, to determine the storage stability or shelf life of enzyme, the laccase enzyme produced from fungal isolate WDP2 in optimised medium was stored at 4^0C in a refrigerator for two months of period. The laccase enzyme showed 76.27% residual laccase activity (144.35 Uml^{-1}) after an incubation period of 7d (**Table 4.13, b**). Further increase in storage period from 7d, the enzyme activity started to decline with residual activity of 43.33% (82Uml^{-1}) on 14d of incubation period. Thereafter the enzyme activity was nearly constant (residual activities range from 36.99 to 31.97%) up to 56d of incubation period (**Fig 4.18**). Reports are there showing shelf life of 25d for the laccase of *Cerrena unicolor* (abasidiomycetous fungi) (**Nam–Seok *et al.*, 2008**). Upon storage, the laccase activity decreased gradually as incubation time was increased. It was concluded from the study that the enzyme was stable for a long period of time (up to 2 months), retaining ~40% enzyme activity at 4^0C.

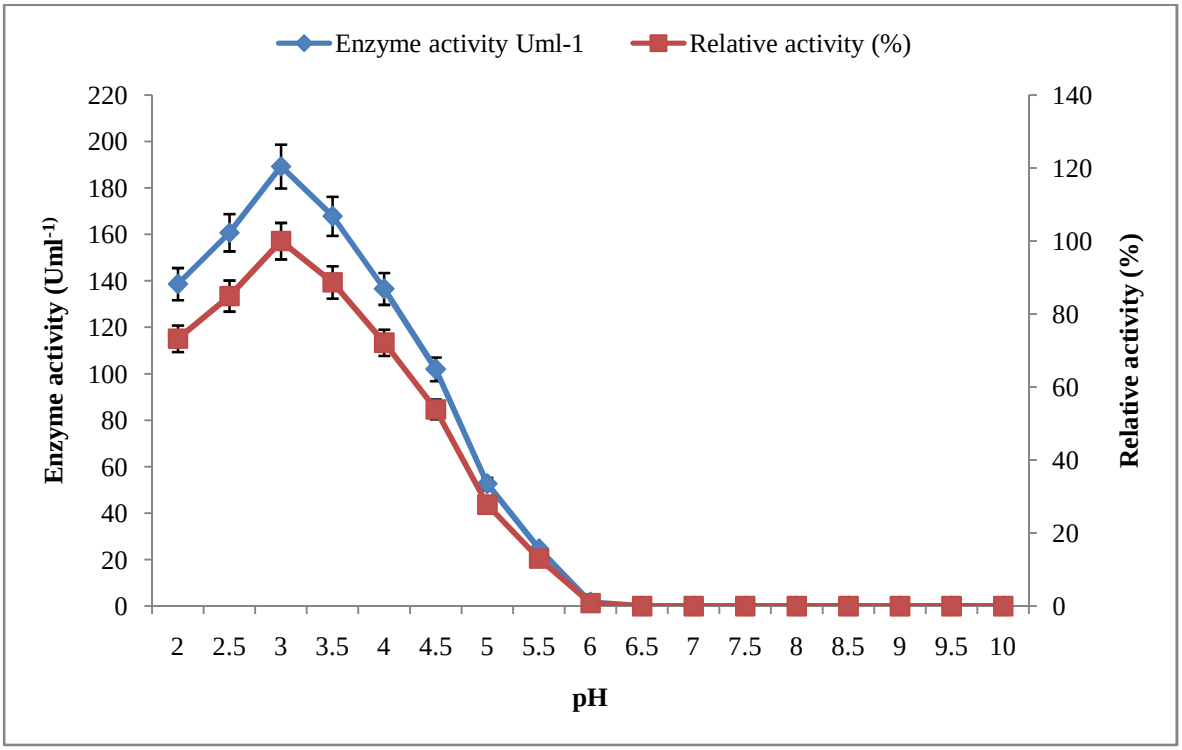


Fig 4.14: Impact of pH on activity and relative activity of extracellular laccase enzyme produced by the fungal isolate WDP2

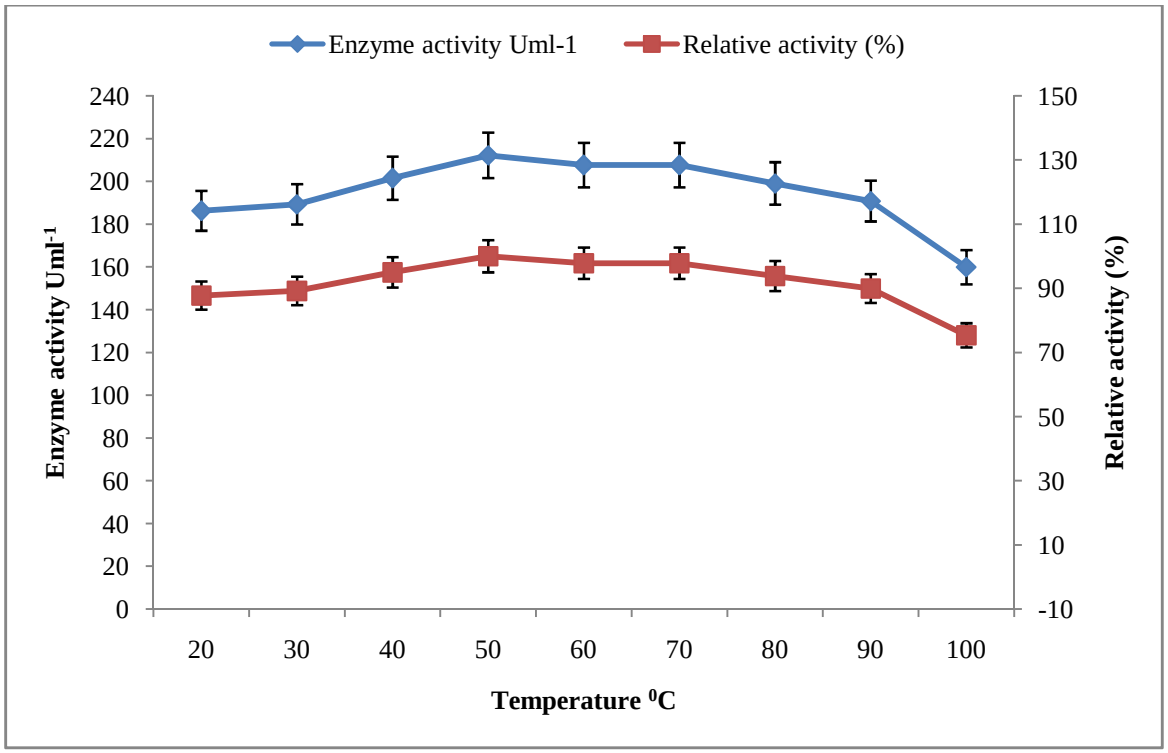


Fig 4.15: Impact of temperature on activity and relative activity of extracellular laccase enzyme produced by fungal isolate WDP2

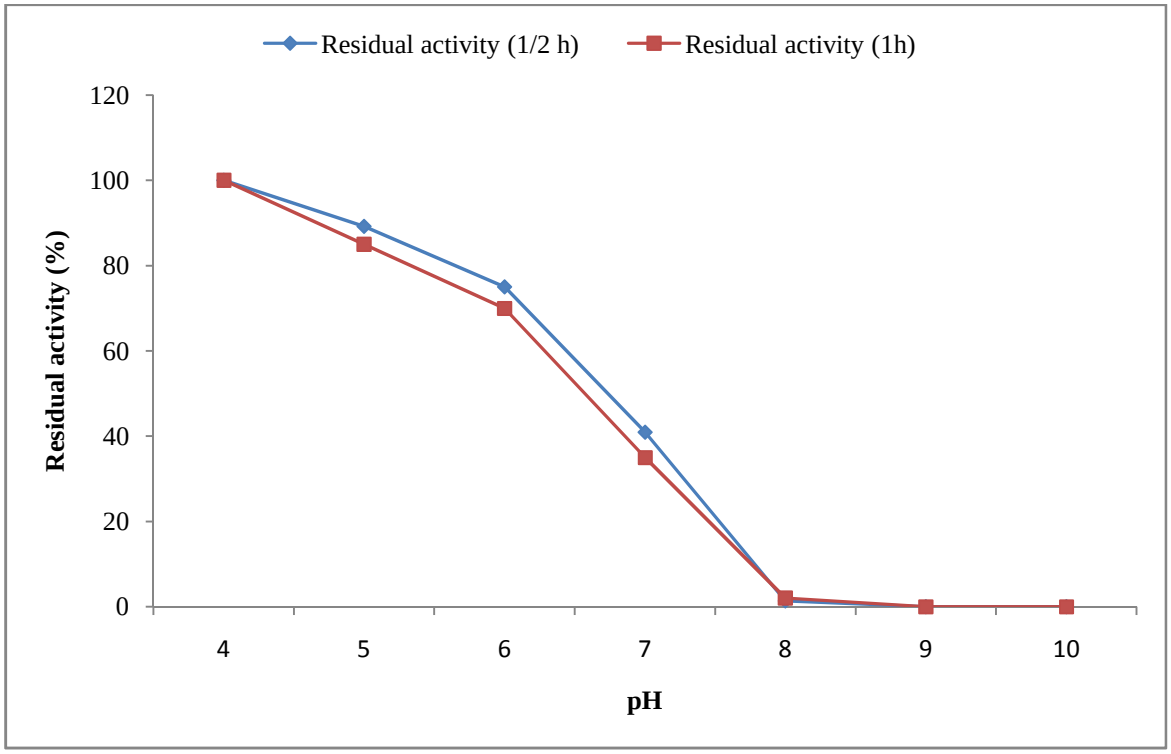


Fig 4.16: Effect of pH on the stability of the extracellular laccase enzyme from fungal isolate WDP2

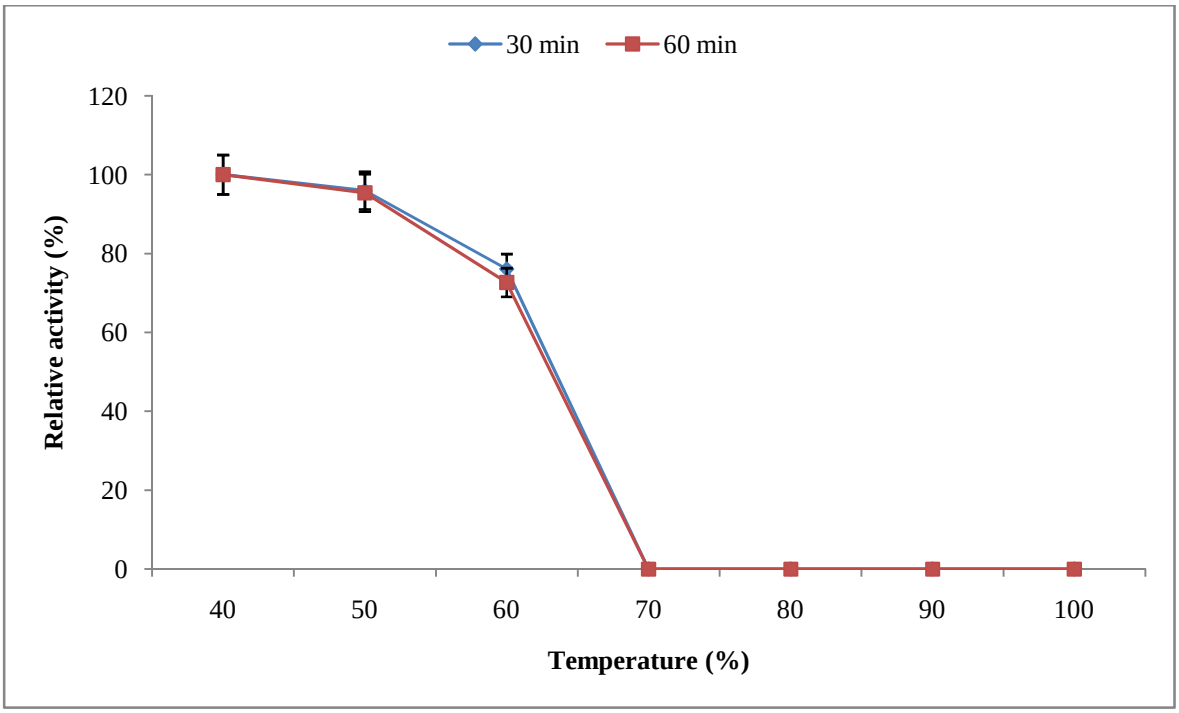


Fig 4.17: Effect of temperature on stability of the extracellular laccase enzyme from the fungal isolate WDP2

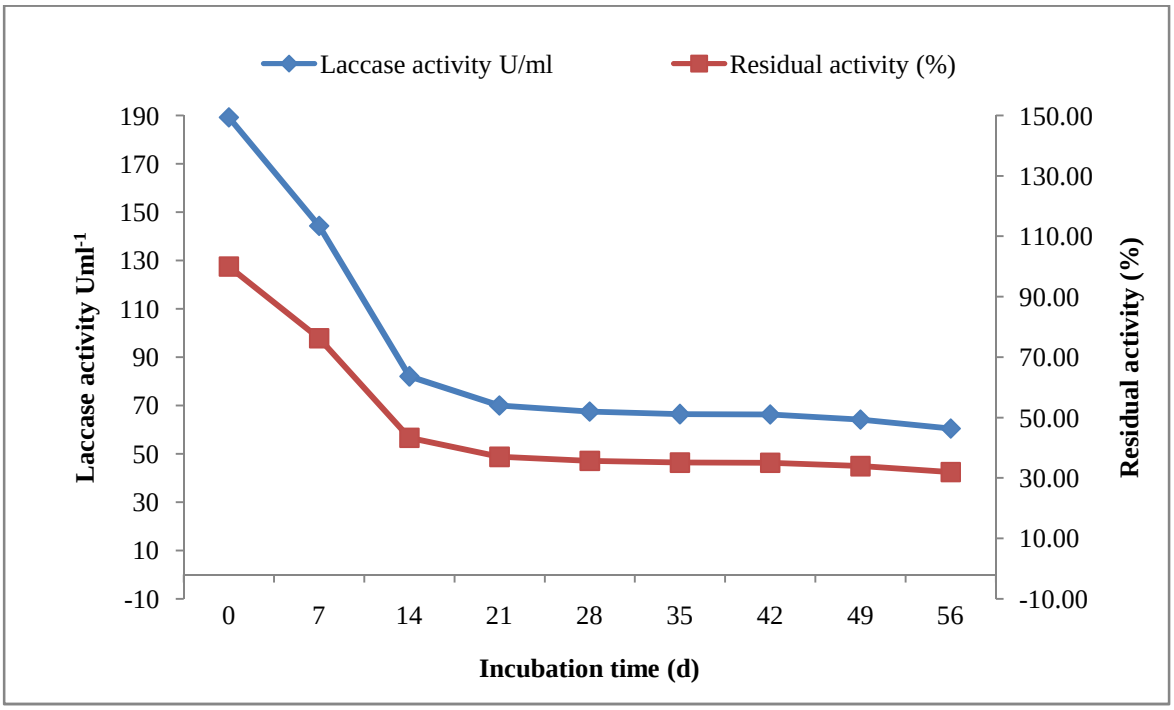


Fig 4.18: Storage stability of extracellular laccase enzyme from fungal isolate WDP2 stored at 4^0C in a refrigerator

Table 4.13 (a): Effect of pH and temperature on activity of extracellular laccase enzyme produced by fungal isolate WDP2

S.No.	Effect of pH		Effect of Temperature	
	pH	Laccase activity (Uml^{-1})	Temperature	Laccase activity (Uml^{-1})
1	2.0	138.66	20	186.23
2	2.5	160.8	30	189.28
3	3.0	189.25	40	201.50
4	3.5	167.85	50	212.18
5	4.0	136.58	60	207.60
6	4.5	102.01	70	207.60
7	5.0	52.647	80	199.04
8	5.5	24.684	90	190.81
9	6.0	1.644	100	159.90
SEm±		2.387		1.993
CD@5%		3.99		2.58

Table 4.13 (b): Effect of pH, temperature and storage on stability extracellular laccase enzyme produced by fungal isolate WDP2

S.No.	Effect of pH			Effect of Temperature			Effect of Storage	
	pH	Relative activity (%)		Tempe -rature	Relative activity (%)		Time period (d)	Relative activity (%)
		½ h	1h		½ h	1h		
1	4.0	100	100	40	100	100	0	100.00
2	5.0	89.22	85.00	50	95.97	95.39	7	76.27
3	6.0	75.00	70.00	60	76.06	84.83	14	43.33
4	7.0	40.89	35.02	70	0.00	0.00	21	36.99
5	8.0	1.33	2.01	80	0.00	0.00	28	35.67
6	9.0	0	0.00	90	0.00	0.00	35	35.09
7	10.0	0	0.00	100	0.00	0.00	42	35.05
SEm±		1.274	1.66		1.125	0.987		1.22
CD@5%		1.869	2.34		1.494	1.029		2.22

4.9 Molecular Characterization of Laccase Enzyme

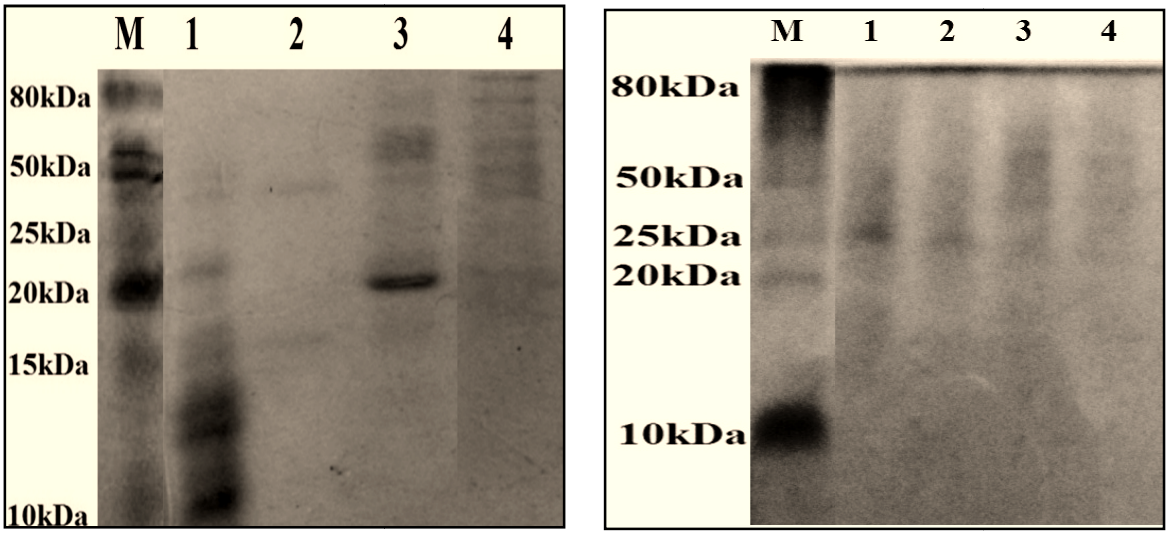
4.9.1 Native/SDS PAGE Analysis

The three potential laccase producing fungal isolates (WFPn1, WDP2, and AAFP5) and standard culture of *Phanerochaete chrysosporium* were selected for laccase enzyme production. The extracellular enzymes were partially purified using chilled acetone precipitation followed by dialysis using 50 mM citrate buffer (pH 3.0). The partially purified laccase enzymes were then used for extracellular protein profiling. The molecular masses of extracellular proteins were determined through SDS-PAGE (**Plate 4.6, a**) and Native-PAGE (**Plate 4.6, b**). The molecular weight of various extracellular proteins varied from 10 to 90kDa. The extracellular protein profiling revealed clarly the presence of different types of proteins that could be visualised on gel. The isolates also showed protein bands with differential intensities that indicated expression of different extracellular proteins with different rates. The lowest number of bands were produced by the standard culture of *P. chrysosporium* (**Plate 4.6, a & b, Lane 2**). Although, *P. chrysosporium* is a well known lignolytic fungi, but, the lower intensity banding pattern might be correlated withthe less efficiency of the strain for laccase production as is also evident by lowesr enzyme (laccase) activity shown by the culture even under optimised conditions.

4.9.2 Laccase Isozyme Analysis

The polyacrylamide gel analysis under non-denaturing conditions (Native-PAGE) through activity staining was used to identify and determine the molecular weight of the laccase enzymes from the selected fungal isolates and compared with standard culture (*P. chrysosporium*). The extracellular protein samples when analysed for laccase enzyme using ABTS *i.e.* 2, 2-azinobis (3-ethylbenzthiazoline-6- sulfonic acid) as a substrate during native-PAGE, different number of green coloured bands were obtained for different fungal strains. Zymogram analysis clearly indicated the production of only one isoform of laccase enzyme by wood rot fungal isolate WFPn1 (**Plate 4.7, lane 1**). The molecular mass of native laccase from fungal isolate WFPn1was found ≈50 kDa. The wood rot fungal isolate AAFP5 was observed to produce two different isoforms of laccase enzyme, and their molecular weights were found to vary from (about) 50 kDa to 75 kDa (**Plate 4.7, lane 2**).

The most potential wood rot fungal isolate WDP2 that produced maximum laccase enzyme activity under optimised culture conditions clearly showed the production of three different isoforms of laccase enzyme on native gel during Zymogram analysis. The molecular masses of native laccase isoforms from the fungal isolate WDP2 varied from ≈48 kDa to 75 kDa. Zymogram analysis revealed three active bands of laccase isozymes from the fungal isolate WDP2, corresponding to the molecular size of about 48 kDa, 60 kDa, and 70 kDa (**Plate 4.7, lane 3**). During quantification of laccase enzyme, activity also, the standard culture of *P. chrysosporium* was not observed for significant production of laccase enzyme. A very light laccase enzyme active band of molecular mass near about 70 kDa was observed in case of *P. chrysosporium* (**Plate 4.7, lane 4**). Generally, the laccase enzyme produced by fungi while our results were in the agreement with **Edens *et al.* (1999)** and **Imran *et al.* (2012)**. They have been reported to have molecular weight of laccase ranging from 50-100kDa. The production of laccase and other related enzymes during liquid fermentation, having the same size range of protein bands. **Heinzkill *et al.* (1998)** have also reported the molecular weights of most of the white rot fungal laccases in the range of 60 KDa to 390 kDa. In our findings, the molecular weights of laccase enzymes from all the selected fungal strains varied from 48 kDa to 75 kDa. As accordance with the earlier reports, we also identified the laccase enzyme from the selected fungal cultures with molecular weight in the range of 45-75kDa (**Plate 4.7**). The isolate WDP2 thus, could be identified as the moist potential laccase producing organism both on the basis of enzyme activity and the number of isoforms produced.



(a) **(b)**

Plate 4.6: SDS PAGE analysis (a) and Native PAGE analysis (b) of extracellular proteins of various lignolytic fungal cultures; Lane (M) protein marker; lane (1) isolate WFPn1, lane (2) *P. chrysosporium*, lane (3) isolate WDP2, and lane (4) isolate AAFP5.

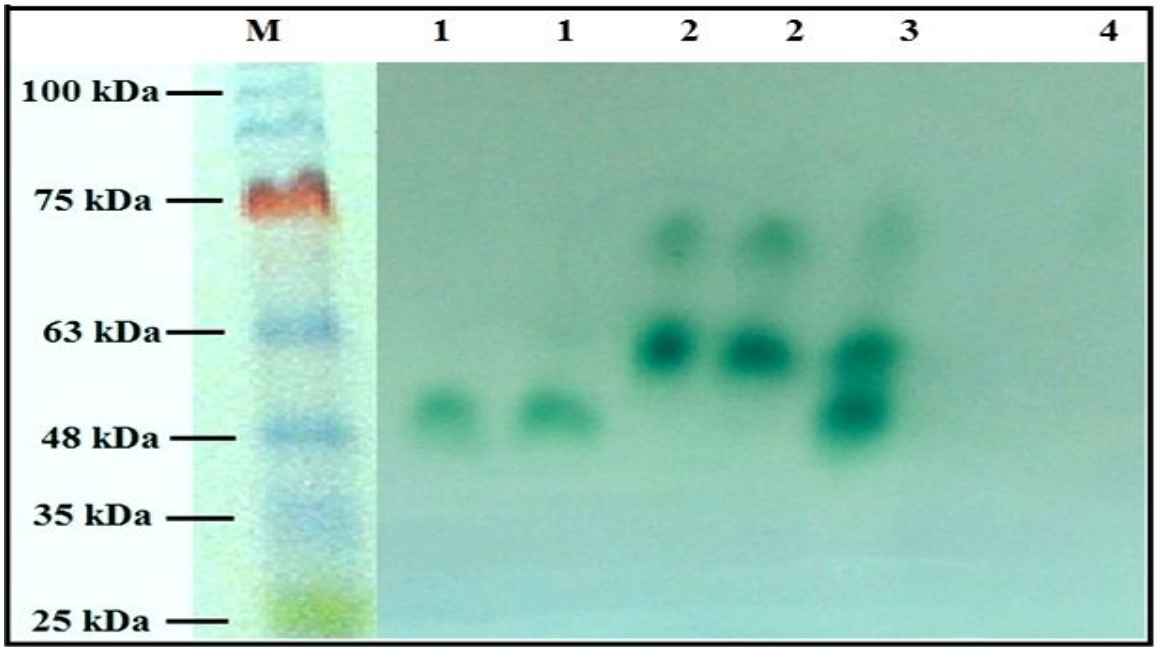


Plate 4.7: Zymogram analysis of extracellular laccase enzyme produced by the selected fungal isolates. Lane (M) protein marker, lane (1) isolate WFPn1, lane (2) isolate AAFP5, lane (3) isolate WDP2, and lane (4) *P. chrysosporium*.

UNIT III: LIGNOCELLULOSIC BIOMASS: SELECTION &PRETREATMENT

4.10 Physical Processing of Biomass

Bamboo currently considered as an interesting and potential feedstock for advanced production of bioethanol due to the natural biomass abundance, a very rapid growth in marginal lands or poor nutrition land, perennial nature and low requirement for management once planted (**Littlewood *et al*., 2013**). The cell wall of bamboo wood like as in other lignocellulosic biomasses is comprised of the hexose polymeric constituents (cellulose), pentose polymeric substance (hemicellulose) and polyphenoloic recalcitrant constituent (lignin). The tough and complex physical and chemical interactions among these three main cell wall components prevent the accessibility of cellulolytic enzymes for microfibrillar cellulose during the saccharification stage of the conversion into bioethanol; therefore a proper pretreatment technology is required (**Zhao *et al*., 2012; Littlewood *et al*., 2013**). However, the lignocellulosic biomass drawn out from different trees/plants show significant differences in their chemical constituents. Sometimes the differences are very prominent, affecting the potentiality of the substrate for its bioconversion into fermentable sugars. Considering these facts, four different bamboo spp. *viz*., *Bambusa balcooa*, *Dendrocalamus hemiltonii*, *Bambusa nutans*, *Dandrocalamus asper* were taken from Agroforestry Research Centre, G.B. Pant University of Agriculture and Technology Pantnagar, Uttarakhand, India, and immediately weighted as fresh weight for moisture content calculation and further processing.

Brief summary of the processing of bamboo biomass from different genotypes under studu is given in **Plate 4.8**. The bamboo stem from each genotype was first chopped into small pieces and then dried completely in a hot air oven at 65 ± 1^0C. The drying was continued till the constant weight had achieved for each wood sample. Dry weight was used for moisture content calculation and the samples were separately converted in to fine powder (~1-2 mm diameter) using locally available electronic wheal saw (small sawmill). The fine powder was used further compositional analysis.

For efficient bioconversion of lignocellulosic biomass, it is required that before proceeding for any pretreatment, the woody substrate should be physically processed and converted into fine powder through milling or comminution. The process is used to reduce particle size of lignocellulosic biomass, typically applied to most or approximately all biofuel production processes to improve feedstock handling and treatment (**Zheng *et al*., 2014)**. Therefore, first of all drying (sun drying followed by oven drying at 60-70^{0}C) of biomass is reported by many studies to remove total moisture content. After drying, particle size reduction of lignocellulosic biomass is often needed and the particle size must be reduced to or below 1-2 mm to eliminate such limitations for biomass hydrolysis (**Schell and Harwood, 1994**). However, sometimes size reduction is a very expensive operation and may be a limitation for hydrolysis, as it consumes about 33% of the total electricity demand for the whole bioethanol production process (**Zheng *et al*., 2014**).



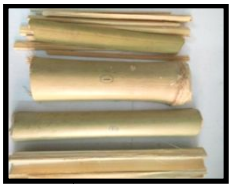
(Bamboo field)



Bamboo stems brought to laboratory



Cutting and chopping



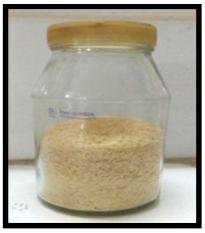
Splitting of samples



(Oven drying & moisture content analysis)



(Grinding to prepare fine powder using electronic wheel saw)



(Fine wood powder (1-2mm) stored in air tight container)

Plate 4.8: Processing of Bamboo biomass from forest to laboratory for bioprocessing studies

4.11 Selection of Potential Bamboo sp.

For any type of lignocellulosic substrate to be a potential source for fermentable sugars for bio-alcohol production, the biomass yield and cellulose content should be high, whereas the moisture, ash and lignin content should be relatively low. Therefore dried powdered biomass of all the four bamboo samples was evaluated for its chemical composition. Out of four bamboo genotypes tested during the study, potential bamboo sp for bioethanol production was selected on the basis of biomass yield, moisture content, cellulose content and lignin content.

4.11.1 Biomass Yield

From each grass-tuft, one complete stem was randomly selected after removing leaflets, chopped into small pieces and weighed. The fresh biomass yield per stem varied from a minimum of 4.13 kg. for *Dandrocalamus asper* to a maximum of 45.87 kg for *Bambusa balcooa* (**Table 4.14**). *Bambusa nutans* also gave good biomass yield (37.89 kg. per branch) followed by *Dendrocalamus hemiltonii* which gave 25.41 kg. Fresh biomass yield per stem. All the samples were taken to laboratory and dried in an oven at 65 ± 2^{0}C till constant weight and moisture content was determined.

4.11.2 Moisture Contents

The total available amount of organic biomass that can be used for bioenergy production depends on moisture content of the wood. The moisture content of any sample was analysed as percentage of moisture present in biomass and calculated using the fresh weight of wood and oven dried final weight of wood. It was observed from the study that half of the fresh weight of *Bambusa balcooa* and *Dendrocalamus hemiltonii* comprised moisture in it. The moisture content for different bamboo genotypes varied from 5.48% for *Dandrocalamus asper* to 66.66% for *Dendrocalamus hemiltonii* (**Table 4.14**). The bamboo genotype *Bambusa balcooa* showed 51.31% of moisture and *Bambusa nutans* contained 46.66% of the moisture. Thus significant differences in the moisture content in the biomass of different genotypes of bamboo wood were recorded. Moisture content of the lignocellulosic biomass is an important factor that can affect the reducing sugar yield **Evanick and Bura (2011)**. Biomass with low moisture can preferably used for the industrial production of bioethanol, because of high biomass yield. Removal of moisture from the biomass is also very important to prevent microbial contamination, and thereby increasing storage stability of biomass (**Bassam, 1998**). Although *Dandrocalamus asper*

seems to be most prominent biomass, but the biomass availability (4.13 kg/stem) compared to *Bambusa balcooa* that showed highest fresh weight (45.87 kg/stem). Furthermore, other factors such as lignin and cellulose contents are more important to be considered. The biomass yield per stem (45.87 kg.) from bamboo genotype *Bambusa balcooa* was significantly very high with moderately low moisture content (51.31%). Thus it seems to be a suitable substrate to be used as a source of fermentable sugars for bioethanol production.

4.11.3 Chemical Composition

The dried powdered biomass from all the four bamboo genotypes was screened for their cellulose, lignin, hemicellulose and ash contents. Only fine wood powder was used for study. The cellulose content of various wood samples varied from 34.83% (*Bambusa nutans*) to 40.74% (*Bambusa balcooa*). No significant difference in the cellulose cotent between maximum biomass producing *Bambusa balcooa* (37.93%) and *Dendrocalamus hemiltonii* (36.74%), was observed (**Table 4.15**). The hemicellulose content of the all wood samples varied from 19.1% to 23.8% no significant difference in hemicellulose content of *B. balcooa, D. Hemiltonii, and B. nutans* was recorded. The lignin contents also varied among wood samples ranging from 25.5% (*Dandrocalamus asper*) to 37.8% (*B. nutans*). The lignin content in *Bambusa balcooa* was found to be 29.5% (**Fig 4.19**).

Table 4.14: Moisture contents of wood biomass from selected bamboo genotypes*

S. No.	Bamboo varieties/ genotypes	Weight of biomass/ stem (Kg.)	Fresh wt. (gm)	Dry wt. (gm)	Moisture content (%)
1	*Bambusa balcooa*	45.87	3830.00	1850.00	51.31
2	*Dendrocalamus hemiltonii*	25.41	1950.00	650.00	66.66
3	*Bambusa nutans*	37.89	1500.00	800.00	46.66
4	*Dandrocalamus asper*	4.13	775.00	500.00	35.48
SEm±		1.15	0.978	1.224	0.004
CD (5%)		3.75	3.991	3.190	0.014

*Samples were taken in triplicates and the average values were used for moisture content determination

Table 4.15: Chemical composition of wood biomass from selected bamboo genotypes used during present study

S.No.	Bamboo varieties/ genotypes	Cellulose Content (%)	Hemi-cellulose (%)	Lignin Content (%)	Ash Content (%)
1	*Bambusa balcooa*	37.93	22.6	29.5	0.018
2	*Dendrocalamus hemiltonii*	36.74	23.2	25.5	0.021
3	*Bambusa nutans*	34.83	23.8	37.8	0.014
4	*Dandrocalamus asper*	40.74	19.1	28.1	0.021
SEm±		0.49	0.2	0.21	0.0008
CD@ 5%		1.59	0.66	0.69	0.002

*Samples were taken in triplicates and the average values were used for compositional analysis

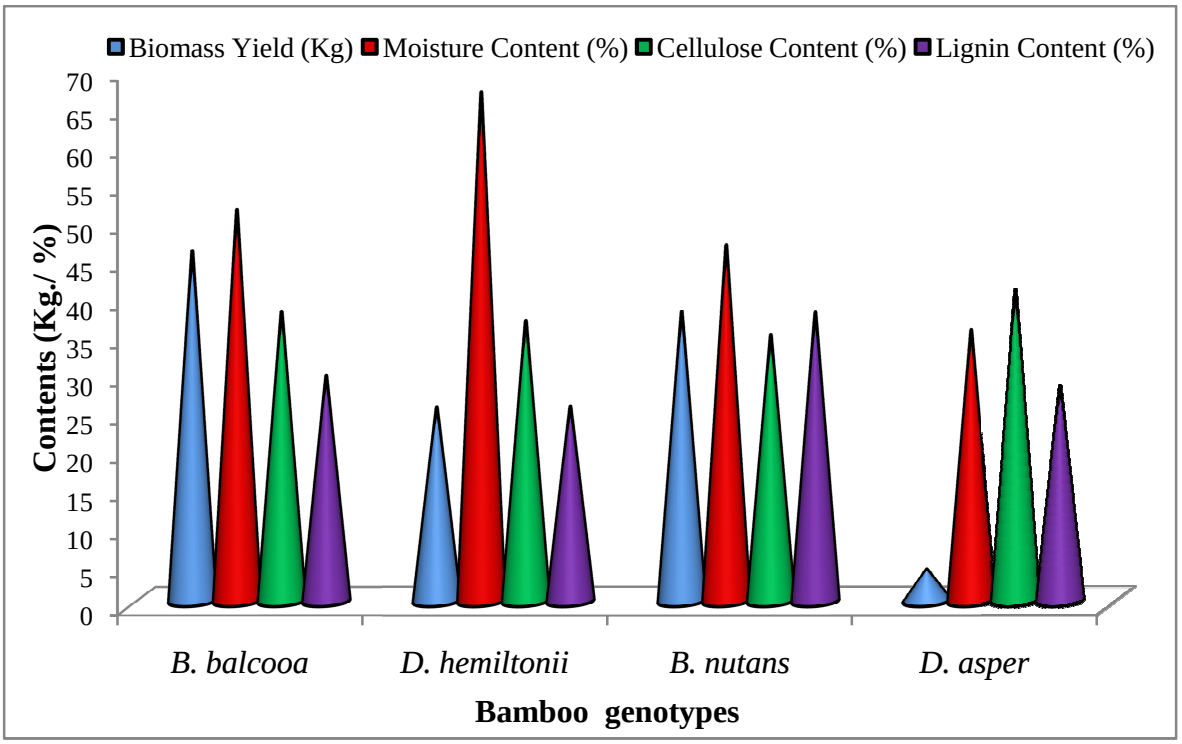


Fig 4.19: Comparative evaluation of wood powder from different bamboo sp for biomass yield & chemical composition

Although, *D. asper* showed maximum cellulose content among all bamboo spp tested but due to the very low biomass yield it was omitted for the further studies. There was no significant difference between the cellulose content of *B. balcooa* and *D. hemiltonii*, and moreover, the lignin content of *D. hemiltonii* (25.5%) was significantly lower than that of the *B. balcooa* (29.5). But due to comparatively very low biomass yield and very high moisture content from *D. hemiltonii* in comparison to *B. balcooa*, it was not selected for further studies. While *B. nutans* maximum lignin content (37.8%) that was significantly higher than the lignin content of rest of the bamboo sp, & hence was not used. *B. balcooa* was thus selected as a potential species for bioethanol production owing to its high biomass, cellulose but low lignin content.

The chemical composition of bamboo grass is very similar to that of other wood *i.e.* the main constituents are cellulose, hemi-cellulose and lignin that make over 90% of the total biomass of bamboo. *Bambusa* sp. and *Dendrocalamus* sp. are the most common in India, of which significant amounts of biomass is generated annually as by products of bamboo processing industries (**Lobovikov *et al.*, 2007; Zhang *et al.*, 2007**). Thus with view of its waste utilization as together with its high cellulose content make it an abundant tropical species of bamboo and is recognized as globally recognised industrially important varieties of bamboo (**Das *et al.*, 2005**). The cellulose and lignin content in various bamboo sp. has been reported to vary from 29.3 to 49.1 % and 23.8 to 26.1% (**Higuchi, 1957; Bhattacharya *et al.*, 2010**). In this way, *Bambus balcooa* appears as an ideal and important crop for energy production.

4.12 Process Optimization for Delignification of Substrate: *Bambusa balcooa*

The dried powdered biomass of *B. balcooa* was subjected to various physical, chemical/physicochemical and biological pretreatments for delignification in order to remove/minimizing the lignin content and break the recalcitrance of the biomass.

4.12.1 Physical/ Physicochemical Pretreatments

(a) Liquid Hot Water (LHW) and LHW with Acid Pretreatment

Compared to other pretreatment methods, liquid hot water (LHW) pretreatment not only make cellulose more accessible to the enzymes but also minimizes the formation of

lignin and other oxidative degradation products that are involved in inhibiting the growth of saccharifying or fermentative microorganisms. With liquid hot water pretreatment, the very less mount of cellulose was exposed, while a significant amount of lignin degradation was achieved in comparison to untreated *Bambusa balcooa* wood powder. The cellulose content reached from 37.93% to 40.37% while lignin content decreased from 29.5% to 10.0% during LHW pretreatment (**Fig 4.20**). Due to the less exposure of cellulose, LHW pretreatment cannot be considered as the effective pretreatment. Recently, **Zhuang *et al.*** (**2016**) reported that the LHW pretreatment having some disadvantages such as high water consumption and energy input, however, the energy consumption problem can be overcome by making LHW pretreatment combined with other pretreatments such as dilute acid. When LHW along with acid was used, surprisingly a significantly higher cellulose yield (59.95%) was obtained as compared to native *Bambusa balcooa* powder, and the lignin content got decreased from 29.5% to 10.2% (**Fig 4.20**). Thus, to obtain good results, a combined pretreatment methods involving LHW and dilute acid together would make this pretreatment more acceptable for industrial application (**Bacovsky *et al.*, 2013**; **Zhuang *et al.*, 2016**).

(b) Autoclaving and Acid Catalysed Autoclaving

The exposure of wood sample to a very high pressure (steam) under wet conditions also leads to lignin modification and cellulose exposure. Steam explosion was introduced and patented as a biomass pre-treatment process in 1926 by **Mason *et al.* (1926)**. In this process, they used wood chips that were steam heated at a very high temperature of about 285°C and a very high pressure of 3.5 MPa for about 2 min. The pressure is then increased rapidly to 7 MPa (70 bar) for 5-6 seconds, and then the wood chips are explode at atmospheric pressure into pulp. The use of diluted acid within the steam explosion process was also known to improve the hemicellulose hydrolysis and cellulose digestibility further. Here a prolonged autoclaving of the powdered biomass has been done for 60 min, both alone and with acid (as substitute for steam explosion). Among all the physical pretreatments given to lignocellulosic selected bamboo biomass, the autoclaving has given a significantly higher cellulose exposure, and a significant degradation of lignin (**Fig 4.20**). during autoclaving without acid the cellulose content increased from 37.93% to 64.5%, while the lignin content decreased from 29.5% to 14.6%. Although the lignin content after the pretreatment was not lesser as compared to LHW pretreatment, but due to release of a

very high amount of desired cellulose, this pretreatment could be excellent and efficient. Disappointing results were obtained when the *B. balcooa* powder was pretreated with autoclaving under acidic conditions. During acid catalysis, the lignin content decrement was near about same (from 29.5% to 49.2%) as that in normal autoclaving, but the cellulose content was increased to a lesser extent (from 37.93% to 44.22%). This comparatively lower increment of cellulose can be due the degradation of exposed cellulose by acid. Efficient increase in exposed cellulose content by various physical pretreatments of lignocellulosic biomass has been well reported by several workers (**Cui *et al.*, 2012; Tutt *et al.*, 2014**). But most of these methods are not very desirable because of requirement of costly equipments and additional energy requirements as operational costs.

(c) Microwave and Ultra-Violet Pretreatments

Microwave heatingimplies heat which directly applies an electromagnetic field to the structural molecules for production of physical, chemical or biological reactions of degradation or modifications mostly by causing swelling and fragmentation inside the biomaterial (**Peng *et al.*, 2014**). Following the pretreatment, the cellulose content reached to 59.29% from an initial of 37.93% of cellulose and the lignin content was decreased from 29.5% to 13.6% (**Fig 4.20**). Although another combination of microwave heating with alkaline pretreatment was reported, but during the present study it did not give good results. Comparatively lower cellulose content (~ 50%) was recorded after microwave +alkali pretreatment as compared to that after microwave heating alone. This decreased cellulose content may be due to hydrolysis (dissilution) of cellulose by alkali during this treatment. The pretreatment with ultraviolet light was found to be least effective among various physical pretreatments as it did not increase the cellulose content rather the cellulose content decreased from an initial (37.93%) value to 23.27% in the UV treated wood samples. Although lignin content decreased from 29.5% (control) to 13.8% in treated samples

Hydrocarbon materials such as wood powder can be good microwave absorbers, thus pretreatment with microwave reduces processing time and the energy demand for generating conditions required for hydrolysis of cell wall polysaccharides. The use of different solvents such as glycerol, ethylene glycol and tetrahydrofurfuryl alcohol for microwave pretreatment have been reported (**Matsumoto *et al.*, 2011**). Microwave

pretreatment using glycerol as solvent has been observed a safe and efficient way to pass the heat quickly in lignocellulose (**de Souza Moretti *et al.*, 2014**). Although **Diaz *et al.* (2015**) have reported no efficient increment in cellulose content with microwave heating when water used as solvent, but during present study an efficient amount of cellulose was exposed while using water as the only solvent in microwave heating. The results of UV pretreatment are in agreement with their of **Kang and Kim (2012)**. They have reported the pretreatment of lignocellulosic biomass with UV light. The combination of UV with TiO_2 used for rice straw pretreatment to facilitate its hydrolysis by hydroxyl radicals (OH·) generated duringpretreatment by TiO_2/UV system that could degrade lignin and the cell wall carbohydrates. Here, the UV light causes a significant degradation of lignin, but it was also accompanied with a significant cellulose loss due to its hydrolysis also.

Thus on the basis of maximum exposed cellulose content (64.5%) autoclaving of lignocellulosic bamboo biomass for 60 min was found as the best pretreatment among various physical/ physicochemical methods during this study.

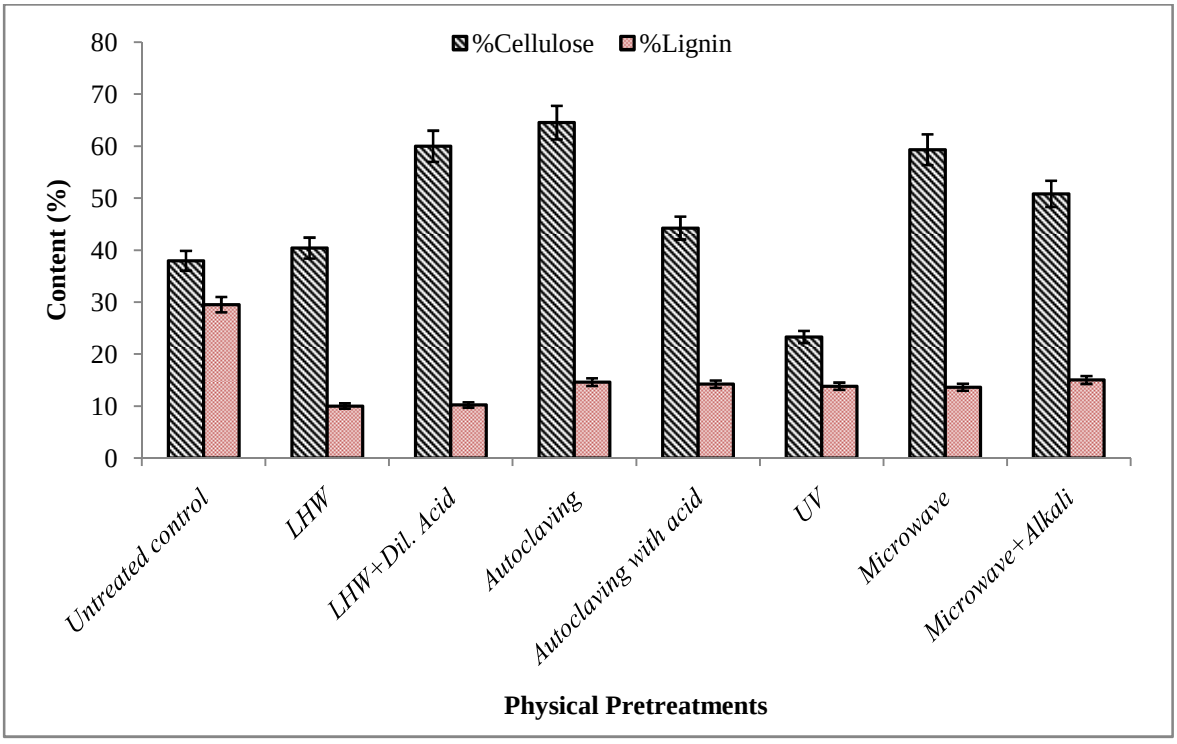


Fig 4.20: Influence of various physical and physicochemical pretreatments on cellulose and lignin content of woody biomass of the selected bamboo sp. (*B. balcooa*)

4.12.2 Chemical Pretreatments

(a) Alkali Pretreatment

Sodium hydroxide and calcium hydroxide were used for the pretreatment of selected bamboo biomass (*B. balcooa*). The pretreatment was carried out at normal room temperature and very effective results were obtained. Although, efficient lignin degradation was achieved with calcium hydroxide, the pretreatment also led to degrade the native cellulose. The total cellulose content of wood after the NaOH pretreatment was increased from 37.93% to 69.48%, while and the lignin content decreased from 29.5% to 11.6% (**Fig 4.21**). Considerably high concentration (2%) of alkali was used to pretreat the biomass, therefore it does not seem to be very desirable method from environment pollution point of view. Although somewhat higher removal of lignin (72.32%) with the same concentration (2%) NaOH from lignocellulosic biomaas has been reported by earlier workers, but it was achieved at very high temperature (**Wang *et al*., 2010**).

(b) Sulphite Pretreatment to Overcome Recalcitrance of Lignin (SPORL)

During the SPORL process, better results were obtained as compared to NaOH or other physicochemical pretreatments. The cellulose content from 37.95% increased up to 77.28% and lignin content decreased from 29.5% to 11.0% (**Fig 4.21**). For sulfite pretreatment to overcome recalcitrance of lignocellulose, earlier workers have reported the combination of SPORL and sulfuric acid at acidic pH (2.0 to 4.5), for 30 min, can completely removed hemicellulose and also more than 90% of cellulose converted to glucose after 10-h enzymatic saccharification (**Wang *et al*., 2009**). Furthermore, the use of SPORL process leads to less formation of fermentation inhibitors like HMF and furfural. They also reported that effective SPORL pretreatment can be achieved even without the addition of acid.

(c) Ammonia Fibre Explosion (AFEX)

Ammonia fibre explosion is a promising method that decreases the crystalinity of cellulose, hydrolyse the hemicellulose component, removes and depolymerises the polyphenolic lignin contents, thereby increasing the size and number of microscopic pores

in the cell wall significantly increases the rate of enzymatic hydrolysis of the pretreated biomass (**Mosier *et al.*, 2005**). The fine bamboo wood powder was pretreated using both commercially available absolute liquid ammonia (dry process) and aqueous ammonia solution (wet process) (**Fig 4.21**). The dry process gave comparatively better results than the wet process, showing significantly higher exposure of cellulose content from 37.93 to 80.43% with percent decrease of lignin from 29.5% to 11.8%. Lignin was poorly degraded using wet ammonia pretreatment (from 29.5% to 24.8%), with increase in cellulose content from 37.93 to 64.97%. **Bals *et al.* (2010)** have reported the release of fermentable sugars effectively from switch-grass using AFEX pretreatment with the combination of different enzyme including xylanases and pectinases. Although they reported that a relatively mild pretreatment gave high reducing sugar yield and thus high alcohol yield in early harvested lignocellulosic biomass while more severe pretreatment conditions (such as absolute ammonia) are necessary for later harvested biomass.

(d) Photocatalytic Pretreatment Using Nanoparticls

Various studies indicate efficient photo-catalytic degradation of lignin or related compounds using titanium dioxide nano-powder photo-catalyst (**Kansal *et al.*, 2008; Ma *et al.*, 2008; Tanaka *et al.*, 1999**). During present study, three different pretreatment experiments were carried out under sunlight using TiO_2 photo-catalyst nano-powder on *Bambusa balcooa* fine wood powder. Among all cases, maximum cellulose recovery and lignin removal were obtained using the combination of TiO_2 and NaOH under sunlight (**Fig 4.21**). The cellulose content increased from an initial 37.93% to 78.78% while the lignin content decreased from 29.25% to 12%. Other combinations did not given satisfactory results (**Table 4.16**).

(e) Hydrogen Peroxide Pretreatment with Different Combinations

Delignification of the bamboo lignocellulosic biomass was also studied by treating the biomass using an oxidising agent such as hydrogen peroxide. In order to make the process more effective various combinations of hydrogen peroxide pretreatment were prepared. Earlier workers have reported use of oxidative pretreatment of the lignocellulosic biomass. But during present study the biomass was subjected to oxidative pretreatment

under sunlight. The various combinations and the results of these pretreatments are given in **Table 4.17**. As per the results, significantly higher cellulose exposure (from 37.93 to 89.8%) was observed when the biomass was pretreated with alkaline hydrogen peroxide, followed by pretreatment with hydrogen peroxide with copper shavings (from 37.93 to 84.512%), and only H_2O_2 (from 37.93 to 80.192%). Similar results were obtained for lignin degradation also, showing maximum (from 29.5 to 8.2%) with hydrogen peroxide+ copper shavings, followed by pretreatment with alkaline hydrogen peroxide (from 29.5 to 8.6%) (**Fig 4.21**).

Thus, results indicated clearly that among various oxidative pretreatments, alkaline hydrogen peroxide pretreatment was found best for the cellulose exposure and delignification of *Bambusa balcooa* biomass. Many times ozone can also be used and, surprisingly, sometimes the air (oxygen) can also be used for pretreatment purposes (**Harmsen *et al.*, 2010**). The oxidising chemicals are highly reactive and thus react with aromatic rings present in the lignin to give effective delignification. Many studies showed the peroxide treatment using a combination of an alkali with H_2O_2 (**Diaz *et al.*, 2013; Gray, 2013; Wi *et al.*, 2015**). **Diaz *et al.* (2013)** have also reported significant improvement in enzymatic saccharification when the biomass was pretreated with alkaline hydrogen peroxide. They have chosen 7% peroxide concentration attaining the cellulose yield of 77.25 ± 5.61 % at a very high pH (pH=11.5), at 90^0C for 2 h. Comperatively better results with higher delignification and cellulose yield under much mild conditions without expenditure of any energy (electricity) were obtained by us during present study.

Table 4.16: Cellulose and lignin content* after pretreatments using titenium dioxide and its combinations

S. No.	Pretreatment	Cellulose Content (%)	Lignin Content (%)
1.	Control (untreated wood Powder)	37.93	29.5
2.	TiO_2+ *hv***	31.449	14.4
3.	TiO_2+ NaOH+*hv*	78.78	12
4.	TiO_2+ NaOH+ H_2O_2+ *hv*	27.411	17.6
SEm±		0.934	0.855
CD (at 5%)		4.98	2.79

*All values were taken in triplicates and analysed for Standard error of mean and critical difference at 5% level of significance

***hv*= Sunlight of summer season (May and June)

Table 4.17: Influence of oxidative/alkaline oxidative pretreatment using H_2O_2 with or without metallic induction

S. No.	Pretreatment	Cellulose Content (%)	Lignin Content (%)
1.	Control (untreated wood Powder)	37.93	29.5
2.	H_2O_2+*hv***	80.192	14.6
3.	H_2O_2+NaOH+*hv*	89.8	8.6
4.	H_2O_2+Iron shavings+ *hv*	67.231	11
5.	H_2O_2+Aluminium shavings+ *hv*	35.86	20.8
6.	H_2O_2+Zink metal+ *hv*	53.426	21.8
7.	H_2O_2+Copper shavings+ *hv*	84.512	8.2
8.	H_2O_2+Al+Fe+Zn+Cu+ *hv*	70.61	10.2
SEm± CD (at 5%)		0.84 2.56	0.515 1.56

*All values were taken in triplicates and analysed for Standard error of mean and critical difference at 5% level of significance

***hv*= Sunlight of summer season (May and June)

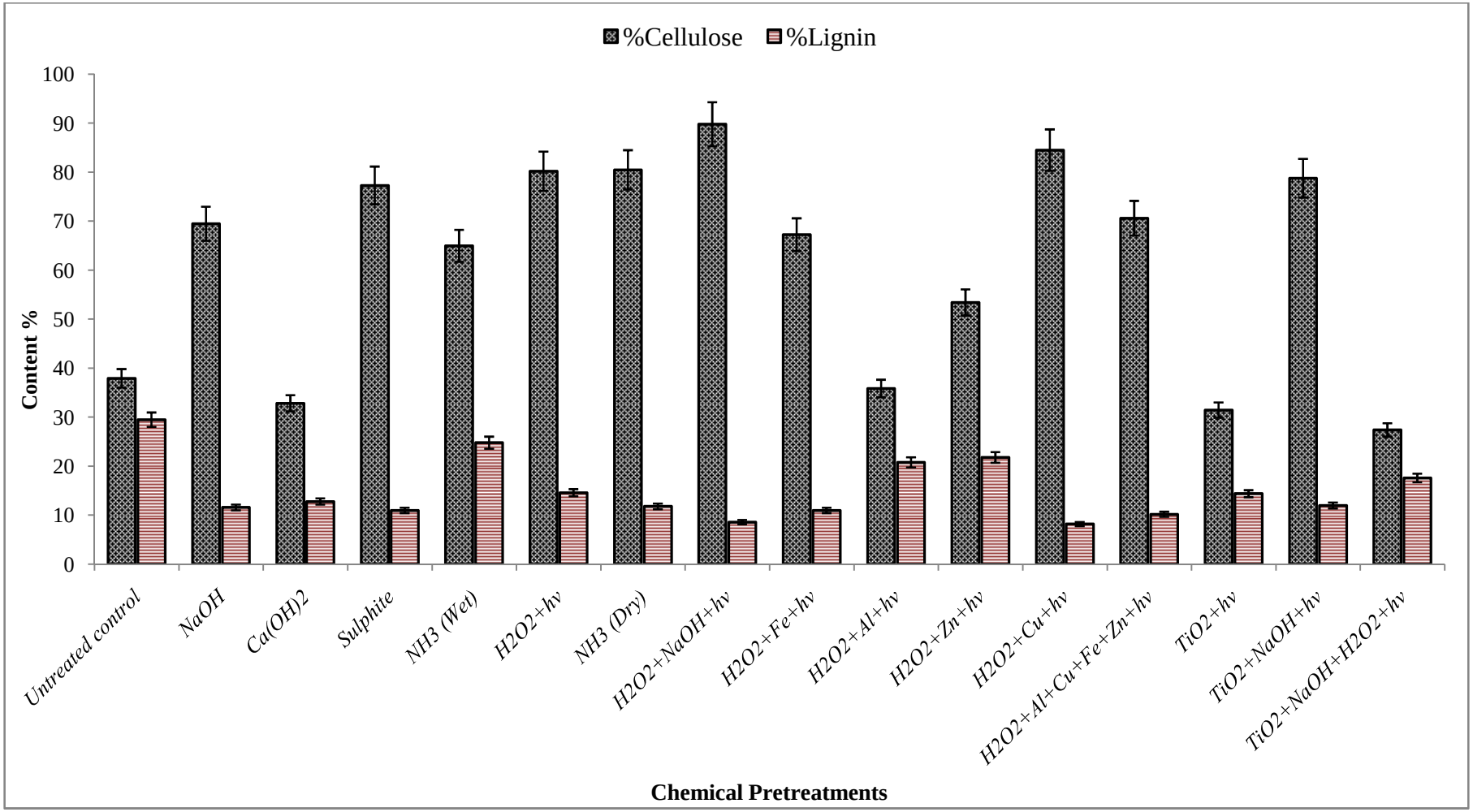


Fig 4.21: Influence of various chemical pretreatments on exposed cellulose and lignin content of the selected bamboo (*B. balcooa*) biomass

4.12.3 Biological Pretreatments

Biological pretreatments were given using both sour (acidic) fruit juices viz. cranberry (water extract), lemon and citrus (absolute juice), and by potential wood rot fungal isolate WDP2. During the pretreatment process, the crude acidic fruit extracts decreased the lignin content in all the cases but at the same time it also degrade the cellulose from the biomass, therefore, could not be considered as suitable biological pretreatment methods (**Fig 4.22**). Lemon juice was found to be the most efficient for delignification that lowered the initial lignin content from (29.25% in control) to 8.4%, in treated samples followed by the cranberry extract (8.6%) and the citrus juice (14.2%). Therefore due to degradation of cellulose during pretreatment, fruit juice treatment was not found as a satisfactory pretreatment method for bamboo biomass pretreatment.

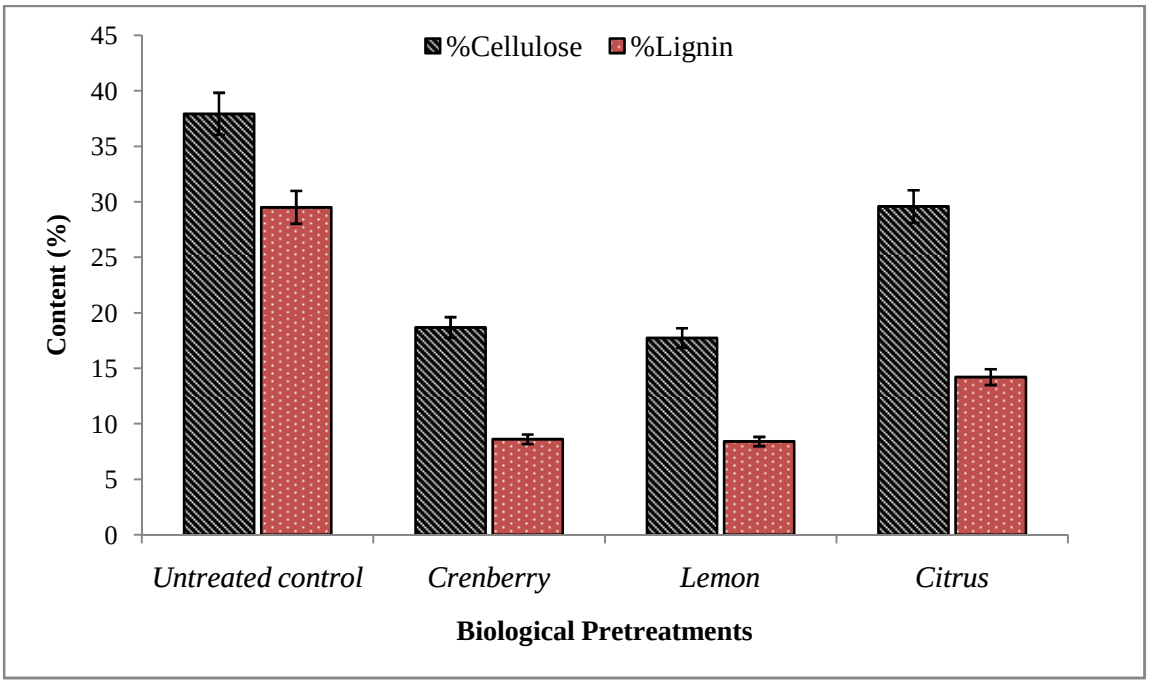


Fig 4.22: Influence of biological pretreatment using fruit juice on cellulose and lignin content of selected bamboo sp. (*B. balcooa*)

For biological pretreatment, the fungal isolate WDP2 as whole cell biocatalyst, the bamboo wood powder was pretreated under three sets of conditions: (I) wood powder+water (without any chemical), (II) wood powder+1/2 concentration of synthetic laccase production medium and (III) wood powder +1/2 conc. of optimised laccase production medium. In all the sets set, 75% moisture content was established. The samples

were withdrawn periodically and analysed for laccase and cellulase activities in the culture filtrate and lignin and cellulose content in the fermented biomass upto 60d. The culture filtrates were also analysed for ethanol production in order to check whether the fungus is ethanolagenic in nature. The experimental setups and mycellial growth of fungal isolate WDP2 in one of the setups are showed in **Plate 4.9**. the extracellular laccase activity was recovered in all the three cases that was followed with a decrease in the lignin content of the biomass (**Table 4.18)**. initially laccase activity increased in all the cases with simultaneous decrease in lignin content upto 24-48d (**Fig 4.23**). it is evident from the results that very high enzyme activity during solid state fermentation was obtained as compared to that liquid state fermentation while using the same optimised medium, indicating that solid state fermentation (SSF) is better then LSF for laccase production. Maximum decrease in lignin content from an initial 29.5% to 8.4% with 42.5 Uml^{-1} laccase activity was achieved in optimised medium at 48d of incubation period, followed by decrease in lignin content from 29.5% to 11.35% at the same incubation period. Thus a significant decrease in lignin content was recorded when optimised enzyme production medium was used for the biological pretreatment of the biomass. It could be due to higher laccase enzyme production in the fermentation medium. The optimised medium with biomass gave highest laccase enzyme production (383.16 U ml^{-1}) at 24th d of incubation followed by only moisture containing medium (301.66 Uml^{-1}) at 36d of incubation period. The pattern of decrease in lignin content in all the three sets was near about same and in all cases, lignin content decreased gradually. The SSF method was reported as best method for many enzyme production processes by several workres(**Reid, 1985; Zadrazil and Isikhuemhen, 1997**). Many previous studies reported that SSF processes have shown to particularly suitable for the production of many industrially important enzymes by using filamentous fungi (**Pandey *et al.*, 1999 a; Pandey *et al.*, 1999b**). It might be correlated with the fact that more favourable natural conditions to the fungi for growth and reproduction (**Moo-Young *et al.*, 1983**), due to which they may be more capable to efficiently reproduce and to produce certain enzymes with high yield in comparison to other methods such as submerged fermentation (SmF).

It is evident from the data that cellulase enzyme production was very less during the process (**Table 4.18**). Maximum FPase enzyme activity (0.18Uml^{-1}) was obtained during the 12d of incubation period in the experiment set that contained synthetic laccase

production medium. Thereafter the activities got decreased and no significant enzyme production was achieved. CMCase enzyme activity is almost negligible in all the cases with the highest production of 0.01 Uml^{-1} at 24d of incubation period within only moisture containing flask. Furthermore, the maximum β-glucosidase enzyme activity (0.455Uml^{-1}) was obtained again at 24d of incubation period in the experimental set that contained only moisture. The production of enzymes in only moisture containing flask can be correlated with the absorption of nutrients only from the bamboo biomass irrespective of the extra supply of minimal salts from outside. This process may industrially be very important due to the no nutrient requirement other than the moisture (distilled water) from outside. Furthermore, most of the lignocellulose degrading enzymes are secondary metabolites (**Elisashvili *et al*., 2009**), since lignin degradation does not provide any energy to the fungus (**Hatti-Kaul and Ibrahim, 2012**). Thus SSF with moisture is a nutrient limiting condition that helps in production of these secondary growth products (**Keyser *et al*., 1978**). The range of cellulose content exposed after fungal pretreatment varied from 47.17% (in biomass+water at 12d of incubation) to 61.59% (in optimised medium at 48d of incubation) as compared to cellulose content of an initial of 37.93%. Thus maximum cellulose was exposed with the minimum lignin content *i.e.* during 48d of incubation period. Following biological pretreatment, very low reducing sugar content was recovered in the fermentation media. It is obvious because the fermenting organism (WDP2) was not having good cellulolytic activity. This low cellulase activity can be correlated with very less reducing sugar loss during pretratement that is desirable for the bioprocess. The maximum sugar release (0.08 mg) was obtained at 24d of incubation period in optimised medium. Almost negligible sugar release indicated that the fungal isolate WDP2 was not able to simultaneously saccharify the delignified biomass. Furthermore, no simultaneous ethanol production was achieved in any set indicating that the test fungus was not ethanolagenic and separate ethanol fermentation step is required. Thus the process of the ethanol production using fungal isolate WDP2 could not be completed in one step. The process utilised separate delignification, saccharification and fermentation, or separate delignification with simultaneous saccharification and fermentation.

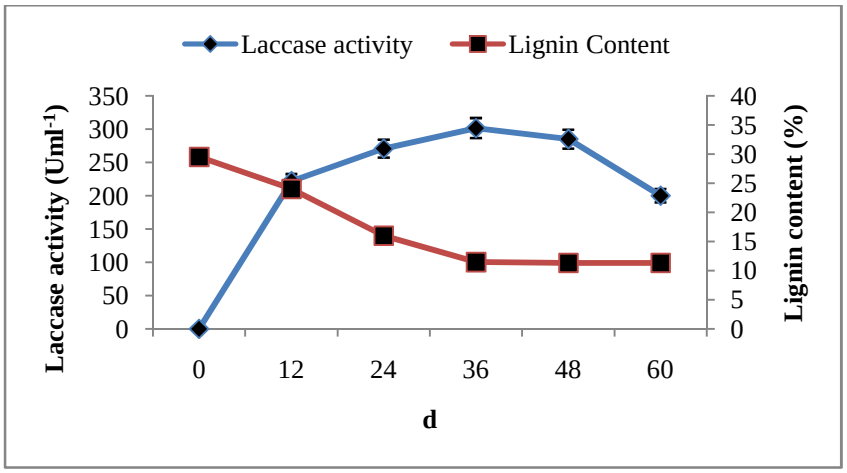


(a)

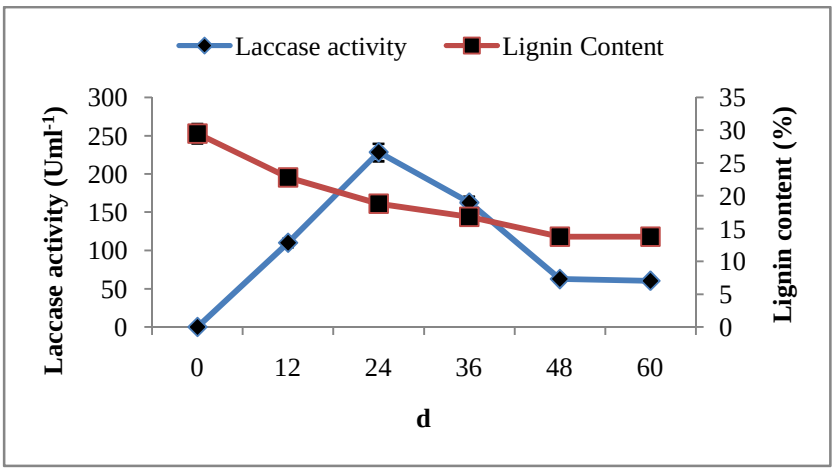


(b)

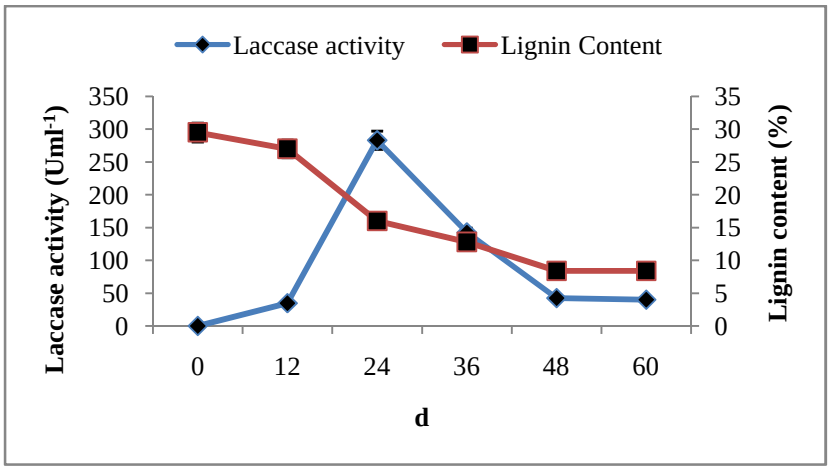
Plate 4.9: Biological pretreatment of (*B. balcooa*) biomass using lignolytic wood rot fungal isolate WDP2 (a), and fungal growth on the bamboo biomass during pretreatment in a flask (b).



(a)



(b)



(c)

Fig 4.23: Influence of biological pretreatment on lignin content of the bamboo wood biomass along with the extracellular laccase production by the fungal isolate WDP2 with fermentation medium containing only water (a), synthetic medium (b), and optimised medium (c)

Table 4.18: Selection of medium for whole cell biological (fungal) pretreatment of *Bambusa balcooa* biomass

Incubation period (d)	Experimental sets	Laccase activity (Uml^{-1})	Lignin content (%)	Cellulase activities			Cellulose content (%)	Reducing Sugar Yield (mg)	Ethanol Production (mg)
				FPase (Uml^{-1})	CMCase (Uml^{-1})	β-glucosidase (Uml^{-1})			
d 12	Set 1	221.41	24.0	0.055	0.00	0.229	47.17	0.04	-
	Set 2	110.00	22.8	0.18	0.004	0.158	50.60	0.022	-
	Set 3	34.58	27.0	0.165	0.002	0.111	48.86	0.021	-
d 24	Set 1	270.83	16.0	0.055	0.01	0.455	51.68	0.038	-
	Set 2	228.33	18.8	0.077	0.002	0.101	50.46	0.08	-
	Set 3	**383.16**	16.0	0.12	0.00	0.121	49.85	0.08	-
d 36	Set 1	301.66	11.45	0.01	0.002	0.359	54.22	0.002	-
	Set 2	162.50	16.8	0.063	0.002	0.071	54.83	0.012	-
	Set 3	142.50	12.8	0.118	0.002	0.101	54.12	0.051	-
d 48	Set 1	285.00	11.3	0.00	0.00	0.159	57.96	0.001	-
	Set 2	62.56	13.77	0.03	0.001	0.06	58.11	0.00	-
	Set 3	42.50	**8.4**	0.00	0.00	0.062	**61.59**	0.00	-
d 60	Set 1	200.00	11.33	0.00	0.00	0.1	41.96	0.00	-
	Set 2	60.29	13.77	0.02	0.001	0.00	57.0	0.00	-
	Set 3	40.34	8.4	0.00	0.00	0.0	50.25	0.00	-
Factors: Time (a) sets (b) Interaction (a*b)		Laccase activity (Uml^{-1})	Lignin content (%)	FPase (Uml^{-1})	CMCase (Uml^{-1})	BGS (Uml^{-1})	Cellulose content	Sugar Yield (mg)	Ethanol Yield
SEm ±		(a) 0.434 (b) 0.565 (a*b) 0.97	(a) 0.131 (b) 0.169 (a*b) 0.293	(a) 0.006 (b) 0.008 (a*b) 0.013	(a) 0.0001 (b) 0.0001 (a*b) 0.0002	(a) 0.003 (b) 0.003 (a*b) 0.006	(a) 0.16 (b) 0.21 (a*b) 0.37	(a) 0.001 (b) 0.002 (a*b) 0.004	-
CD @ 5%		(a) 1.25 (b) 1.61 (a*b) 2.8	(a) 0.379 (b) 0.489 (a*b) 0.848	(a) 0.017 (b) 0.023 (a*b) 0.04	(a) 0.0003 (b) 0.0004 (a*b) 0.0008	(a) 0.008 (b) 0.011 (a*b) 0.019	(a) 0.48 (b) 0.62 (a*b) 1.08	(a) 0.002 (b) 0.002 (a*b) 0.008	-

4.12.4 Delignification Optimization Using Oxidative Alkaline Pretreatment

Alkaline hydrogen peroxide pretreatment was selected as the best chemical pretreatment combination for delignification and maximum exposure of the cellulose. The concentration of alkali used for pretreatment was 2%, which was very high so that could not be used for industrial delignification purposes. Thus, Response Surface Methodology (RSM) was further adopted for the optimization of alkaline hydrogen peroxide pretreatment for the maximum removal of lignin using minimum amount of the alkali. The experiments were designed in such a way by which alkali concentration can be minimised, using high or low concentration of hydrogen peroxide, because unlike alkali, no toxicity of H_2O_2 on our environment. Pretreatment of *B. balcooa* wood powder was performed and analyzed using 17 experiments designed in Design-Expert® version 10 Software. The significance of percent lignin degradation (R1) was studied by considering three factors variables: alkali (sodium hydroxide) concentration (% w/v) A, solid to liquid ratio B, H_2O_2 concentration (% w/v) C. As shown in **Table 4.19**, it was observed that all the three influencing factors considered during the study have a variable effect on residual lignin content (%). Various samples were treated for a 4h of incubation period at various combinations as per experimental design, the minimum lignin content (5.0%) observed for experiment number 2, which having the solid: liquid was 1:14, NaOH concentration was 0.5%, and H_2O_2 concentration was 3.0%. It was observed from the study that a high concentration of H_2O_2 (3.0%) at a relatively low concentration of NaOH (5.0%) at a high solid: liquid (1:14). Although the results are showing a very high lignin removal at a very low alkali concentration, but, as per the ANOVA table for response surface quadratic model for alkaline hydrogen peroxide pretreatment of *B. balcooa* powder, the model comes to be non significant (**Table 1, Appendix II**). The Model F-value of 1.93 implies the model is not significant relative to the noise. There is a 19.95 % chance that F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case AB is a significant model term. Values greater than 0.1 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve our model. The "Lack of Fit F-value" of 97.35 implies the Lack of Fit is significant. There is only a 0.03% chance that a "Lack of Fit F-value" this large could occur due to noise. Significant lack of fit is bad -we want the model to fit. Thus according to the ANOVA table, and significant lack of fit value, we cannot use the model and this design for the optimization of alkali concentration for delignification of *Bambusa balcooa* wood.

Table 4.19: Experimental response of dependent variable (% delignification) after alkaline hydrogen peroxide pretreatment of *Bambus balcooa* powder using Box-Behnken design (BBD).

Expt. No.	Coded variables			(Residual Lignin Content, %)
	A	B	C	
1	-1	-1	0	9.6
2	1	-1	0	**5.0**
3	-1	1	0	5.2
4	1	1	0	8.8
5	-1	0	-1	14.8
6	1	0	-1	8.0
7	-1	0	1	9.2
8	1	0	1	5.8
9	0	-1	-1	12.8
10	0	1	-1	5.4
11	0	-1	1	7.6
12	0	1	1	6.6
13	0	0	0	5.2
14	0	0	0	5.2
15	0	0	0	5.8
16	0	0	0	5.6
17	0	0	0	5.47

A: Solid:Liquid ratio; B: Alkali Concentration; C: Hydrogen peroxide concentration

4.12.5 Manual Optimization of Oxidative Delignification

In order to make process more cost effective and ecofriendly, further experiments were carried out using different concentrations of H_2O_2 and NaOH for biomass pretreatment under intense sunlight of summer season (~temperature 40-45^{0}C) (**Table 3.2**). It is evident from the results that maximum delignification was achieved at 0.5% alkali (NaOH)+ 3.0% of hydrogen peroxide concentration with solid: liquid ratio of 1:14. Better lignin removal was observed when water content was higher. Significantly reduced value (5.0%) of lignin content was recorded under these conditions as compared to other

treatments. The results indicated significant effect of solid: liquid ratio on delignification irrespective of alkali+H_2O_2 concentrations as even in the presence of highest alkali concentration (2%), with 1:6 solid:liquid ratio, lignin content decreased to 14.8% from an initial 29.5%. The results were further proved the involvement of hydrogen peroxide to enhance the delignification by alkali, even at a very low concentration (0.5%). In this way, all the three parameters, *i.e.* solid: liquid ratio, NaOH concentration, and hydrogen peroxide concentration were focused to influence the delignification of biomass to a considerable extent, and a combination of 0.5% NaOH + 3% hydrogen peroxide at 1:14, solid: liquid ratio selected as the best alkaline hydrogen peroxide pretreatment and it can be recommended for further delignification experiments. Our results are in agreement with those of earlierworkers, who have also reported that solid: liquid ratio helps in autohydrolysis of biomass, thereby improving the delignification (**Karp *et al.*, 2013; Vallejos *et al.*, 2015**). Furthermore, **Rocha *et al.* (2012**), developed steam explosion pretreatment for bagasse involving NaOH (1%, w/v), and solid-liquid ratio of 1:10 (w/v). The operation gave efficient lignin removal from the biomass (92.7±3.9%) when carried out 100^0C. Compared to it, we could achieved good (almost 83%) delignification with lower alkali concentration (0.5%) without any extraq energy input, as the experiment was carried out under the natural light source (sunlight).

Table 4.20: Optimization of oxidative pretreatment for delignification of bamboo (*B. balcooa*) biomass

S. No.	Solid:Liquid	NaOH Concentration (%)	H_2O_2 Concentration (%)	(Residual Lignin Content, %)
1.	1:6	0.5	0.3	9.6±0.22
2.	1:6	2.0	1.0	14.8±0.44
3.	1:10	0.5	3.0	9.2±0.35
4.	1:10	2.0	1.0	8.6±0.23
5.	1:14	0.5	3.0	**5.0±0.12**
6.	1:14	2.0	1.0	8.0±0.22
SEm± (Residual Lignin Content, %)				0.288
CD@ 5% (Residual Lignin Content, %)				0.888

UNIT IV: ENZYMATIC SACCHARIFICATION OF PRETREATED BIOMASS & MONITORING WOOD HYDROLYSIS

4.13 Enzymatic Saccharification

Biologically pretreated *B. balcooa* powder using potential laccase producing fungal isolate WDP2 was oven dried 65±2^{0}C. The dried powder then used for optimization of enzymatic saccharification, using partially purified cellulase enzyme from potent cellulase producing fungal isolate PCF1. For optimization, a second order Box-Behnken design (BBD) was used for optimization(**Box *et al*., 1978**).

4.13.1 Process Optimization

It was observed that all the three parameters considered during the study have a variable effect on percent saccharification (**Table 4.21**). The samples were treated for a 48 h in different combinations as per experimental design. Optimization of proce3ss parametets was carried out and contour plots were developed for the selected parameters. Reducing sugar after enzymatic hydrolysis were measured by DNS method and results were represented as saccharification efficiency (%). The maximum saccharification yield (46.255%) was obtained when the substrate at the concentration of 1% was hydrolysed using 15FPU of cellulase/g of substrate at 40^{0}C during the experimental run numbered 1. The corresponding condition for minimum saccharification yield (18.11%) observed during the experiment number 12, included enzyme loading20 FPU/g of substrate, substrate concentration @4%, w/v), and saccharification temperature of 50^{0}C. It was observed from the study that a high substrate concentration, the incubation temperature (above or below 40^{0}C) adversely affected the cellulase enzyme complex, therby reducing the saccharification yield. Minimum saccharification yield was observed during the experimental run no. 4, 5, 7, and 12 that might be correlated with the high substrate concentration in each case, and a lower (<40^{0}C) or higher (>40^{0}C) saccharification temperature (of 30^{0}C and 50^{0}C) in these experiments.

4.13.2 Statistical Analysis of Dependent Variable

The Model F-value (4.05) implies the model is significant. For predicted model, F value is significant at 5% level of significance (LOS), thus it indicated that the model is

accurate. The value of F_{tab} for un-coded variable B *i.e.* substrate concentration (6.962) was significant at 1% LOS, thus as per the **Table 4.22 and Table 4.23**, only concentration of pretreated *B. Balcooa* powder have the major effect on percent saccharification. There is only a 3.94% chance that an F-value this large could occur due to noise. "Values of ""Prob > F"" less than 0.0500 indicate model terms are significant."In this case B is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. "The ""Lack of Fit F-value"" of 4.27 implies there is a 9.74% chance that a ""Lack of Fit F-""value"" this large could occur due to noise. Lack of fit is bad -- we want the model to fit."This relatively low probability (<10%) is troubling.

(a) Effect of Individual Parameter on % Saccharification

Effect of individual influencing factors on saccharification efficiency was calculated using sequential sum of squares and given in **Table 4.23**. It was observed form the F-values, that generated model is highly significant at 5% level of significance, and it was also predicted that the concentration (%, w/v) of substrate (*Bambusa balcooa*) was highly significant at 1% level of significance ($p<0.01$) for achieving maximum percent saccharification. The effect of other factors (enzyme concentration and temperature) was not significant in the design of experiments.

(b) ANOVA for Saccharification (%)

The effects of influencing factors at linear, interactive, and quadratic levels are reported in **Table 4.24**. It was observed that the effect on saccharification efficiency (%) of influencing factor B *i.e.* 'concentration (%, w/v) of substrate (*B. balcooa*)' at liner level is highly significant at 1% level of significance. Thus the highest effect of substrate concentration was found at linear level, thus linear model was generated (**Fig 4.24**).

(c) Numerical Analysis of Percent Saccharification

The percent saccharification of various experimental samples of biologically pretreated *Bambusa balcooa* biomass varied from 18.11% to 46.255%, showed in **Table 4.21**. The values of percent saccharification was maximum for experiment number 1 enzyme concentration (15FPU/g of substrate), substrate concentration (1%, w/v), and incubation temperature (40^0C). A second order regression **Equation 4.1** was fitted into the

percent saccharification data and the results of regression analysis are reported in **Table 4.25**. The model was checked using a numerical method employing the coefficient of determination R^2, and then calculated as shown in **Equation 4.1**. From the regression model of percent saccharification, it was insured a relatively satisfactory adjustment of linear model to the experimental data. It is also showed that the regression coefficients in the model and the significance of each term. It was apparent from the table, the model is significant at 5% level of significance, and a linear model can be generated. The percent saccharification was significantly affected by concentration of substrate (%, w/v), ($p<0.01$) and there was no significant effect of enzyme concentration and the incubation temperature on percent saccharification. The influencing factor as substrate concentration (%, w/v) have highest effect on percent saccharification at 1% level of significance at linear level with no effect was found in case of interactive or quadratic levels.

Table 4.21: Experimental response of dependent variable (saccharification efficiency) after enzymatic saccharification of alkaline hydrogen peroxide pretreatment of *B. balcooa* powder using Box-Behnken design (BBD).

Expt. No.	Coded variables			Response (% saccharification)
	A	B	C	
1	-1	-1	0	**46.255**
2	1	-1	0	34.2
3	-1	1	0	12.65
4	1	1	0	18.15
5	-1	0	-1	18.4
6	1	0	-1	23.58
7	-1	0	1	18.95
8	1	0	1	31.8
9	0	-1	-1	36.1
10	0	1	-1	21.52
11	0	-1	1	32.26
12	0	1	1	**18.11**
13	0	0	0	39.33
14	0	0	0	32.418
15	0	0	0	30.114
16	0	0	0	32.562
17	0	0	0	31.59

X1: Cellulase loading (FPU g^{-1} substrate)**; X2:** substrate concentration (% w/v)**; X3:** temperature (^{0}C)

Table 4.22: ANOVA for response surface quadratic model (analysis of variance table)

Source	Sum of Squares	df	Mean Square	F Value	P-value Prob > F	
Model	1108.68	9	123.19	4.05	0.0394	**Significant**
A-Enzyme (FPU/g substrate)	16.46	1	16.46	0.54	0.4860	
B-Substrate Conc. (%)	768.03	1	768.03	25.23	0.0015	
C-Temperature (^{0}C)	0.29	1	0.29	9.488E-003	0.9251	
AB	77.04	1	77.04	2.53	0.1556	
AC	14.71	1	14.71	0.48	0.5094	
BC	0.046	1	0.046	1.519E-003	0.9700	
A^2	89.17	1	89.17	2.93	0.1307	
B^2	2.61	1	2.61	0.086	0.7782	
C^2	123.61	1	123.61	4.06	0.0837	
Residual	213.08	7	30.44			
Lack of Fit	162.36	3	54.12	4.27	0.0974	N.S.*
Pure Error	50.71	4	12.68			
Cor Total	**1321.75**	**16**				

*Not significant

Table 4.23: Effect of individual parameters on saccharification efficiency

Source	df	Sum of Squares (SS) =(SS A+SS AB+SSAC+SS A^2)	Mean Square (MS) =(SS/df)	F-value =(MS/MS Residual)	
Model	9	1108.68	123.186	4.046**	Significant
A	4	197.38	49.345	1.621	
B	4	847.726	211.931	6.962*	
C	4	138.656	34.664	1.138	
Residual	7	213.08	30.44		
Lack of Fit	3	162.36	54.12	4.27	N.S.

*, **, and *** are representation for 1, 5, 10% level of significance, respectively.

At 1% F_{tab} (0.01, 4, 7) = 7.84

At5% F_{tab} (0.05, 4, 7) = 4.12

At 10% (0.1, 4, 7) = 2.96.

Table 4.24 ANOVA for saccharification efficiency

Source	df	Sum of Squares (SS)	Mean Square (MS)	F-value	
Model	9	1108.68	123.186	4.046**	Significant
Linear	3	784.78	261.59	8.59*	
Interactive	3	91.796	30.59	1.01	
Quadratic	3	215.39	71.79	2.35	
Residual	7	213.08	30.44		
Lack of Fit	3	162.36	54.12	4.27	N.S.

*, **, and *** are representation for 1, 5, 10% level of significance, respectively.

Model:

At 1% F_{tab} (0.01, 9, 7) = 6.718

At 5% F_{tab} (0.05, 9, 7) = 3.67

At 10% (0.1, 9, 7) = 2.72.

Linear, interactive, quadratic,

At 1% F_{tab} (0.01, 9, 7) = 6.718;

At 5% F_{tab} (0.05, 9, 7) = 3.67;

At 10% (0.1, 9, 7) = 2.72.

Table 4.25 Results of regression model for percent saccharification

Factor	% Saccharification	
	Coefficient	P-value (Prob>F)
Intercept	33.20	0.0394**
A-Enzyme (FPU/g substrate)	1.43	0.4860
B-Substrate Conc. (%)	-9.80	0.0015*
C-Temperature (^{0}C)	0.19	0.9251
AB	4.39	0.1556
AC	1.92	0.5094
BC	0.11	0.9700
A^2	-4.60	0.1307
B^2	-0.79	0.7782
C^2	-5.42	0.0837***
R^2	0.8388	
Lack of Fit	N.S.	

***, **, and *** are representation for 1, 5, 10% level of significance, respectively.**

Predicted regression equation for % saccharification is as follows:

% Saccharification (Y) = $33.20+1.43A-9.80B+0.19C+4.39AB+1.92AC+0.11BC-4.60A^2-0.79B^2-5.42C^2$ ..... (**4.1**)

Where A, B, C are Enzyme (FPU/g substrate), substrate conc. (%, w/v), and incubation temperature (^{0}C), respectively. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the centre of the design space.

3.13.4 Calculation of Optimum Values of Selected Parameters

The coded independent variable were A, B, C for cellulase enzyme (FPU/g of substrate), substrate concentration (%, w/v), and temperature (^{0}C). The actual values after optimization experiments were calculated using formulas given in **Section 3.12.3**. Before obtaining actual optimised values of various independent variables, the goal of response (% saccharification) was maximised as we required maximum saccharification (**Table 4.26**). The coded values for all the three parameters were -0.332, -1, and -0.051, for enzyme (FPU/g substrate), substrate conc. (%), temperature (^{0}C), respectively (**Table 4.27**). The optimum values for maximum saccharification were obtained by putting coded values in formula. The optimum conditions for maximum saccharification were: cellulase enzyme 18.4 Uml^{-1} FPU/g substrate, substrate concentration 1.0%, and temperature 39.49^{0}C, that repeated again to confirm maximum % saccharification (**Table 4.27**). The linear surface (**Fig 4.24 a, b**) and 3D surface contour plots (**Fig 4.25 a, b**) showing the interaction of various parameters during saccharification of *B. balcooa* powder. Again, only substrate concentration (influencing factor) has shown highest effect on percent saccharification at linear level with no effect was found in case of interactive or quadratic levels.

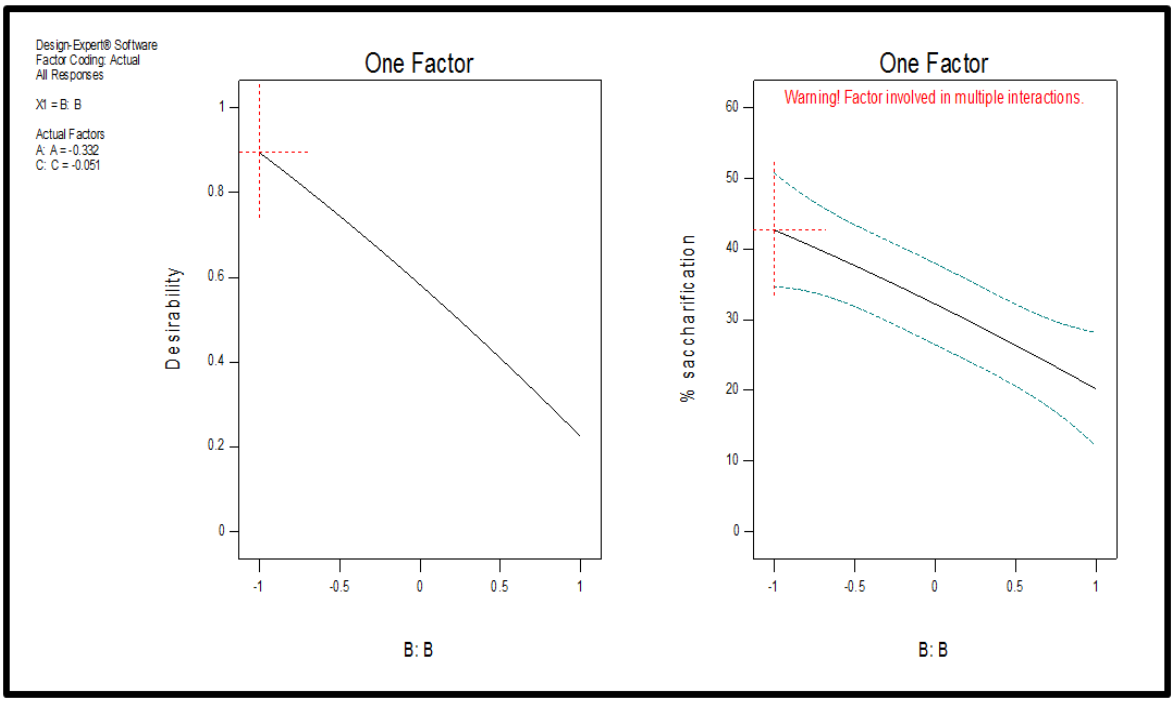
Response surface methodology (RSM) is a statistical method that is used to study and collect the data about effect of several important and functioning variables on a process (**Kim *et al.*, 2008**). Use of RSM for optimisation of any method can reduce the number of experiments and thus time period to evaluate the interactions between many of experimental parameters and their different values (**Singh and Bishnoi, 2012**). Optimization of enzymatic saccharification using response surface methodology (RSM) was reported by previous studies also (**Pandiyan *et al.*, 2014; Das *et al.*, 2015**). Central composite design (CCD) in Response surface methodology (RSM) was followed by **Sopajarn and Sangwichien, (2015)**. They used the method to optimize cellulase saccharification process in order to obtain high glucose from cattail material (wetland plants) for bioethanol production.

Table 4.26 Constraints for optimization of variables for saccharification efficiency

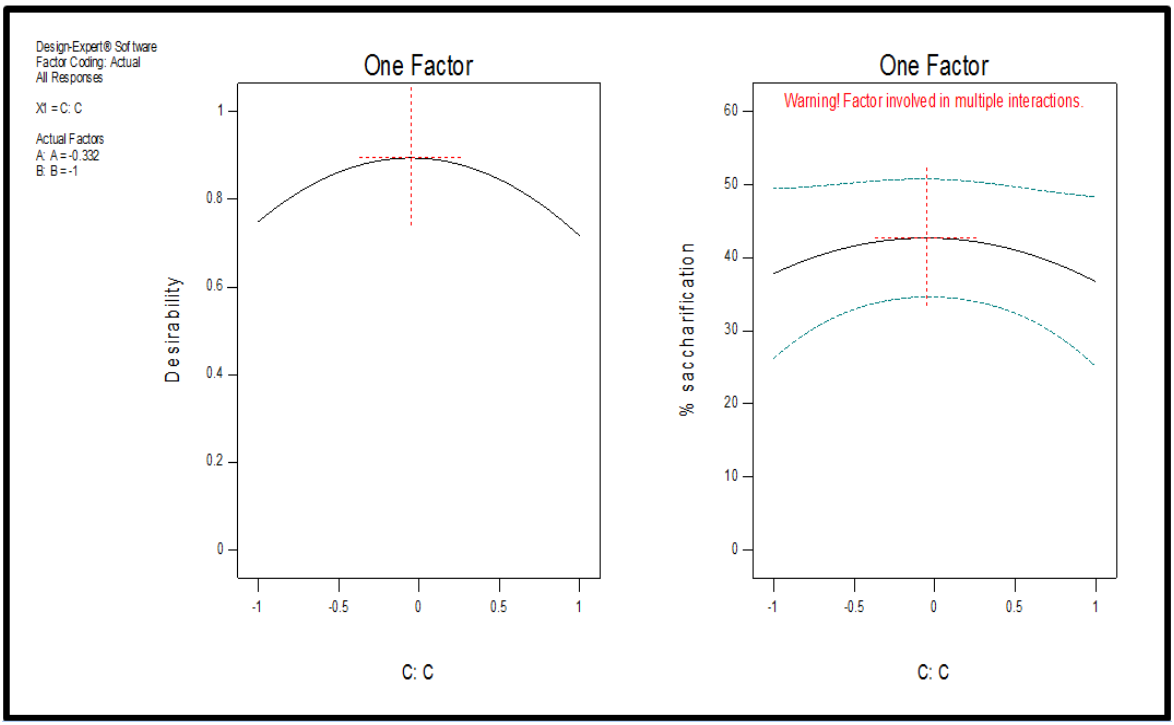
Factor	Goal	Lower Limit	Upper Limit
A-Enzyme (FPU/g substrate)	In range	-1	+1
B-Substrate Conc. (%, w/v)	In range	-1	+1
C-Temperature (^{0}C)	In range	-1	+1
% Saccharification	maximize	12.65	46.25

Table 4.27 Optimum values of parameters for percent saccharification

Parameter	Coded Value	Actual Value
A-Enzyme (FPU/g substrate)	-0.332	18.4
B-Substrate Conc. (%)	-1	1.0
C-Temperature (^{0}C)	-0.051	39.49

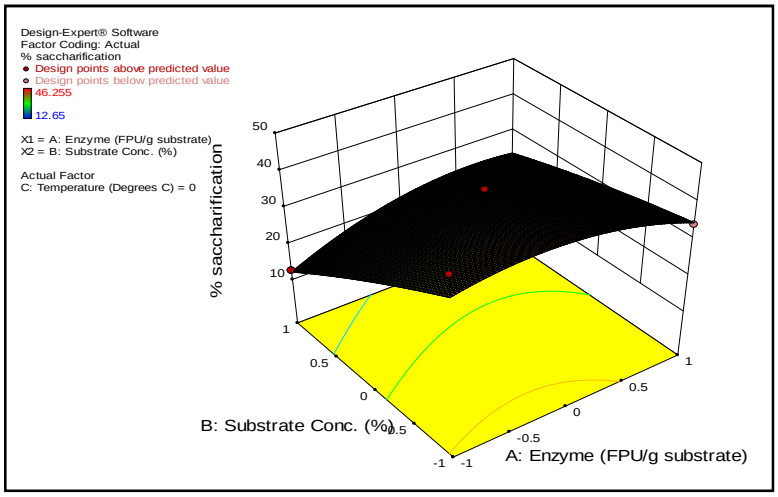


(a)

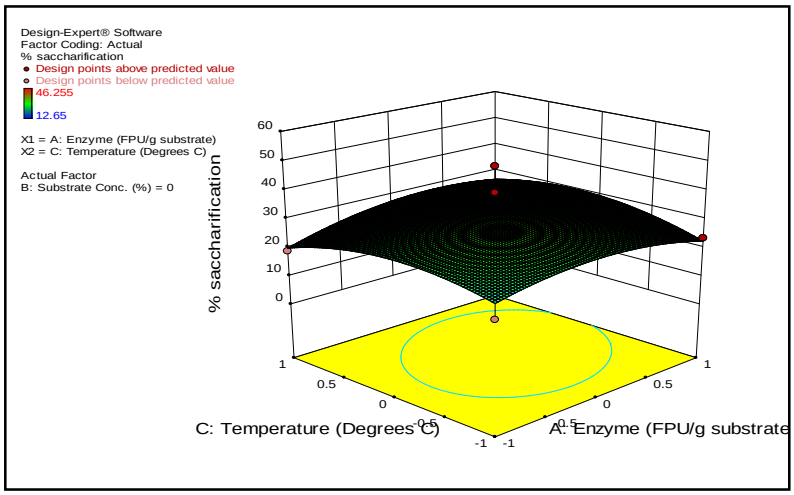


(b)

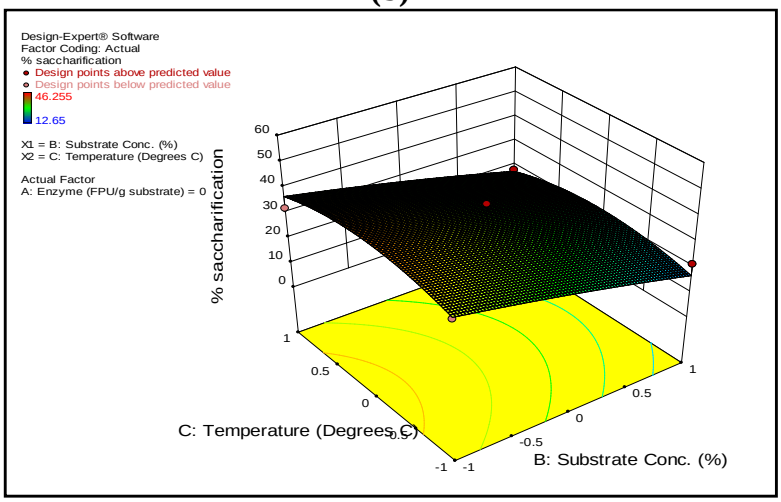
Fig 4.24: Linear surface contour plots (a, b) showing the interaction of various parameters during saccharification of *B. balcooa* powder



(a)



(b)



(c)

Fig 4.25: 3D surface contour plots (a, b and c) showing the interaction of various parameters during saccharification of *B. balcooa* powder

4.14 Wood Hydrolysis Monitoring

4.14.1 Changes in Chemical Composition

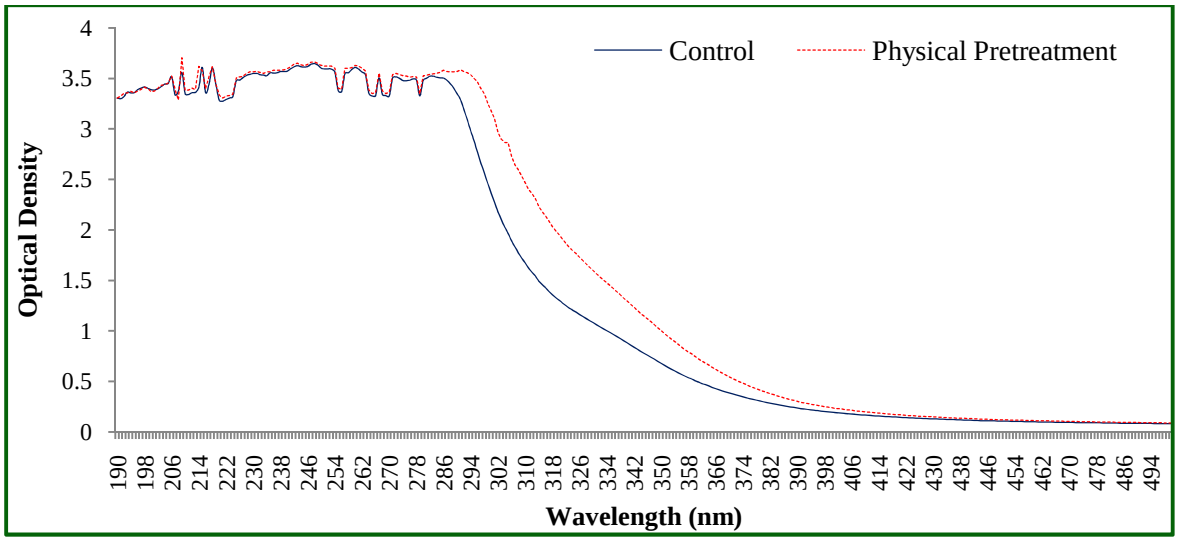
Lignin is most important chemical constituents in the plant cell walls of wood that limits enzymatic hydrolysis of lignocellulosic biomass by the formation of cross links with cellulose and hemicellulose fibres (**Vinzant *et al.*, 1997**). Lignin content reduction is well accomplished by various pretreatment methods that led to expose the highly ordered cellulosic fibres as well as removal of crystalinity of cellulose. It is apparent from the results that the initial cellulose of untreated *B. balcooa* powder was exposed with percent increase of 41.19%, 57.76% and 38.41% with physical, chemical, and biological pretreatments respectively (**Table 4.28**), whereas, initial lignin content got degraded with percent decrease of 50.5, 70.84, and 71.52 % with physical chemical and biological pretreatments respectively. Although, best results were obtained with alkaline H_2O_2 pretreatment in context of cellulose exposure, but the biological pretreatment gave maximum delignification (71.52%) with 38.41 % increase in cellulose content. Various studies reported varying results about lignin removal (%) after fungal pretreatment. **Lee *et al.* (2007)** reported pretreatment of softwood using potential lignin degrading white rot fungi *Celacerata* sp., which gave only 13.1 % lignin removal with enhanced cellulose exposure. Only 12% lignin removal of bamboo clums using potent ligninase producer *Trametes versicolor* was reported by **Zhang *et* al. (2007)**, while a maximum 45.6 % lignin removal of Chinese willow (hard wood) was reported on 120th day of incubation period with *Echinodontium taxodii* 2538 (**Yu *et al.*, 2009**). In our findings, we have found 71.52% lignin removal within 48th d of incubation using wood rot fungal isolate WDP2.

Table 4.28 Compositional changes in *B. balcooa* wood after various pretreatments

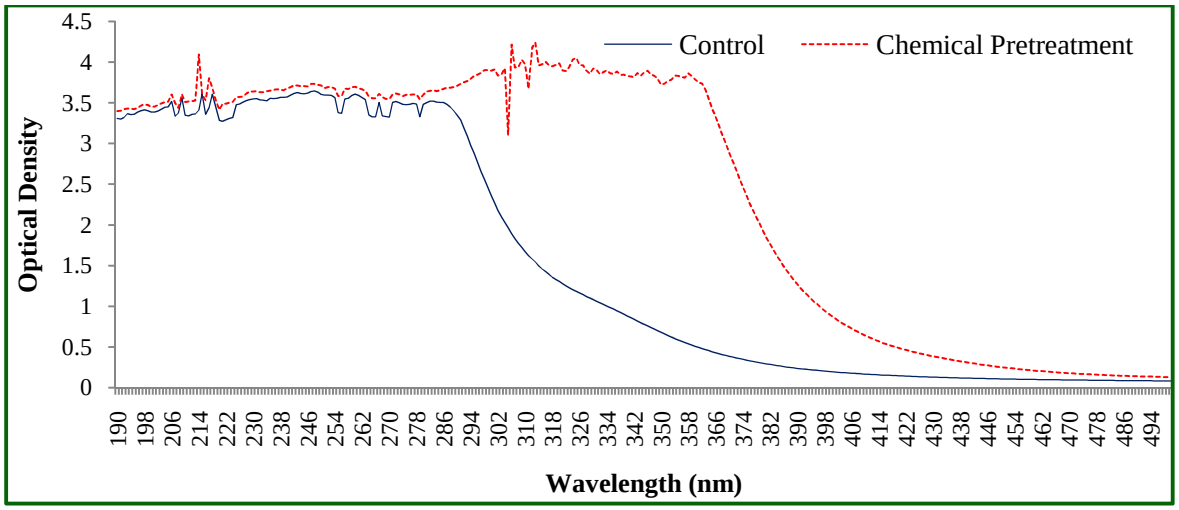
Pretreatments	Cellulose		Lignin	
	Content (%)	% Increase	Content (%)	% Decrease
Untreated (control)	37.93	-	29.5	-
Physical Pretreatment (Steam)	64.5	41.19	14.6	50.5
Chemical Pretreatment (Alkaline H_2O_2 in sunlight)	89.8	57.76	8.6	70.84
Biological Pretreatment (Fungal isolate WDP2)	61.59	38.41	8.4	71.52

4.14.2 UV-Vis Spectroscopic Studies

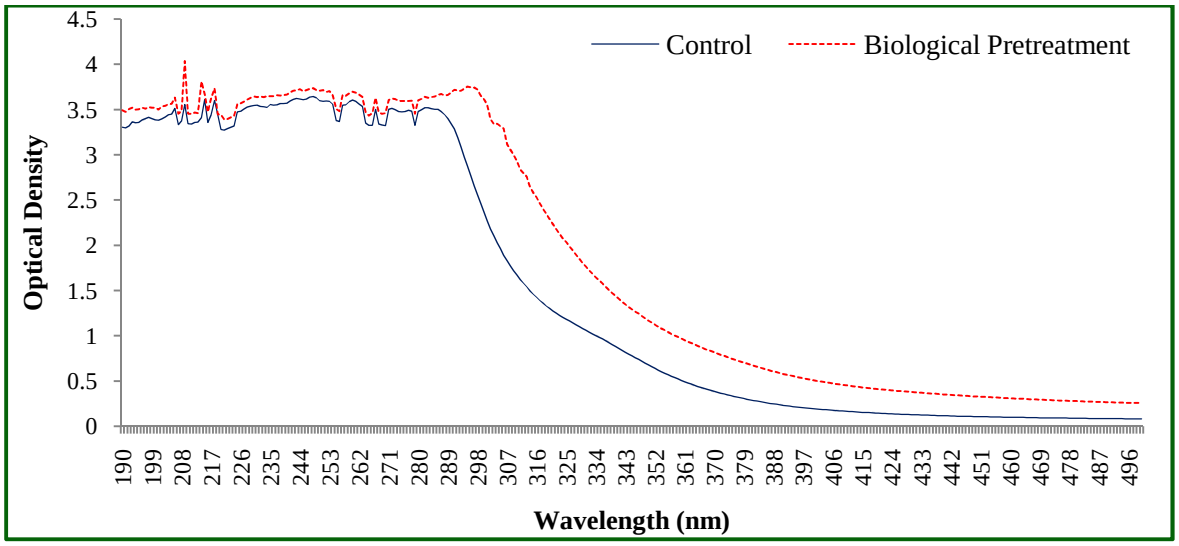
Spectroscopic techniques used to measure interaction of molecules with electromagnetic radiation. The spectrum is obtained when the light absorption is measured as a function of its frequency or wavelength (**Schmid, 2001**). Aromatic molecules with delocalised electrons often absorb electromagnetic radiation (light) in UV (150–400 nm) or the visible (400–800 nm) region of electromagnetic spectrum. UV spectrophotometry is an analytical tool that could used to explain structural modifications in pretreated wood by studying the peak shift/formation in supernatant, moreover the conjugated double bond systems (as in aromatic molecules such as benzene and phenol), have a large influence on peak wavelengths and absorption. (**http://www.shimadzu.com**). Therefore, during present investigation the clean filtrate of all three best pretreatments were scanned in a UV-Vis spectrophotometer (Thermo) using wavelengths ranged from 190-500nm. Clear differences were observed in UV-Vis spectra of filtrates of various pretreatments when compared with clear filtrate of untreated control (**Fig 4.26 a, b, c**). Various kinds of chemical shifts (bathochromic, hypsochromic, hyperchromic, and hypochromic) were observed for various pretreatment experiments. During the study, bathochromic shift (peak shift to longer wavelength) or peak formation was observed in all the cases, remarkably for chemical pretreatment, at wavelengths ranged from 304-370nm. The peaks appeared around the wavelengths ranged from 280-380 nm in each case of pretreatment may belongs to phenolic *o*, *p*-dihydroxystilbenes (λmax = 378 nm), p,p-dihydroxystilbenes (λmax = 356 nm), phenolic α- carbonyl units (λmax >>350nm), anthracene (λmax = 375 nm), p-nitrophenol (λmax = 300-400 nm), phenol (peaks between 220-300 nm), and possible other chromophores (**Lin, 1992; http://www.shimadzu.com, 2016; Kaijanen *et al.*, 2015**). Maximum peaks were formed while clean filtrate of alkaline oxidative pretreatment was scanned. The hyper-chromic shift (spectrum shifts toward greater absorbance) was prominent in case of only chemical and biological pretreatment (wavelength ranged from 202-220 nm), with a slight shift in case of physical pretreatment. Destruction of various peaks was also observed in case of chemical and biological pretreatment (wavelength ranged from 250-280 nm), with a slight destruction in case of physical pretreatment. Slight disturbed peaks in wavelengths ranged from 210-275 nm was also observed. The wavelength range belongs to the presence of sugars (glucose, and xylose) in the filtrate, although in negligible amounts (**Kaijanen *et al.*, 2015**). Therefore, UV-Vis scanning results indicated the release of some new compounds in filtrate/washing of various pretreatments, further and final confirmation of structural modifications can be done using FTIR and scanning electron microscopy of fine wood powder pretreated using various pretreatments (**Lin, 1992**). During the study, the absorption band shift to longer wavelengths or to greater absorbance. Many previous studies reported that the ionization of phenolic hydroxyl groups in polyphenolic moity (degraded compounds present in washing/filtrate) is one of the major causes for these kinds of shifts (**Lin, 1992; Pasco & Suckling, 1998; Dyer & Ragauskas, 2006**).



(a)



(b)



(c)

Fig 4.26 UV-Vis spectra of the hydrolysate of the wood samples subjected to physical (a), chemical (b), and biological (c) pretreated showing chemical bonding shifts as compared to untreated (control) wood filtrates.

4.14.3 FTIR Analysis

FTIR spectral analysis is one of the major techniques used to investigate the effects of the pretreatments on the lignocellulosic biomaterial as well as the enzymatic or microbial hydrolysis (saccharification) (**Faix and Böttcher, 1992; Karimi & Taherzadeh, 2016**). Since structure of lignocellulosic plant biomass is too complicated, FTIR analysis thus used to describe structural changes in lignin and carbohydrate components during pretreatment of lignocellulosic substrate. The FTIR spectrum study of physically, chemically, and biologically pretreated *B. balcooa* wood powder showed in **Fig 4.27**. It is apparent from all the three spectrums that certain functional groups in wood were changed significantly in all the cases. In physically pretreated wood sample, reduction of various bands was observed between 700-1000cm^{-1}, that attributed to glycosidic linkages and C-O vibration in biomass components (**Fig 4.27 a**). A significant reduction in 2354.54 cm^{-1} and 3234.67 cm^{-1} band positions, that corresponds to C-H and O-H stretching in lignin of wood, were also observed. Moreover, other peaks destruction and formation was observed from 3500-4000 cm^{-1} band positions. These all peaks are prominent in untreated sample suggesting the degradation of lignin as well as hemicellulose fractions in wood sample after physical pretreatment (**Table 4.29**). Related results were obtained after alkaline H_2O_2 (chemical) pretreatment. Degradation of various peaks in 3500-4000 cm^{-1} was observed prominently that attributed to C-H and O-H stretching in lignin of wood, thus showed more degradation of lignin (**Fig 4.27 b**). Reduction of peak at 1259 cm^{-1} in chemically pretreated sample can also be correlated to C-C, C-O stretching, aromatic ring vibration, and C-H bending, that all are related to decrease in lignin content and cellulose crystalinity in wood. In physically pretreated wood sample, same kind of bands reduction was observed between 700-1000cm^{-1}, that is again a sign of distruction in glycosidic linkages and C-O vibration in biomass components (**Fig 4.27 c**). Moreover, formation of new band at 1750.50 cm^{-1} band position, results due to ketone aldehyde C=O stretch, or formation of free esters from hemicellulose polymer, thus could be correlated to hemicellulose degradation also. Reduction of 3458.38 cm^{-1} band position in biologically pretreated wood can also be a good finding because that leads to the O-H stretching in lignin of wood. A reduction in 2354.54 cm^{-1} band position was also observed, that corresponds to C-H in lignin of wood. Many previous studies reported the significant reduction in the peaks after various pretreatments that are same as observed during present study (**Faix, 1991; Kline *et al*., 2010; Fan *et al*., 2012; Xu *et al*., 2013**). Fourier transform mid-infrared spectroscopic analysis is rapid and non-destructive technique for the determination of biomass components **(Tucker *et* al., 2001**). Although sample preparation for FTIR study is critical, because of water absorption by sample and complexity of biomaas, but it provides good information about certain components or change in these components in the plant cell wall through absorbance bands (**Mouille *et al*., 2003; Smith-Moritz *et al*., 2011**).

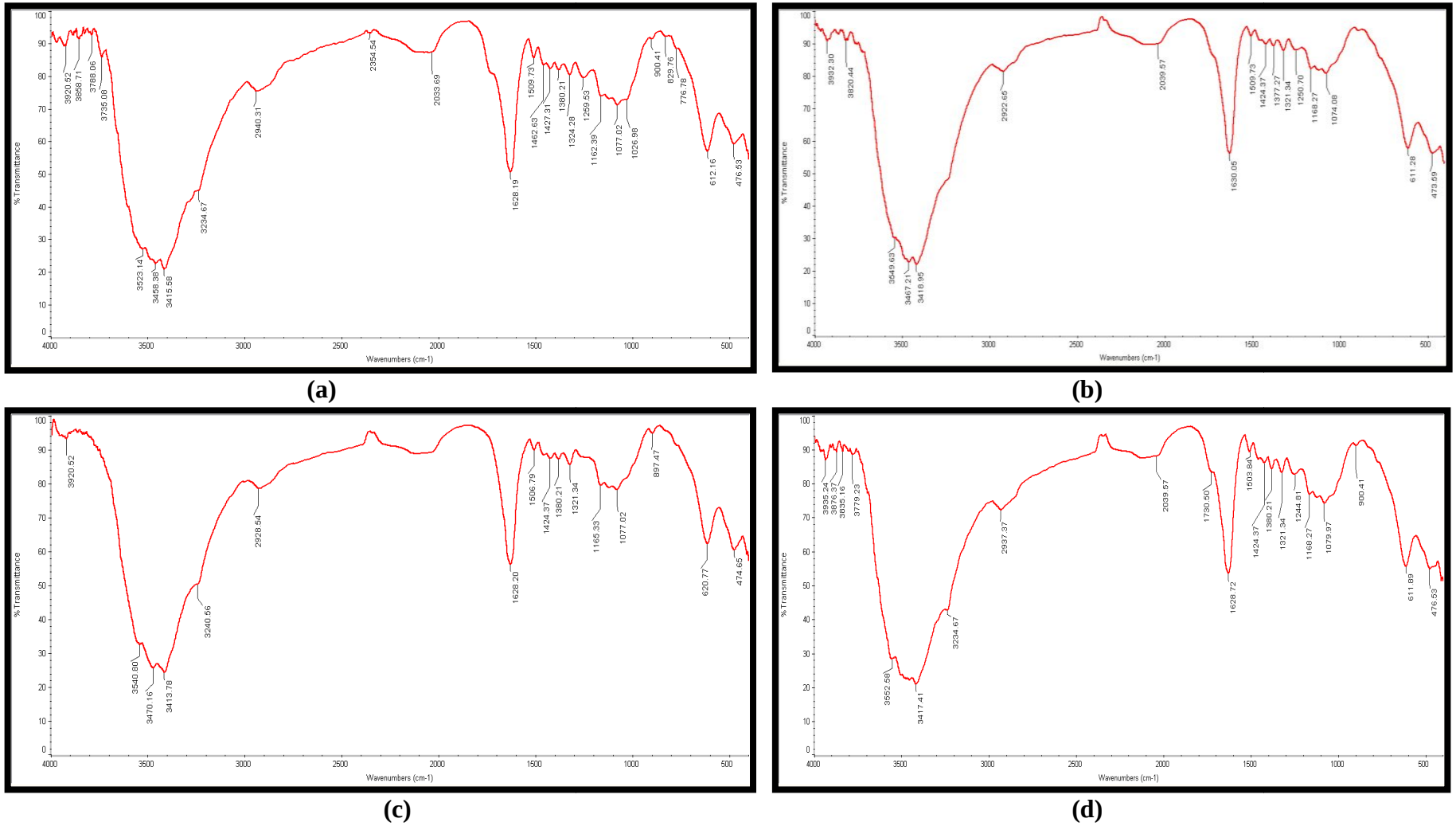


Fig 4.27: FTIR spectra of *B. balcooa* samples before (a) and after physical (b), chemical (c) and fungal pretreatment (d)

Table 4.29: The reported chemical changes in the functional groups of lignocellulosic biomass as observed through FTIR analysis based on absorbance bands (*Source*: Kline *et al*., 2010; Xu *et al*., 2013)

Band Position	Peak Assignment/ Functional Group	Polymers
755	Asymmetric bending of HC CH group	Lignin
875	Glycosidic linkage	Hemicellulose
930	Glycosidic linkage	Cellulose, hemicellulose
990	C-O valence vibration	Cellulose
1035	C-O, C=C, and C-C-O stretching	Cellulose, hemicellulose, lignin
1160	C-O-C asymmetrical stretching	Cellulose, hemicellulose
1200	O-H bending	Cellulose, hemicellulose
1215	C-C + C-O	Stretch Lignin (wood)
1270	Aromatic ring vibration	Guaicyl lignin
1280	C-H bending	Crystalline cellulose
1310	CH_2 wagging	Cellulose, hemicellulose
1327	C-O of syringyl ring	Lignin (wood)
1335	C-H vibration, O-H in-plane bending	Cellulose, hemicellulose, lignin
1380	C-H bending	Cellulose, hemicellulose lignin
1425	C-H in-plane deformation	Lignin (wood)
1440	O-H in-plane bending	Cellulose, hemicellulose, lignin
1465	C-H deformation	Lignin
1500	Aromatic ring vibration	Lignin
1595	Aromatic ring vibration+ C=O stretch	Lignin
1682	C=O stretching (unconjugated)	Lignin (wood)
1730	Ketone/aldehyde C=O stretch	Hemicellulose
1750	Free ester	Hemicellulose
2840, 2937	C-H stretching	Lignin (wood)
3421	O-H stretching	Lignin (wood)

4.14.4 Ultra-structural Changes

Scanning electron microscopy (SEM) permits a successful visualization of structural changes on *B. balcooa* wood powder after various pretreatments as well as saccharification. During present study, the SEM study of differentially pretreated and saccharified bamboo biomass revealed visual details on tissue damage and ultra structural changes occurring as resulted by physical, chemical, and eco-friendly biological pretreatments (**Plate 4.10**), and cellulase hydrolysis of biologically pretreated bamboo wood powder (**Plate 4.11**). Untreated sample seem to have many deposits on its surface that may include lignin polymer, hemicellulose, waxes and other binding materials. Moreover, the study further indicated the presence of intact vascular bundles, epidermis, and lumen (**Plate 4.10 a, b**), but negligible disintegration was observed due to initial processing for particle size reduction. A very good response of bamboo wood powder to different pretreatments and saccharification processes was observed in comparison to untreated sample. As per the observed results, overall same degradation pattern in physically **(Plate 4.10 c, d)**, chemically **(Plate 4.10 e, f)**, and biologically **(Plate 4.10 g, h)** pretreated wood powder was observed through SEM analysis. The cracks were developed that were heterogeneous and finely scattered throughout the biomass. Development of cracks throughout the biomass particle under study could be a sign of good results of pretreatment that was applied, and hence, can increase the surface area to facilitate fragmentation and disruption of lignocellulose. Moreover, some thread like structures were also observed on the biologically pretreated wood powder, which may be the mycellia of attached fungi to biomass. Same degradation pattern were observed while fungal pretreated *B. balcooa* wood powder was saccharified using cellulase enzyme from potential cellulase producer PCF1 (**Plate 4.11 a, b**). However, increased size of cracks and presence of more pores on biomass particle was supported further degradation (saccharification) of bamboo wood powder. Similar observations were also reported by previous workers (**Kumagai *et al*., 2014; Pooja and Padmaja, 2014; Shafiei *et al*., 2014**) for SEM analysis of pretreated wood. The cracks/pits were observed in pretreated wood samples, would obviously favour the contact of steam/chemical/enzyme(s) to inner linkages of wood, hence can accelerating the biodegradation process. Cracks and pit formation can be due to the removal of a large

proportion of lignin, hemicellulose and crystalinity of cellulose after pretreatment (**Kumar *et al*., 2010; Kumagai *et al*., 2014**). Thus all the pretreatments resulted in an increase in surface area for enzymatic saccharification of pretreated wood powder due to partial degradation of lignin polymer. However, increased number of pits (pores) was observed in biologically pretreated and saccharified wood as compared to physical and chemical pretreated wood. The fungal isolate had negligible cellulase enzyme production, thus more pit formation can also correlated to lignin degradation due to very high laccase enzyme production, or cellulose crystalinity removal (**Kumagai *et al*., 2014; Kumar *et al*., 2010; Pooja and Padmaja, 2014**). Furthermore, saccharification of biologically pretreated bamboo powder using cellulase enzyme from potential cellulase producer enhanced the pore formation and number of cracks. Increase in the surface area also responsible for cellulase accessible to cellulosic part of delignified wood thus surface plays an important role in the improvement wood digestion. The increased number of pits/pores and the damaged structures that observed during the study on cell walls help the hydrolyzing microbes/ enzymes to act more efficiently on the pretreated wood powder (**Taherzadeh and Karimi, 2008**).

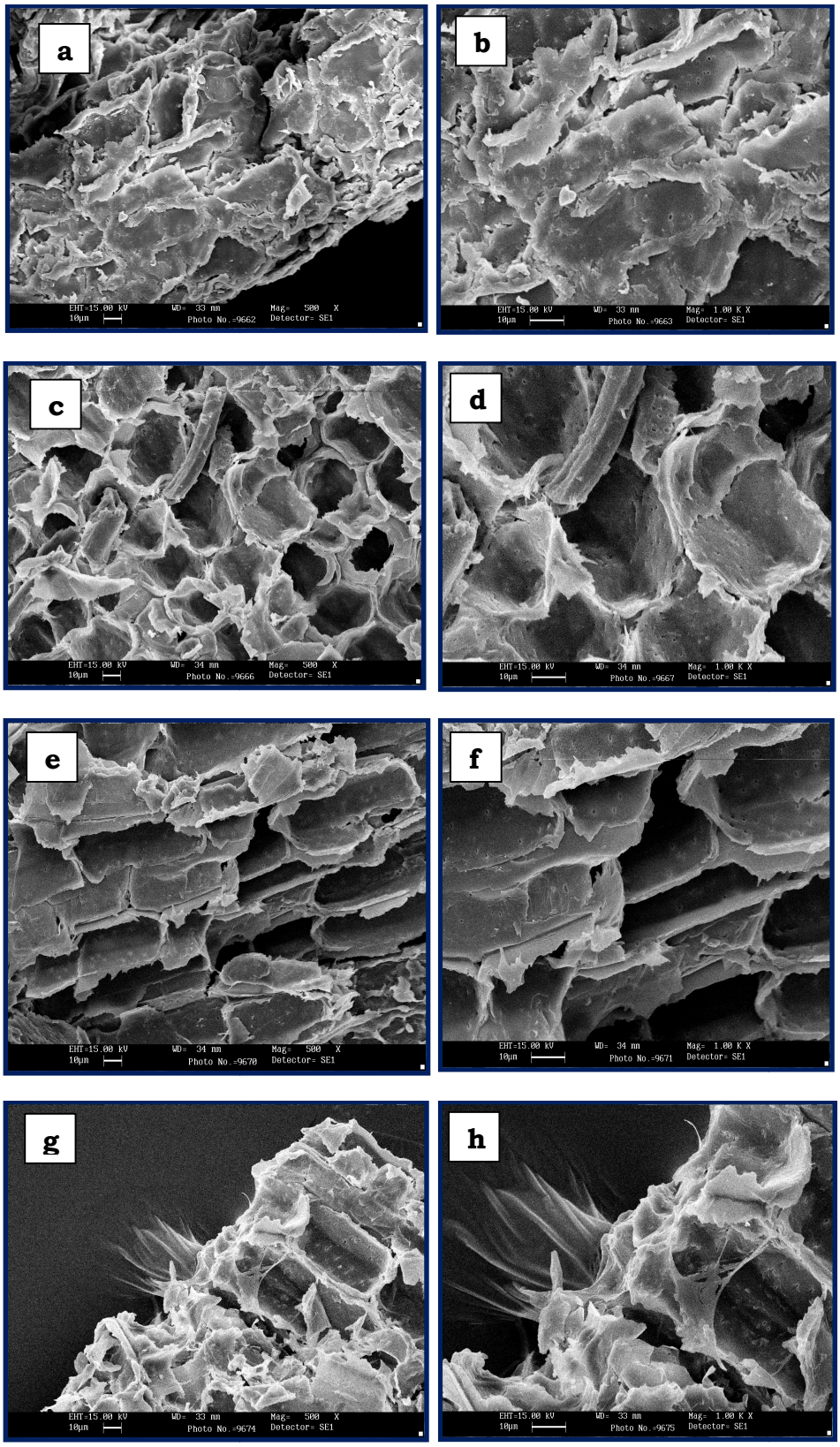
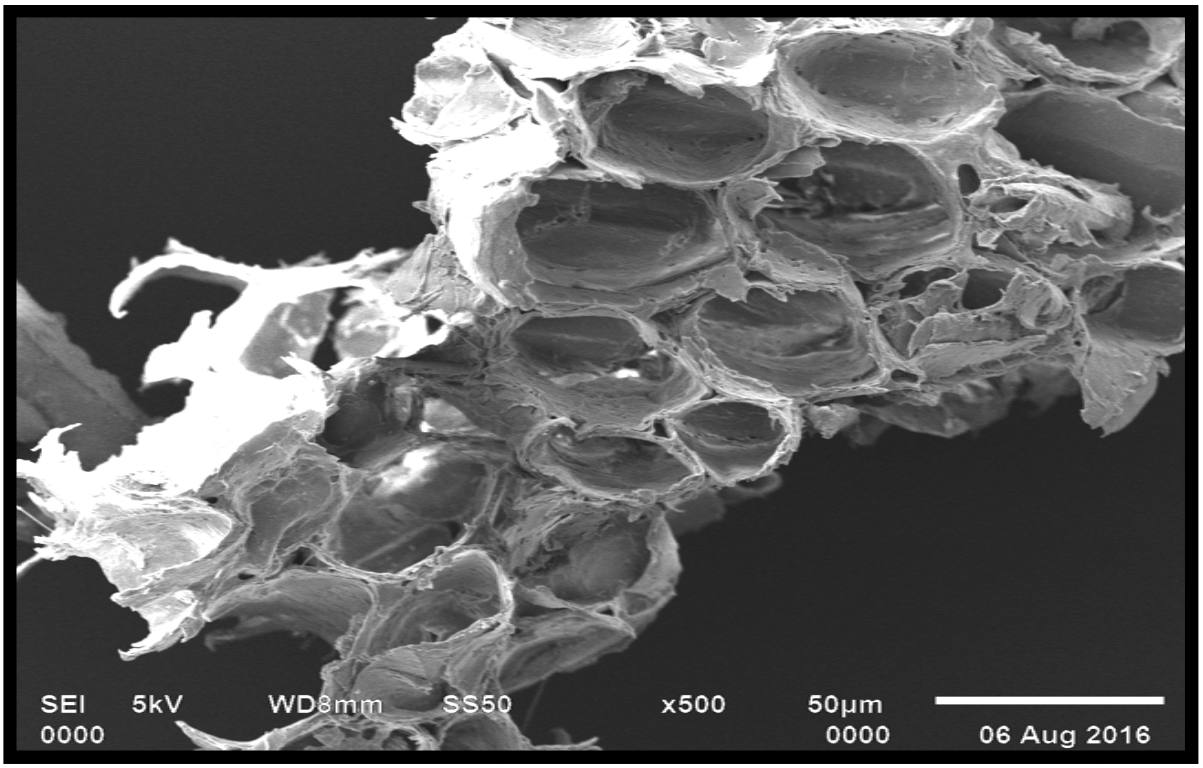
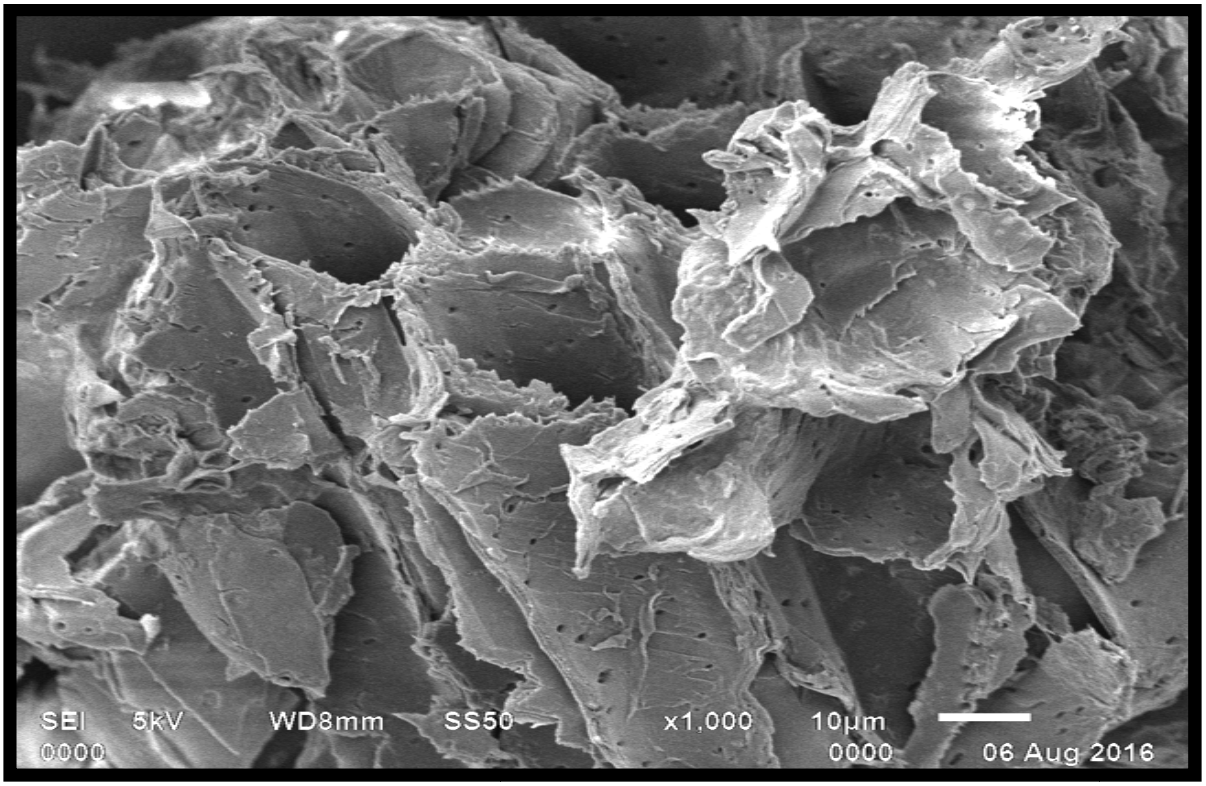


Plate 4.10: Scanning electron photomicrographs of *B. balcooa* wood powder: untreated at 500X (a) and at 1000X (b); physically pretreated at 500X (c) and 1000X (d); chemically pretreated at 500X (e) and 1000X (f); biologically (fungal) pretreated at 500X (g) and 1000X (h).



(a)



(b)

Plate 4.11: Scanning electron photomicrographs of biologically (fungal) pretreated *B. balcooa* wood sample after enzymatic saccharification at 500X (a) and at 1000X (b).

UNIT V: IDENTIFICATION AND CHARACTERIZATION OF LIGNOLYTIC FUNGAL CULTURES

4.15 Characterization of Lignolytic Isolates

Purity of microbial isolates is very important for successful operation of bioprocess. Thus the cultures should routinely be checked and sub-cultured at the part of preservation and during the whole process. For quality measures, potential microbial cultures should be properly characterised using phenotypic, biochemical, and molecular markers; and identified using modern tools and techniques. Therefore all four potent laccase producing wood rotting fungal isolates (WDP2, WFPn1, AAFP5, and TAFP4), were characterised at both phenotypic and biochemical levels. Out of them, two fungal isolates (WDP2, WFPn1) further characterised at molecular level also using molecular markers.

4.15.1 Morphological Characterization

The selected fungal fruiting bodies, from their natural habitat (**Plate 4.12**) were brought to laboratory for further work in airtight sterile poly-bags to minimize the chance of further contamination and moisture loss. Variation in fruiting body structure and colour was observed. The isolates WFPn1, and AAFP5 having almost same kidney shaped dark brown stalked fruiting body, while the other two potential fungi having yellowish white, un-stalked fruiting body structures. The fungal isolate WDP2 having porous surface at one side of fruiting body. For isolation of mycellial form small pieces of fruiting bodies were surface sterilized with 70% ethanol, followed by several washings with sterile distilled water. The sterilized pieces were than inoculated in the centre of PDA plates contained antibiotic and incubated at 28 ± 2^{0}C for 7d for the mycellial growth. The fungal isolates showed no pigmentation in mycelium and forming white to off white coloured colonies with differential mycellial pattern (**Plate 4.13**). The phenotypic characteristics of all the four fungal isolates are presented in **Table 4.30**. Potential isolate WDP2 was observed to form irregular, raised, rough, and white coloured mycellial colony (with primary and secondary growth), whereas isolate WFPn1 and AAFP5 showed typical creamy white (old cultures were saffron to white), irregular, flat, smooth, and filamentous fungal colony. Isolate TAFP41 having very complex mycellial structure with white (old cultures were yellow white), irregular, convex, rough, and irregular colony that was very difficult to remove from agar surface. Based on unstained live slide preparations under phase contrast

microscopy, all selected isolates showed septate mycelium with no spore formation (**Plate 4.13**). Our results are in agreements with those of previous findings that indicated most of the similar fruiting body and agar plate characteristics of wood rotting fungi (**Van Der Westhuizen, 1958; Liu *et al*., 2009**).

4.15.2 Biochemical Characterstics

The selected four lignolytic fungal cultures were further tested for their sugar (mono-, di-, polysaccharides), sugar alcohols and organic compounds utilizing efficiency during growth using HiCarbohydrate kit KB009 (HiMedia) (**Plate 4.14**). All cultures varied in their abilities to utilise various monosaccharides such as hexoses (fructose, dextrose, galactose, mannose sorbose), pentoses (xylose, L-arabinose, D-arabinose, ribose, and rhamnose); disaccharides (lactose, maltose, trehalose, melibiose, sucrose, and cellobiose); trisaccharides (raffinose, and melezitose); polysaccharides (inulin, cellulose, CMC); sugar alcohols (dulcitol, inocitol, sorbitol, mannitol, adonitol, and xylitol); organic compounds (sodium gluconate, glycrol, salicin, glucosamine, α-methyl-D-glucoside, α-methyl-D-mannoside, ONPG, esculin, citrate, and malonate); extracellular enzyme activities (laccase, overall ligninase, lignin peroxidase, FPase, CMCase, β-glucosidase, and xylanase); and synthetic dye (congo red, malachite green, brilliant green crystal violet, fuchsine basic) removing efficiency.

It is apparent from the results that the all selected fungal isolates were able to utilise D-arabinose, cellulose, CMC, mannitol, xylitol, ONPG, esculin, citrate, and malonate. Fungal isolate AAFP5 was found to with assimilation of all the disaccharides provided indicating broad substrate utilising efficiency of the isolate. The fungal isolate WFPn1 and AAFP5 utilised the provided compounds in approximate similar manner. When tested for extracellular enzyme production production, none of the isolate could show CMCase activities, lignin peroxidase activity was shown by only two isolates WFPn1, and WDP2; while extracellular laccase , overall ligninase, FPase, β-glucosidase, and xylanase activities were shown by all the four test cultures. All the four test cultureswere found to have dye decolourizing efficiency towards congo red, malachite green, and brilliant green dyes. The cultures were found to produce extracellular lignin modifying/ degrading enzyme activities while characterized biochemically. Various previous findings reported ability of wood rot fungi to secret such enzymes for microbial degradation lignin of dead plant materials into

natural environment (**Bonnen *et al*., 1994; Eggert *et al*., 1996; Homma *et al*., 2007; Kalyani *et al*., 2013; Fen *et al*., 2014; Woo *et al*., 2014; Sindhu *et al*., 2016)**. Although cultures were cellulase positive, but very less cellulase enzyme production was reported while quantification of cellulase was performed, due to which less sugar release was observed at the time of biological delignification experiment. Other studies reported variability in utilization of various sugars by wood rot fungi (**Saheed *et al*., 2016; Schmidt *et al*., 2016**).

4.15.3 Molecular Characterization and Identification of Fungal Isolates

Two potential laccase producing wood rot fungal isolates (WDP2 and WFPn1) were characterised based on the molecular study of 5.8S ITS r-RNA gene analysis.

I. 5.8S ITS r-RNA Gene Amplification

PCR amplification of 5.8S ITS r-RNA gene was carried out using the ITS 1and ITS4 primers. The PCR products ~700-750 nucleotide bp were generated from the two fungal isolates **(Plate 4.15).**The PCR amplicon was purified and sequenced through Yaazh Xenomics Pvt. Ltd. Chennei, India. The process for submission of gene sequences to NCBI genbank is under process

II. Homology Search

The 5.8S ITS rRNA gene sequence of two selected cultures were analised using nucleotide basic local alignment search tool (BLAST N) and gene sequences available in the National Centre for Boitechnology Information (NCBI) database (**http://blast.ncbi.nlm.nih.gov/**). Program MUSCLE 3.7 was used for multiple alignments of sequences and Gblocks was used for elimination of poorly aligned part. PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

Using BLAST search among 5.8S ITS rRNA gene sequences of potential wood rot laccase producing fungal isolates (WDP2, and WFPn1) and other closely related fungal genera existing in database, percent identity were determined (**Table 4.32**). It is apparent from the table that the wood rot fungal isolate WDP2 and WFPn1 belong to the genus *Lengites* and *Ganoderma* respectively. The 5.8S ITS r RNA sequence was blast using NCBI blast similarity search tool.

III. Phylogenetic Tree Construction and Identification

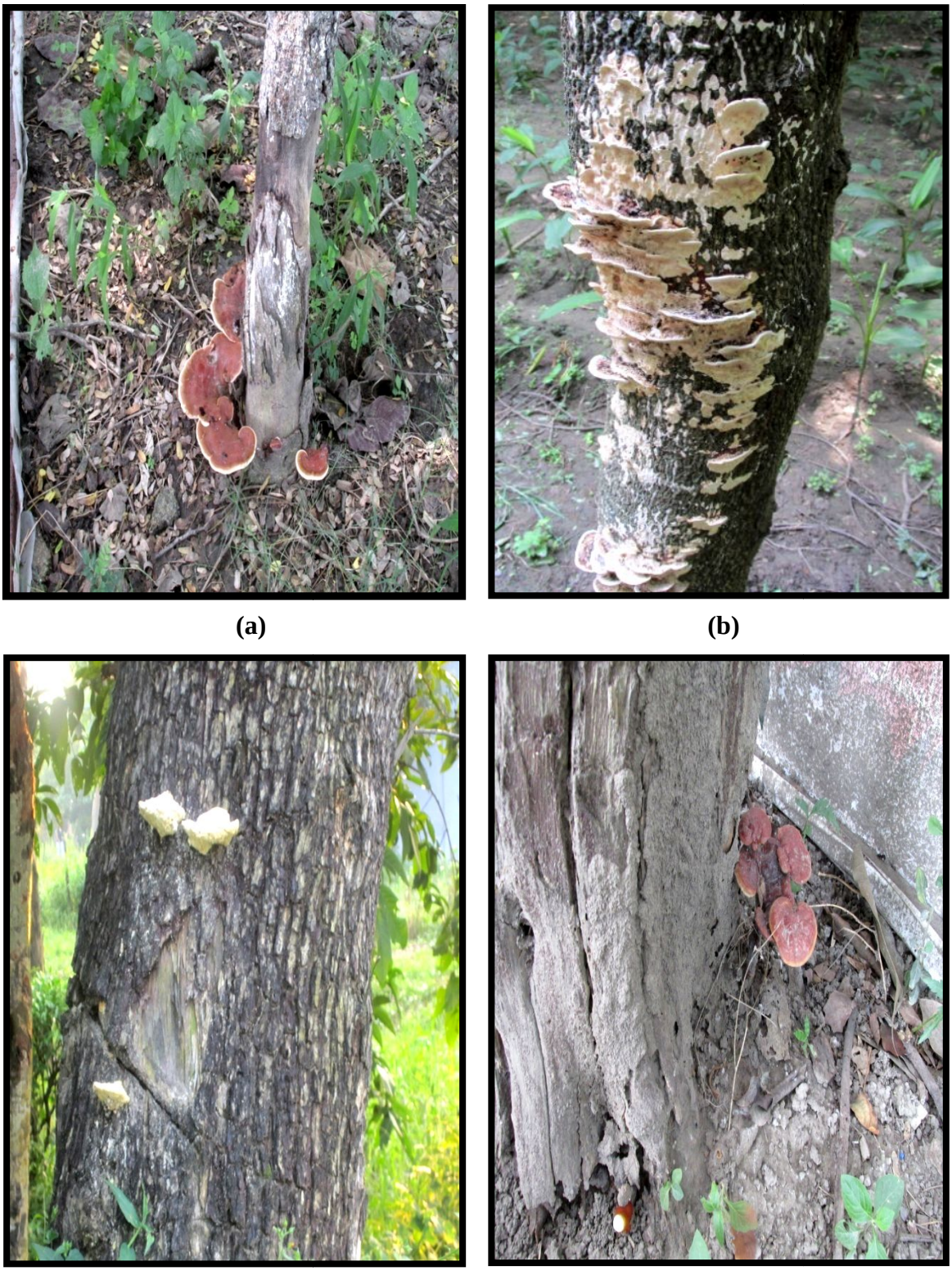
For preparation of phylogenetic tree to study the evolutionary relatedness of selected wood rot fungal isolates with other wood rot fungal cultures, the retrieved sequences using BLAST search were aligned using Mega 6.0 bioinformatics software. It is apparent from phylogenetic tree constructed for the both fungus, the isolate WDP2 showed maximum identity (99%) and highest query coverage (37%) with *Lengites elegans* isolate PCT.22, whereas isolate WFPn1 was observed to reveal maximum identity (99%) with *Ganoderma lucidum* strain BCRC 37043 (**Fig 4.28 & Fig 4.29**).

The program MUSCLE 3.7 was used by previous workers for multiple sequence alignment (**Castresana, 2000; Edgar, 2004**). For alignment curing (alignment noise removal), programme Gblocks can be used to eliminates poorly aligned positions and divergent regions (**Talavera and Castresana, 2007**). The characterstic features of fruiting bodies of the isolates WDP2 and WFPn1 could be correlated with molecular study. The porous structure f the fruiting body at ventral side as observed by us for the isolate WDP2 has also been reported as the characteristic feature of fruiting body of wood rot fungus *Lengites* (**Im *et al.*, 2016**). Therefore this fungus is also known as “gilled polypore”. Moreover, morphologically similar fruiting body structure like fungal isolate WFPn1 was reported by various studies (**Seo *et al.*, 2000; Hapuarachchi *et al.*, 2015**). Thus, the isolate WDP2 showed maximum identity with *Lengites elegans* isolate PCT. 22 (HQ248217.1), while the isolate WDPn1 revealed maximum similarities with the *Ganoderma lucidum* strain BCRC 37043 (EU021460.1).

Table 4.30: Phenotypic (fruiting body/colony/mycelial) characteristics of selected wood rot laccase producing fungal cultures*

S. No.	Characteristics	Fungal Cultures			
		WFPn1	**WDP2**	**AAFP5**	**TAFP41**
I. Fruiting Body					
i.	**Appearance**	Dark brown, kidney shaped, stacked, non porous	Porous, upper surface orange brown, lower surface white, un-stacked	Dark brown, kidney shaped, stacked, non porous	Orange white to creamish white, porous, un-stacked
II. Colony Characteristics					
ii.	**Chromogenesis**	Creamy white, radial growth (Old cultures- saffron white)	White radial growth (compact growth in old cultures)	White, radial growth (Old cultures- saffron white)	White (Old cultures- yellow white)
iii.	**Shape**	Irregular	Irregular	Irregular	Irregular
iv.	**Elevation**	Flat	Raised	Flat	Convex
v.	**Surface**	Smooth	Rough	Smooth	Rough
vi.	**Form**	Filamentous	Irregular	Filamentous	Irregular
vii.	**Margin**	Filiform	Undulate	Filliform	Filliform
III. Cellular Characteristics					
viii.	**Mycelial structure**	Compact mycellia, with septate hyphae	Compact mycellia, with septate hyphae	Compact mycellia, with septate hyphae	Compact mycellia, with septate hyphae
ix.	**Spore formation during mycelial growth**	**-**	**-**	**-**	**-**

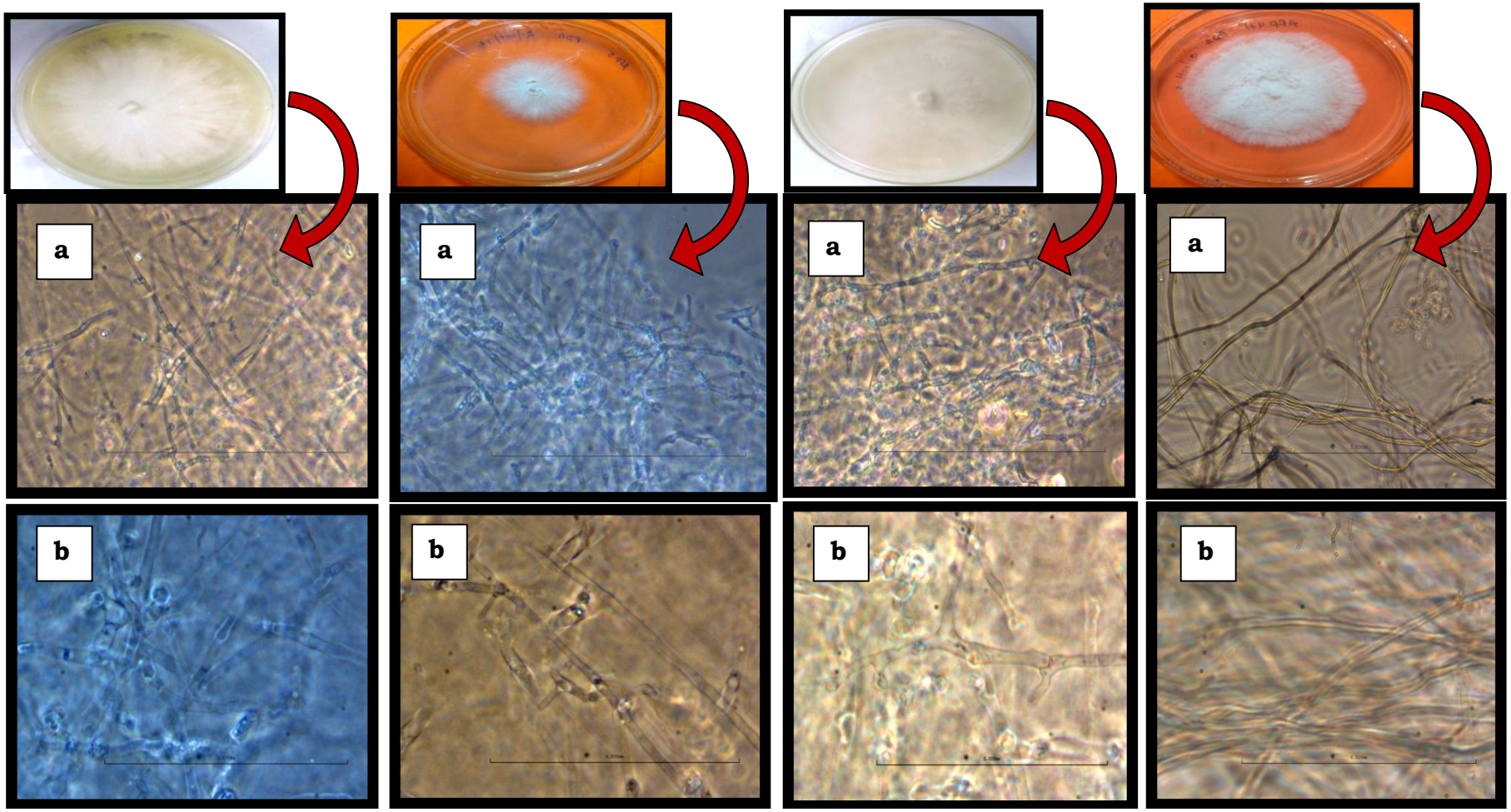
*** The cultures were grown on potato dextrose agar (PDA) for 7d at 28±1^{0}C.**



(a) **(b)**

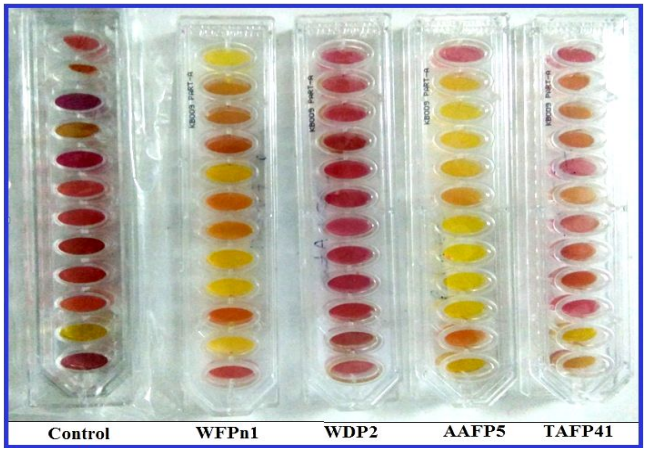
(c) **(d)**

Plate 4.12: Fruiting body morphology of the selected wood rot fungal cultures at their natural habitat. WFPn1 (a), TAFP41 (b), WDP2 (c), and TAFP5 (d)

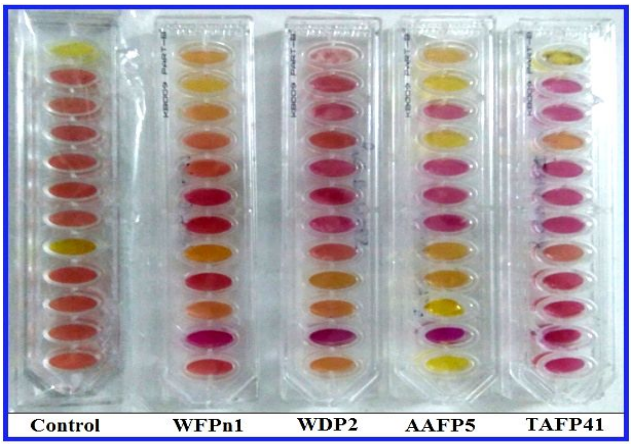


WDP2 AAFP5 WFPn1 TAFP4

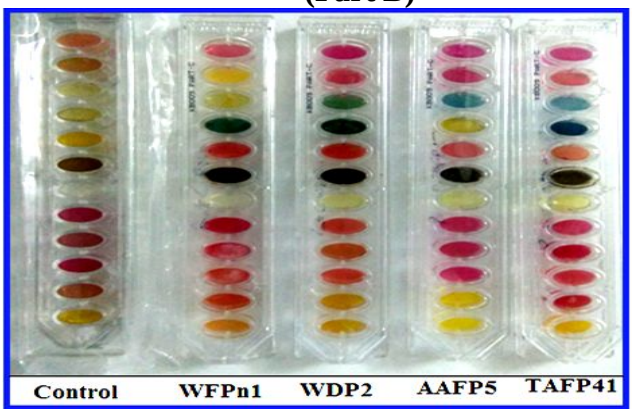
Plate 4.13: Phase contrast photomicrographs showing mycelial structures of the unstained live fungal preparations at 40X (a) and 100X (b)



(Part A)



(Part B)



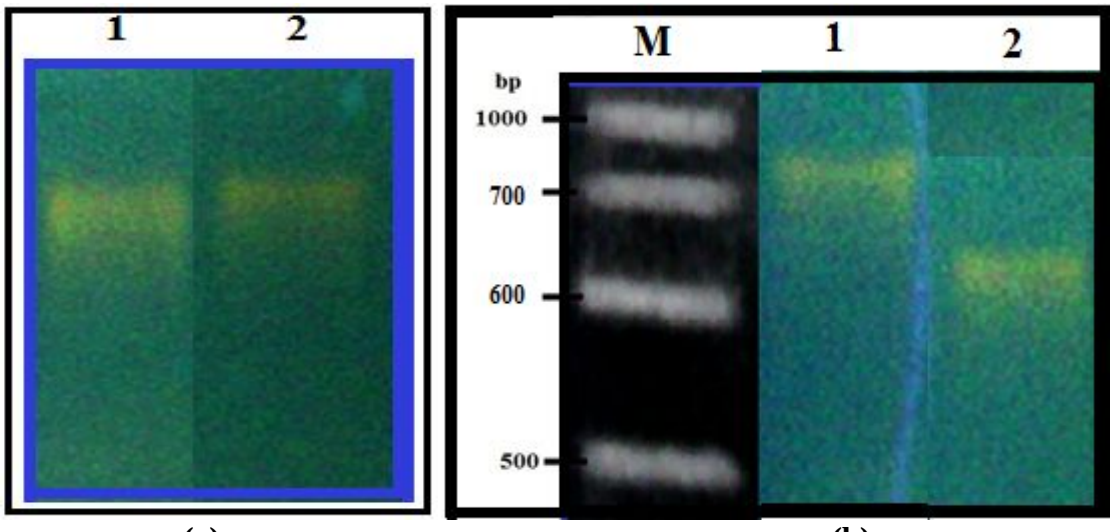
(Part C)

Plate 4.14: Sugar/ organic compound assimilation efficiency of selected fungal cultures. Part A contained (from down to up) lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-areabinose, and mannose. Part B contained (from down to up) inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inocitol, sorbitol, mannitol, adonitol, α-methyl-D-glucoside, and ribose. Part C contained (from down to up) rhamnose, cellobiose, melezitose, α-methyl-D-Mannoside, xylitol, ONPG, esculin, D-arabinose, citrate, malonate, sorbose, and last one is negative control.

Table 4.31: Biochemical characteristics of selected fungal cultures

I. Sugar assimilatory efficiency	Cultures			
	WFPn1	**WDP2**	**AAFP5**	**TAFP41**
Monosaccharides (hexoses)				
Fructose	+	-	+	-
Dextrose	+	-	+	-
Galactose	+	-	+	-
Mannose	+	-	-	-
Sorbose	-	-	-	-
Monosaccharides (pentoses)				
Xylose	-	-	+	+
L-arabinose	+	-	+	+
D-arabinose	+	+	+	+
Ribose	+	-	+	+
Rhamnose	+	+	+	+
Disaccharides				
Lactose	-	-	+	+
Maltose	+	-	+	-
Trehalose	+	-	+	-
Melibiose	-	-	+	+
Sucrose	+	-	+	+
Cellobiose	-	+	+	-
Trisaccharides				
Raffinose	+	-	+	+
Melezitose	-	+	-	-
Polysaccharides				
Inulin	-	-	+	-
Cellulose	+	+	+	+
CMC	+	+	+	+
II. Sugar alcohols				
Dulcitol	-	-	-	-
Inocitol	-	-	-	-
Sorbitol	-	+	-	-
Mannitol	+	+	+	+
Adonitol	+	-	-	-
Xylitol	+	+	+	+
III. Organic compound utilising efficiency				
Sodium gluconate	-	-	-	-
Glycrol	+	-	+	-

Salicin	-	-	+	-
Glucosamine	-	-	+	-
α-methyl-D-glucoside	+	-	+	-
α-methyl-D- mannoside	-	+	-	-
ONPG	+	+	+	+
Esculin	+	+	+	+
Citrate	+	+	+	+
Malonate	+	+	+	+
IV. Extracellular enzyme activities				
Laccase	+	+	+	+
Overall ligninase	+	+	+	+
Lignin Peroxidase	+	+	-	-
FPase	+	+	+	+
CMCase	-	-	-	-
β-glucosidase	+	+	+	+
Xylanase	+	+	+	+
V. Synthetic dye decolourizing efficiency				
Congo red	+	+	+	+
Malachite green	+	+	+	+
Brilliant green	+	+	+	+
Crystal violet	-	-	-	-
Fuchsine basic	-	-	-	-



(a) **(b)**

Plate 4.15: Electrophoretic separation of genomic DNA on agarose gel (a), PCR amplicon of 5.8S ITS rRNA gene (b) of two selected lignolytic fungal cultures. Lane M: Marker (500 bp DNA ladder); Lane 1: WFPn1; Lane2: WDP2.

Table 4.32: 5.8S ITS r-RNA gene sequences retrieved from NCBI database showing identity (%) with that of selected laccase producing fungal isolates

Strain	(Accession no.)	Strand	Gaps	Query Cover (%)	E-value	Length (bp)	Identity (bp)	Identity (%)	Organism
WDP2	HQ248217.1	Plus	2/619 (0%)	37	0.0	619	608	99	*Lengites elegans* isolate PCT.22
	JX082361.1	Plus	0/617 (0%)	36	0.0	617	616	99	*Lengites* sp. BRFM 1079
	KT210101.1	Plus	1/616 (0%)	36	0.0	616	614	99	*Lengites* sp. DK14
	JF689829.1	Plus	0/602 (0%)	35	0.0	602	601	99	*Lengites* sp. FGTR13
WFPn1	GQ249884.1	Plus	10/692 (1%)	74	0.0	692	677	98	*Ganoderma lucidum* isolate CAW-17
	GQ249880.1	Plus	19/726 (2%)	77	0.0	726	702	97	*Ganoderma lucidum* isolate CAW-1
	EU021460.1	Plus	2/638 (0%)	68	0.0	632	638	99	*Ganoderma lucidum* strain BCRC 37043
	KR154928.1	Plus	6/651 (0%)	69	0.0	641	651	98	*Ganoderma* sp. BAB 4558

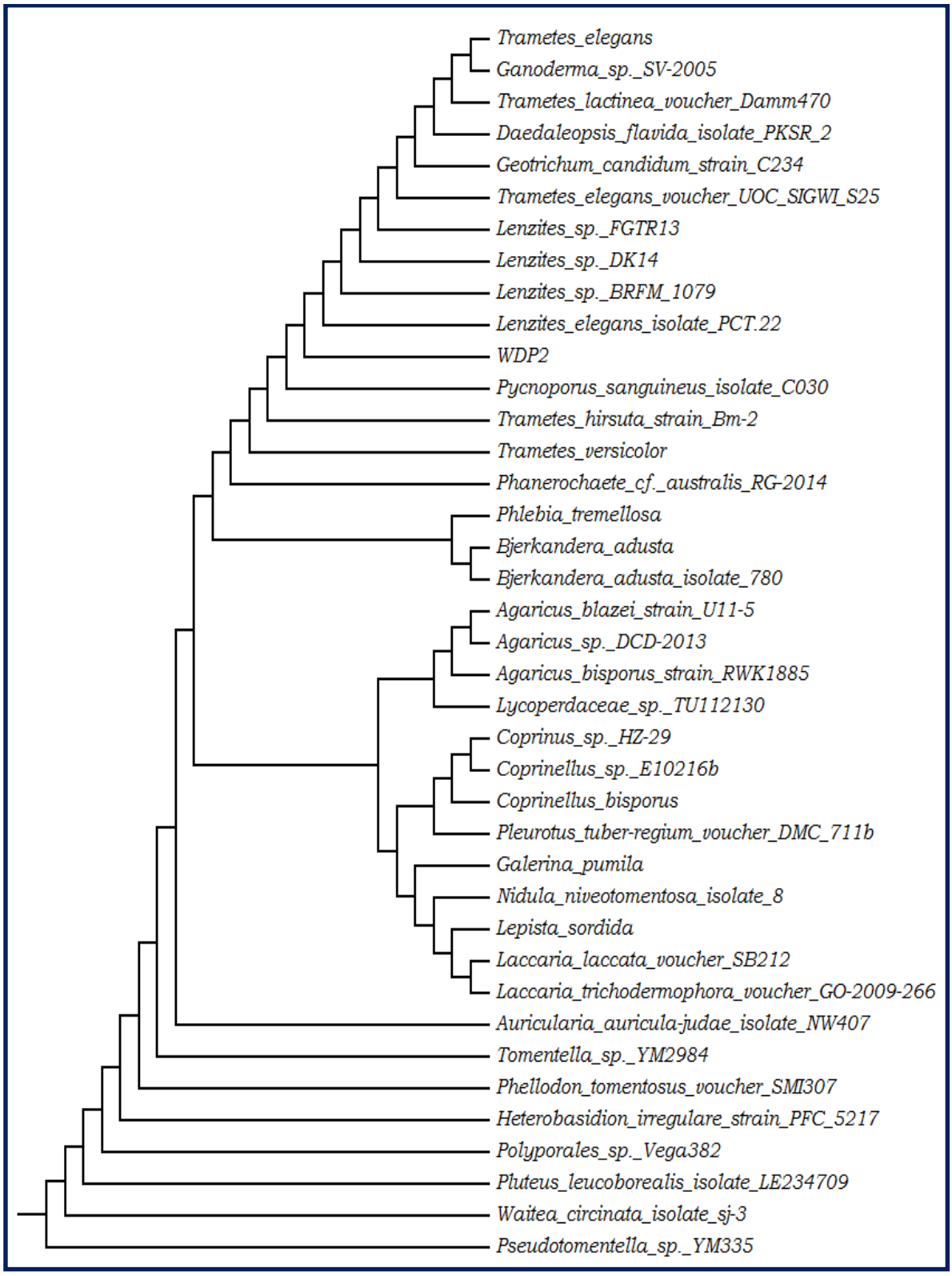


Fig 4.28: Boot strapped consensus phylogenetic tree showing evolutionary relatedness of selected wood rot fungal isolate WDP2 with other wood rot fungi based on 5.8S ITS r-RNA gene sequences.

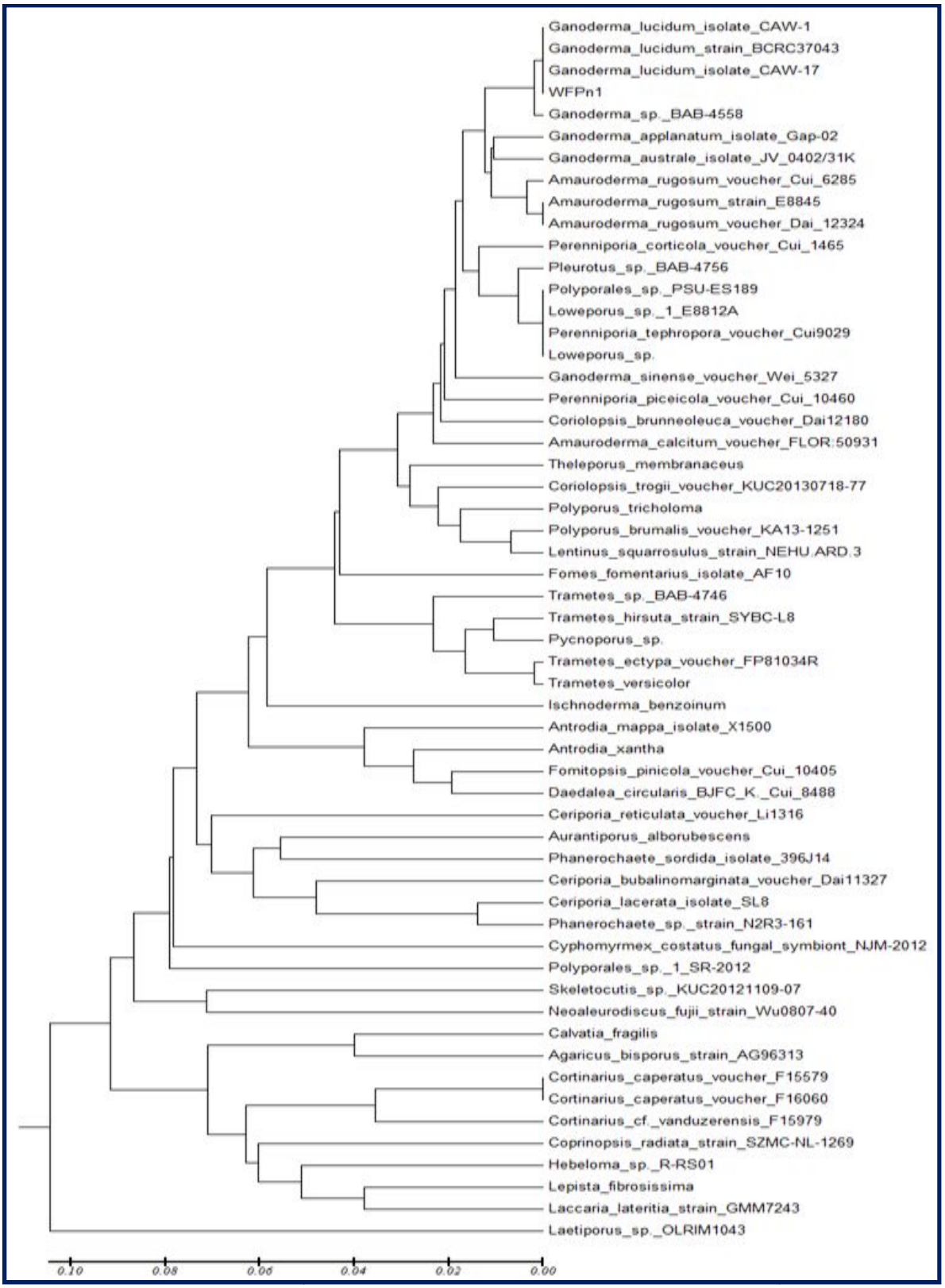


Fig 4.29: Phylogenetic tree showing evolutionary relatedness of selected wood rot fungal isolate WFPn1 with other wood rot fungi based on 5.8S ITS r-RNA gene sequences

Summary and Conclusions

Chapter 5 SUMMARY & CONCLUSIONS

The rising concerns about the rapid depletion of fossil fuel reservoirs, increase in air pollution due to large emission of green house gases by combustion of fossil fuels, have resulted in increased focus on the use of eco-friendly lignocellulosic biomass based liquid transportation fuel. Biomass recalcitrance of lignocellulosic substrates is a major constraint that can be removed efficiently through enzymatic process using laccase and cellulase enzymes. However, there is scarcity of a proper microbial enzyme technology for efficient delignification and saccharification & bioconversion of cellulosic biomass into bioethanol. Therefore, present investigation was carried out for optimization of conditions for maximizing lignolytic enzyme production and biological pretreatment strategies for effective delignification in a best possible way. Moreover, the characterization of the lignolytic laccase enzymes for pH and temperature stability and the shelf life was also done as per need for industrial use of enzymes. The salient findings are summarised below:

1. A total of forty one bacterial cultures and seventy three fungal cultures (both mushrooms and non mushroom fungi) were isolated from diverse sources and screened qualitatively for laccase and cellulase enzyme production. Six cultures including standard culture of *P. chrysosporium* were selected for quantification of lignolytic enzyme (laccase), seven cultures including the standard culture of *A. niger* (for cellulase enzyme production) were selected for quantification of cellulase enzyme. The laccase production was further correlated to decolourization of synthetic industrial dyes.

2. Out of six isolates tested quantitatively for laccase production, the isolate WDP2 showing highest laccase enzyme activity (156.793 Uml^{-1}) in production medium was selected for further study. The fungal isolate PCF1 showing maximum FPase-0.732 Uml^{-1}, CMCase- 0.03 Uml^{-1}, and β-glucosidase-0.295 Uml^{-1} was selected for saccharification experiments.

3. Maximum laccase production (189.26 Uml^{-1}) was achieved 28±2^{0}C by the fungal isolate WDP2 in 12d period in modified minimal salt medium (pH 7.0) containing

wheat bran (2%, w/v) and in optimised culture conditions. Activity profile of laccase enzyme exhibited maximum laccase enzyme activity at pH 3.0 with temperature optima at 50^0C.

4. Studies on thermal and pH stability of laccase enzyme revealed that the laccase enzyme from the fungal isolate WDP2 was highly stable at acidic pH (pH 3.0), and it retained 100% activity at 40^0C and >95% activity at 50^0C up to one hour. It was also concluded from the study that the enzyme was stable for a long period of time (up to 2 months), retaining~40% enzyme activity at 4^0C.
5. Zymogram analysis of partially purified laccase enzyme produced by selected lignolytic cultures (WDP2, AAFP5, WFPn1, and *P. chrysosporium*) revealed different isoforms in each case. Three active bands of laccase isozymes from the fungal isolate WDP2, corresponding to the molecular size of about 48 kDa, 60 kDa, and 70 kDa were visualised during activity staining by Native PAGE. Laccase activity bands of molecular mass~70 kDa was observed in case of *P. chrysosporium*, while one and two active bands (48-75 kDa) were observed in case of fungal isolate WFPn1, and AAFP5 respectively.
6. *Bambusa balcooa* biomass was selected as the substrate for bioethanol production on the basis of its high biomass yield/stem (45.87 Kg./stem), good cellulose content (37.93 %), and relatively low lignin content (29.5%). out of various physical and chemical pretreatments given to the selected biomass, prolonged autoclaving and alkaline hydrogen peroxide pretreatment were observed as the best physical and chemical pretreatments respectively. Optimization of alkaline hydrogen peroxide pretreatment for lowering the NaOH use revealed a significant reduction inlignin content (from 29.5% to 5.0%).
7. The whole cell biological pretreatment to the bamboo biomass was given using the fungal isolate WDP2 under solid state fermentation. Significant decrease in lignin content from an initial (29.5%) to 8.4% with 42.5 Uml^{-1} laccase enzyme was achieved when the biomass was fungal pretreated in half concentration of optimised medium for 48d.

8. Optimization of bioprocess for enzymatic saccharification of biologically (fungal) pretreated *B. balcooa* wood powder was done using Box Behnken design (BBD) in RSM. Maximum percent saccharification (46.255%) was achieved with the enzyme loading of 15FPU/g of substrate, 1% substrate concentration at 40^0C, that were near about to the conditions predicted from the model.
9. The changes in chemical structure of *B. balcooa* wood powder after physical, chemical, and biological (fungal) pretreatments were observed through UV-Vis scanning, and FTIR spectroscopy. UV-VIS scanning results indicated the release of some new compounds in filtrate/washing after various pretreatments. It is apparent from FTIR spectrums that certain functional groups in wood were changed significantly in all the cases. In physically and chemically, and biologically pretreated wood sample, reduction of various bands was observed that indicated decrease in lignin recalcitrance and cellulose crystalinity in wood.
10. The ultrastructural changes and tissue damage in pretreated and saccharified *B. balcooa* wood were observed through scanning electron microscopic (SEM) studies. Same degradation pattern were observed while fungal pretreated *B. balcooa* wood powder was saccharified using cellulase enzyme from potential cellulase producer PCF1.
11. Morphological characterization of potential wood rot fungal isolates (WDP2, AAFP5, TAFP4, and WFPn1) was done on the basis of fruiting body structures, and colony and cellular characteristics. The isolates were further biochemically characterised on the bases of utilization of various carbohydrates and other compounds, extracellular enzyme activities, and synthetic dye removal capacity. All the four cultures were belong to mushroom group of fungi and showed variability in morphological and biochemical characteristics.
12. For molecular characterization of two selected cultures (WDP2 and WFPn1), 5.8S ITS r-RNA gene sequence analysis was done. The isolate WDP2 was identified as *Lengites elegans* WDP2 based on maximum (99%) percent identity with the same fungus, while, the isolate WFPn1 revealed maximum identity (99%) with *Ganoderma lucidum* and hence identified as *Ganoderma lucidum* WFPn1.

The present investigation reveals that lignolytic and laccase producing ability is mainly confined to the mushroom fungi. It may be concluded that the thermoacidophilic laccase enzyme with longer shelf life from the potential lignolytic culture *Lengites elegans* WDP2 has a great potential for decreasing the recalcitrance and crystalinity of lignocellulosic biomass and thereby enhancing the accessibility of cellulase enzyme for saccharification. Hence the fungal culture *Lengites elegans* isolate WDP2, appears to have significant biotechnological applications in various industrial processes.

Literature Cited

LITERATURE CITED

Aftab, Z. and Ahmed, S. 2015. *Ganoderma lucidum*: a case study for laccase biosynthesis. *Pakistan Journal of Phytopathology*. 27(1): 95-103.

Ahammed, S. 2002. Production and characterization of lignin peroxidases from mangrove ascomvcetes. Thesis. Doctor of Philosophy in Biotechnology. Cochin University of Science and Technology. Trivandrum. Pp 39-41.

Akhtar, M. S., Saleem, M. and Akhtar, M.W. 2001. Saccharification of lignocellulosic materials by the cellulases of *Bacillus subtilis*. *International Journal of Agriculture and Biology*. 3: 199-202.

Alley, E. R. 2007. Water quality control handbook. New York, N.Y.: McGraw-Hill.

Alvira, P., Tomás-Pejó, E., Ballesteros, M. and Negro, M. J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource Technology*. 101 (13): 4851-4861.

Appels, L., Impe, J. V. and Dewil, R. 2012. Oxidizing agents and organic solvents as pretreatment for anaerobic digestion. Biogas Production: Pretreatment Methods in Anaerobic Digestion. Hoboken. New Jersey: John Wiley & Sons, Inc. pp199-214.

Arantes, V. and Saddler, J. N. 2011. Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. *Biotechnology for Biofuels*. 4(1):1.

Arulmani, M., Murugesan, K., Arumugam, P., Dhandapani, R. and Kalaichelvan P. T. 2005. Decolorization of basic dyes methyl violet and emerald green by *Pleurotus sajorcaju* and its effluent decolorization activity, *Indian Journal of Applied Microbiology*. 1: 47–52.

Aswathy, U.S., Sukumaran, R.K., Devi, G.L., Rajasree, K.P., Singhania, R.R. and Pandey, A. 2010. Bio-ethanol from water hyacinth biomass: an evaluation of enzymatic saccharification strategy. *Bioresource Technology*. 101(3): 925-930.

Atalla, M. M., Zeinab, H. K., Eman, R. H., Amani, A. Y., & Abeer, A. A. E. A. 2013. Characterization and kinetic properties of the purified *Trematosphaeria mangrovei* laccase enzyme. *Saudi Journal of Biological Sciences*. 20(4): 373-381.

Atri, N.S. and Sharma, S.K. 2012. Qualitative estimation of cellulases and lignin modifying enzymes in five wild *Lentinus* species selected from North West India. *World Journal of Fungal and Plant Biology*. 3 (1): 13-17.

Bacovsky, D., Ludwiczek, N., Ognissanto, M., Wörgetter, M. 2013. Status of advanced biofuels demonstration facilities in 2012. A Report to IEA Bioenergy Task. 39:1– 209.

Badshah, M., Lam, D.M., Liu, J. and Mattiasson, B. 2012. Use of an automatic methane potential test system for evaluating the biomethane potential of sugarcane bagasse after different treatments. *Bioresource Technology*. 114: 262-269.

Bailey, P. S. 1982. Ozonation in organic chemistry. New York, N.Y.: Academic press, Inc.

Bajpai P. 1999. Application of enzymes in the pulp and paper industry. *Biotechnol Progress*. 15:147–57.

Bakkiyaraj, S., Aravindan, R., Arrivukkarasan, S. and Viruthagiri, T. 2013. Enhance laccase production by *Trametes hirsuta* using wheat bran under submerged fermentation. *International Journal of ChemTech Research*.5:1224-38.

Baldrian, P., 2004. Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. *Applied Microbiology and Biotechnology*. 63(5): 560-563.

Bals, B., Rogers, C., Jin, M., Balan, V. and Dale, B. 2010. Evaluation of ammonia fibre expansion (AFEX) pretreatment for enzymatic hydrolysis of switchgrass harvested in different seasons and locations. *Biotechnology for Biofuels*. 3(1):1-11.

Barl, B., Biliaderis, C.G., Murray, E.D. Macgregor, A.W. 1991. Combined chemical and enzymic treatments of corn husk lignocellulosics. *Journal of the Science of Food and Agriculture*. 56 (2): 195-214.

Bassam, N. 1998. Energy plant species: their use and impact on environment and development. James & James, London, UK.

Bayer, E.A., Chanzy, H., Lamed, R., Shoham, Y. 1998. Cellulose, cellulases and cellulosomes. *Current Opinion in Structural Biology*. 8: 548–557.

Becker, H. G. & Sinitsyn, A. P. 1993. Mn-peroxidase from *Pleurotus ostreatus*: the action on the lignin. *Biotechnol Letters*. 15:289– 94.

Ben Younes, S., Mechichi, T., Sayadi, S., 2007. Purification and characterization of the laccase secreted by the white rot fungus *Perenniporia tephropora* and its role in the decolourization of synthetic dyes. *Journal of Applied Microbiology*. 102: 1033–1042.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S. Saddler, J. 2006. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. *Journal of Biotechnology*. 125 (2): 198-209.

Binod, P., Janu, K.U., Sindhu, R. and Pandey, A. 2011. Hydrolysis of lignocellulosic biomass for bioethanol production. Biofuels: alternative feedstocks and conversion processes: 229-250.

Bisaria, V.S. and Martin, A.M. 1991. Bioprocessing of agro-residues to glucose and chemicals. *Bioconversion of waste materials to industrial products*. 187-223.

Bochek, A.M. 2003. Effect of hydrogen bonding on cellulose solubility in aqueous and nonaqueous solvents. *Russian Journal of Applied Chemistry*. 76 (11): 1711-1719.

Bonnen, A. M., Anton, L. H., and Orth, A. B.1994. Lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus. Applied and Environmental Microbiology*. 60 (3): 960-965.

Bothast, R., J. and Saha, B.C. 1997. Ethanol production from agricultural biomass substrates. *Advances in Applied Microbiology*. 44: 261-286.

Box , G. E. P., Hunter, W. G. And Hunter, J., S. 1978. Statistics for experimenters. John Wiley & Sons, Inc. pp653.

Brennan, L. and Owende, P., 2010. Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews*. 14 (2): 557-577.

Brennana, L., and Owendea, P. 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and coproducts. *Renewable Sustainable Energy Rev*. 14:557–577.

Browning, B. L. 1975. The chemistry of wood. Robert E. Krieger Publishing Company.

Buswell, J. A., and E. Odier. 1987. Lignin biodegradation. *Critical Reviews in Biotechnology*. 6:1–60.

Cai, Y.J., Buswell, J.A. and Chang, S.T. 1994. Production of cellulases and hemicellulases by the straw mushroom, *Volvariella volvacea*. *Mycological Research*. 98(9): 1019-1024.

Call, H.P. and Mücke, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). *Journal of Biotechnology*. 53(2): 163-202.

Campbell, C.J. and Laherrère, J.H. 1998. The end of cheap oil. Scientific American. 278 (3): 60-5.

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A. and Alfani, F. 2004. Effect of inhibitors released during steam-explosion treatment of poplar wood on subsequent enzymatic hydrolysis and SSF. *Biotechnology Progress*. 20(1): 200-206.

Carere, C.R., Sparling, R., Cicek, N. and Levin, D.B. 2008. Third generation biofuels via direct cellulose fermentation. *International Journal of Molecular Sciences*. 9(7): 1342-1360.

Castresana, J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*. 17: 540-552.

Caulfield, D.F. and Moore, W.E. 1974. Effect of varying crystallinity of cellulose on enzymic hydrolysis. *Wood Science*. 6 (4): 375–379.

Cavaco-Paulo, A.R.T.U.R. and Gübitz, G. 2003. Catalysis and processing.Textile Processing with Enzymes. England. Woodhead Publishing Ltd. p86.

Chakroun, H., Mechichi, T., Martinez, M.J., Dhouib, A., Sayadi, S. 2010. Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: application on bioremediation of phenolic compounds. *Process Biochemistry*. 45: 507–513.

Chander, M. and Arora, D. S. 2007. Evaluation of some white-rot fungi for their potential to decolourise industrial dyes. *Dyes and Pigments*. 72: 192–198.

Chang, V.S., and Holtzapple, M.T. 2000. Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry Biotechnology*. 84:5–37.

Chefetz, B., Chen, Y., & Hadar, Y. 1998. Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. *Applied and Environmental Microbiology*. *64*(9): 3175-3179.

Chen, H. 2014. Chemical composition and structure of natural lignocellulose. *In* Biotechnology of Lignocellulose (pp. 25-71). Springer Netherlands.

Chen, X. 2012. Development of effective pretreatment and bioconversion systems for converting organic residuals to bioenergy. Ph.D. Dissertation. Davis: University of California.

Chu, B. C., and Lee, H. 2007. Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. *Biotechnol Advances*. 2007. 25 (5): 425-41.

Chundawat, S.P.S., Beckham, G.T., Himmel, M.E. et al. 2011. Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annual Review of Chemical and Biomolecular Engineering.* 2:6.1–6.25.

Cui, L., Liu, Z., Si, C., Hui, L., Kang, N. and Zhao, T. 2012. Influence of steam explosion pretreatment on the composition and structure of wheat straw. *Bioresources*. 7(3): 4202-4213.

Cui, X., Zhao, X., Zeng, J., Loh, S.K., Choo, Y.M. and Liu, D. 2014. Robust enzymatic hydrolysis of formiline-pretreated oil palm empty fruit bunches (EFB) for efficient conversion of polysaccharide to sugars and ethanol. *Bioresource Technology*. 166: 584-591.

Czernik, S. and Bridgwater, A.V. 2004. Overview of applications of biomass fast pyrolysis oil. *Energy & Fuels*. 18(2): 590-598.

D'souza, T. M., Merritt, C. S. and Reddy, C. A., 1999. Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. *Applied and Environmental Microbiology*. 65(12), pp.5307-5313.

D'Souza-Ticlo, D., Garg, S., & Raghukumar, C. 2009. Effects and interactions of medium components on laccase from a marine-derived fungus using response surface methodology. *Marine Drugs*. 7(4): 672-688.

da Costa Sousa, L., Chundawat, S.P.S., Balan, V. et al. 2009. 'Cradle-to-grave' assessment of existing lignocellulose pretreatment technologies. *Current Opinion in Biotechnology.* 20:339–347.

Das M., Bhattacharya S. and Pal A. 2005. Generation and characterization of SCARs by cloning and sequencing of RAPD products: A strategy for species-specific marker development in bamboo. *Annals of Botany*. 95: 835–841.

Das, A., Paul, T., Halder, S. K., Jana, A., Maity, C., Mohapatra, P. K. D., Pati, B. R. and Mondal, K. C. 2013. Production of cellulolytic enzymes by *Aspergillus fumigatus* ABK9 in wheat bran-rice straw mixed substrate and use of cocktail enzymes for deinking of waste office paper pulp. *Bioresource Technology*. 128: 290-296.

Das, S., Bhattacharya, A., Haldar, S., Ganguly, A., Gu, S., Ting, Y.P. and Chatterjee, P.K. 2015. Optimization of enzymatic saccharification of water hyacinth biomass for bio-ethanol: Comparison between artificial neural network and response surface methodology. *Sustainable Materials and Technologies*. 3: 17-28.

Dashtban, M., Schraft, H., Syed, T. A., & Qin, W. 2010. Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemistry and Molecular Biology*. 1(1): 36-50.

de Souza Moretti, M. M., Bocchini-Martins, D. A., Nunes, C. D. C. C., Villena, M. A., Perrone, O. M., da Silva, R., Boscolo, M. and Gomes, E. 2014. Pretreatment of sugarcane bagasse with microwaves irradiation and its effects on the structure and on enzymatic hydrolysis. *Applied Energy*. 122: 189-195.

De Souza, W.R. 2013. Microbial degradation of lignocellulosic biomass. Chandel A, Da Silva, S. Sustainable degradation of lignocellulosic biomass-techniques, applications and commercialization. Brazil: InTech.

De Wild, P., Reith, H. and Heeres, E. 2011. Biomass pyrolysis for chemicals. *Biofuels*. 2(2): 185-208.

Dekker, R., F., & Barbosa, A., M. 2001. The effects of aeration and veratryl alcohol on the production of two laccases by the ascomycete *Botryosphaeria* sp. *Enzyme and Microbial Technology*. 28 (1): 81-88.

Demirbas, A. 2005. Bioethanol from cellulosic materials: a renewable motor fuel from biomass. *Energy Source*. 27:327:37.

Demirbas, E., Kobya, M. and Sulak, M. T., 2008. Adsorption kinetics of a basic dye from aqueous solutions onto apricot stone activated carbon. *Bioresource Technology*. 99 (13): 5368-5373.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M. and Claverie, J. M. 2008. Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*. 36(suppl 2): W465-W469.

Desai, S. S., Tennali, G. B., Channur, N., Anup, A. C., Deshpande, G., and Murtuza, B. A. 2011. Isolation of laccase producing fungi and partial characterization of laccase. *Biotechnology, Bioinformatics and Bioengineering*, 1(4), 543-549.

Devi, M.C.Kumar, and M.S., 2012. Isolation and screening of lignocellulose hydrolytic saprophytic fungi from dairy manure soil. *Annals of Biological Research*, 3(2): 1145-1152.

Diaz, A. B., de Souza Moretti, M. M., Bezerra-Bussoli, C., Nunes, C. D. C. C., Blandino, A., da Silva, R., & Gomes, E. 2015. Evaluation of microwave-assisted pretreatment of lignocellulosic biomass immersed in alkaline glycerol for fermentable sugars production. *Bioresource Technology*. 185: 316-323.

Diaz, A., Le Toullec, J., Blandino, A., De Ory, I. and Caro, I. 2013. Pretreatment of rice hulls with alkaline peroxide to enhance enzyme hydrolysis for ethanol production. *Chemical Engineering Transactions*. 32: 949-954.

Dittmer, J. K., Patel, N. J., Dhawale, S. W., & Dhawale, S. S. 1997. Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. *FEMS Microbiology Letters*. 149 (1): 65-70.

Donghai, S.U., Junshe, S., Ping, L.I.U. and Yanping, L.Ü., 2006. Effects of different pretreatment modes on the enzymatic digestibility of corn leaf and corn stalk. *Chinese Journal of Chemical Engineering.* 14 (6): 796-801.

Dragone, G., Fernandes, B.D., Vicente, A.A. and Teixeira, J.A. 2010. Third generation biofuels from microalgae. *Current research, technology and education topics in applied microbiology and microbial biotechnology*. 2: 1355-1366.

Du, M., Huang, S., Zhang, J., Wang, J., Hu, L., and Jiang, J. 2013. Isolation of total saponins from *Sapindus mukorossi* Gaerth. *Open Journal of Forestry*. 4(1):24-27.

Dyer, T. J., and Ragauskas, A. J. 2006. Deconvoluting chromophore formation and removal during kraft pulping:influence of metal cations. *Appita Journal*. 59 (6): 452-458.

Edens, W.A., Goins, T.Q., Dooley, D., and Henson, J.M. 1999. Purification and characterization of a secreted laccase of Gaeumannomyces gramminis var. tritici. *Applied and Environmental Microbiology*. 65: 3071–3074.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*. 32(5): 1792-1797.

Egan, B. Z., Lee, D. D. and McWhirter, D. A., 1988. Solvent extraction and recovery of ethanol from aqueous solutions. *Industrial & Engineering Chemistry Research*. 27 (7): 1330-1332.

Eggert, C., Temp, U., and Eriksson, K. E. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied and Environmental Microbiology.* 62 (4): 1151-1158.

El Monssef, R. A. A., Hassan, E. A., & Ramadan, E. M. 2016. Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. *Annals of Agricultural Sciences*. 61 (1): 145-154.

El-Batal, A. I., ElKenawy, N. M., Yassin, A. S., & Amin, M. A. 2015. Laccase production by *Pleurotus ostreatus* and its application in synthesis of gold nanoparticles. *Biotechnology Reports*. 5: 31-39.

Elisashvili, V., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Khardziani, T. and Agathos, S.N. 2009. Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the forests of Georgia. *World Journal of Microbiology and Biotechnology*. 25 (2): 331-339.

Elliott, D.C., 2007. Historical developments in hydroprocessing bio-oils. *Energy & Fuels*. 21(3): 1792-1815.

El-Shishtawy, R.M., Mohamed, S.A., Asiri, A.M., Abu-bakr, M.G., Ibrahim, I.H. and Al-Talhi, H.A. 2015. Saccharification and hydrolytic enzyme production of alkali pre-treated wheat bran by *Trichoderma virens* under solid state fermentation. *BMC Biotechnology*. 15(1): 1-13.

Eskicioglu, C., Terzian, N., Kennedy, K.J., Droste, R.L. and Hamoda, M. 2007. Athermal microwave effects for enhancing digestibility of waste activated sludge. *Water Research*. 41 (11): 2457-2466.

Eugenio, M. E., Carbajo, J. M., Martín, J. A., Martín-Sampedro, R., González, A. E., & Villar, J. C. 2010. Synergic effect of inductors on laccase production by *Pycnoporus sanguineus*. *Afinidad*. 67. (546).

Ewanick, S. and Bura, R. 2011. The effect of biomass moisture content on bioethanol yields from steam pretreated switchgrass and sugarcane bagasse. *Bioresource Technology*. 102(3): 2651-2658.

Faix, O. and Böttcher, J. H. 1992. The influence of particle size and concentration in transmission and diffuse reflectance spectroscopy of wood. *Holz als Roh-und Werkstoff*. 50(6), pp.221-226.

Faix, O., 1991. Classification of lignins from different botanical origins by FT-IR spectroscopy. Holzforschung-*International Journal of the Biology, Chemistry, Physics and Technology of Wood*. 45 (s1): 21-28.

Fan, M., Dai, D., and Huang, B. 2012. Fourier transform infrared spectroscopy for natural fibres. *In*: Mohammed Salih, Salih (Ed.), Fourier Transform – Materials Analysis. InTech. http://dx.doi.org/10.5772/35482.

Farnet, A. M., Criquet, S., Tagger, S., Gil, G., & Petit, J. L. 2000. Purification, partial characterization, and reactivity with aromatic compounds of two laccases from *Marasmius quercophilus* strain 17. *Canadian Journal of Microbiology*.46 (3): 189-194.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'hare, M. and Kammen, D.M. 2006. Ethanol can contribute to energy and environmental goals. *Science*. 311(5760): 506-508.

Fen, L., Xuwei, Z., Nanyi, L., Puyu, Z., Shuang, Z., Xue, Z.,and Haiping, L.2014. Screening of lignocellulose-degrading superior mushroom strains and determination of their CMCase and laccase activity. *The Scientific World Journal*. 2014.

Flores, C., Vidal, C., Trejo-Hernández, M.R., Galindo, E. and Serrano-Carreón, L. 2009. Selection of *Trichoderma* strains capable of increasing laccase production by Pleurotus *ostreatus* and *Agaricus bisporus* in dual cultures. *Journal of Applied Microbiology*. 106(1): 249-257.

Forootanfar, H., Faramarzi, M., A., Shahverdi, A., R., Yazdi, M., T. 2011. Purification and biochemical characterization of extracellular laccase from the ascomycete *Paraconiothyrium variabile*. *Bioresource Technology*. 102: 1808–1814.

Galazka, J. M., Tian, C. G., Beeson, W. T., Martinez, B., Glass, N. L., Cate, J. H. D. 2010. Cellodextrin transport in yeast for improved biofuel production. *Science*. 330(6000): 84-86.

Galbe, M. and Zacchi, G. 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production. *Advances in Biochemical Engineering/Biotechnology*. 108: 41–65.

Galhaup, C., Wagner, H., Hinterstoisser, B. and Haltrich, D. 2002. Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme and Microbial Technology*. 30(4): 529-536.

Gao, H., Wang, Y., Zhang, W., Wang, W., & Mu, Z. 2011. Isolation, identification and application in lignin degradation of an ascomycete GHJ-4. *African Journal of Biotechnology*. 10 (20): 4166-4174.

García-Aparicio, M.P., Ballesteros, I., González, A., Oliva, J.M., Ballesteros, M. and Negro, M.J. 2006. Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. InTwenty-Seventh Symposium on Biotechnology for Fuels and Chemicals. Humana Press. pp278-288.

Gautam, S.P., Bundela, P.S., Pandey, A.K., Awasthi, M.K. and Sarsaiya, S. 2010. Optimization of the medium for the production of cellulase by the *Trichoderma viride* using submerged fermentation. *International Journal of Environmental Sciences*. 1 (4): 656.

Gayazov, R. and J. Rodakiewicz-Nowak, 1996. Semi-continuous production of laccase by *P.radiata* in different culture media. *Folia Microbiologica*. 41: 480-484.

Gettemy, J. M., Ma, B., Alic, M., and Gold, M. H. 1998. Reverse transcription-PCR analysis of the regulation of the manganese peroxidase gene family. *Applied and Environmental Microbiology*. 64 (2): 569-74.

Ghose, T.K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry*. 59(2): 257-268.

Gianfreda, L., Xu, F. and Bollag, J.M. 1999. Laccases: a useful group of oxidoreductive enzymes. *Bioremediation Journal*. 3(1): 1-26.

Gianfreda, L., Xu, F., and Bollag, J-M. 1999. Laccases: a useful group of oxidoreductive enzymes. *Bioremediation Journal*. 3 :1– 26.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., Sannia, G. 2010. Laccases: a never-ending story. *Cellular and Molecular Life Sciences*. 67 (3): 369-85.

Glenn, J. K. & Gold, M. H. 1983. Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*. 45:1741–7.

Gochev, V. K., & Krastanov, A. I. 2007. Isolation of laccase producing *Trichoderma* spp. *Bulgarian Journal of Agricultural Science*. 13 (2): 171.

Gold, M. H., and M. Alic 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Microbiological. Reviews*. 57:605–622.

Goldemberg, J. 2007. Ethanol for a sustainable energy future. *Science*. 315:808–810.

Gratani, L., Crescente, M. F., Varone, L., Fabrini, G., Digiulio, E. 2008. Growth pattern and photosynthetic activity of different bamboo species growing in the botanical garden of Rome. *Flora*. 203: 77-84.

Gray, K.A., Zhao, L. and Emptage, M. 2006. Bioethanol. *Current Opinion in Chemical Biology*. 10 (2), pp.141-146.

Gray, M.K. 2013. Alkaline hydrogen peroxide pretreatment for its use in an on-farm bioprocessing facility. Thesis. Biosystems and Agricultural Engineering. Lexington, Kentucky. Pp 4-8.

Gressel, J. 2008. Transgenics are imperative for biofuel crops. *Plant Science*. 174:246–263.

Grethlein, H.E. 1985. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulose substrates. *Nature Biotechnology*. 3 (2): 155–160.

Grous, W.R., Converse, A.O., Grethlein, H.E. 1986. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. *Enzyme and Microbial Technology*. 8 (5) 274–280.

Hahn-Hagerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Lidén, G. and Zacchi, G., 2006. Bio-ethanol–the fuel of tomorrow from the residues of today. *Trends in Biotechnology*. 24(12): 549-556.

Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M. F. 2007. Towards industrial pentose-fermenting yeast strains. *Applied Microbiology and Biotechnology*. 74 (5) :937-53.

Hansen, A.C., Zhang, Q., Lyne, P.W. 2005. Ethanol–diesel fuel blends—a review. Bioresource Technology, 96(3): 277-285.

Hapuarachchi, K. K., Wen, T. C., Deng, C. Y., Kang, J. C. and Hyde, K. D., 2015. Mycosphere Essays 1: Taxonomic Confusion in the *Ganoderma lucidum* Species Complex. *Mycosphere*. 6 (5): 542-559.

Hariharan, S., Nambisan, P. 2013. Optimization of lignin peroxidase, manganese peroxidase, and lac production from *Ganoderma lucidum* under solid state fermentation of pineapple leaf. *Bioresources*. 8 (1):250-271.

Harmsen, P., Huijgen, W., Bermudez, L. and Bakker, R. 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. *Energy Research Centre of the Netherlands*: 10-13.

Hatti-Kaul, R., and Ibrahim, V., 2012. Lignin-Degrading Enzymes: An Overview. Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, *Chemicals, and Polymers*. pp.167-192.

He, J., Ye, X., Ling, Q., & Dong, L. 2014. Enhanced production of an acid-tolerant laccase by cultivation of *Armillariella tabescens*. *Journal of Chemical and Pharmaceutical Research*. 6 (1): 240-245.

He, M. X., Li, Q., Liu, X. Y., Hu, Q. C., Hu, G. Q., Pan, K., Zhu, Q. and Wu, J. 2013. Bio-ethanol production from bamboo residues with lignocellulose fractionation technology (LFT) and separate hydrolysis fermentation (SHF) by *Zymomonas mobilis*. *American Journal of Biomass and Bioenergy*, 1, 1-10.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P. and Anke, T. 1998. Characterization of laccases and peroxidases from wood-rotting fungi (family Coprinaceae). *Applied and Environmental Microbiology*. *64* (5):1601-1606.

Hendriks, A. T. W. M., and Zeeman, G. 2009. "Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*. 100 (1):10-18.

Highley, T.L. 1980. Cellulose degradation by cellulose-clearing and non-cellulose-clearing brown-rot fungi. *Applied and Environmental Microbiology*. 40 (6): 1145-1147.

Higuchi, H. 1957. Biochemical studies of lignin formation, III. *Physiologia Plantarum*. 10: 633-648.

Himmel, M., E., Ding, S., Y., Johnson, D., K., Adney, W., S., Nimlos, M., R., Brady, J., W. and Foust, T., D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels. *Production Science*. 315 (5813): 804-807.

Himmel, M.E., Adney, W.S., Baker, J.O., Nieves, R.A., Thomas, S.R. 1996. Cellulases structure, function and applications. In: Wyman, C.E. (Ed.), Handbook on Bioethanol: Production and Utilization. Taylor and Francis. pp143–161.

Himmelsbach, J.N., Raman, D.R., Anex, R.P., Burns, R.T. and Faulhaber, C.R. 2010. Effect of ammonia soaking pretreatment and enzyme addition on biochemical methane potential of switchgrass. *Transactions of the ASABE*. 53 (6): 1921-1927.

Homma, H., Shinoyama, H., Nobuta, Y., Terashima, Y., Amachi, S., and Fujii, T. 2007. Lignin-degrading activity of edible mushroom *Strobilurus ohshimae* that forms fruiting bodies on buried sugi (*Cryptomeria japonica*) twigs. *Journal of Wood Science*, 53 (1): 80-84.

Hong, K., J., & Tokunaga, S. 2000. Extraction of heavy metals from MSW incinerator fly ash using saponins. *Chemosphere*. 41: 345-352.

Hou, H., Zhou, J., Wang, J., Du, C. and Yan, B. 2004. Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Process Biochemistry*. 39(11): 1415-1419.

Hövel, K., Shallom, D., Niefind, K., Belakhov, V., Shoham, G., Baasov, T., Shoham, Y. and Schomburg, D. 2003. Crystal structure and snapshots along the reaction pathway of a family 51 α-l-arabinofuranosidase. *The EMBO Journal*. 22 (19): 4922-4932.

http://www.accuweather.com/en/in/lalkuan/201534/month/201534?.monyr= Pantnagar/ Lalkua, local, India, whether. 5/06/2015.

http://www.shimadzu.com/an/uv/support/uv/ap/apl.html. 14/11/2016.

https://i2.wp.com/www.bioenergyconsult.com/wpcontent/uploads/2012/06/Ethanol_Manufacture.jpg. 12/03/2015.

Hu, G., Heitmann, J. A., Rojas, O. J. 2008. Feedstock pretreatment strategies for producing ethanol from wood, bark, and forest residues. *Bioresources*, 3(1): 270-294.

Hussain, A., Shrivastav, A., Jain, S.K., Baghel, R.K., Rani, S. and Agrawale, M.K. 2012. Cellulolytic enzymatic activity of soft rot filamentous fungi *Paecilomyces variotii*. *Advances in Bioresearch*. 3: 10-17.

Im, K. H., Nguyen, T. K., Choi, J. and Lee, T. S. 2016. Ethanol production from various sugars and cellulosic biomass by white rot fungus *Lenzites betulinus*. *Mycobiology*. 44(1): 48-53.

Imai, M., Ikari, K. and Suzuki, I. 2004. High-performance hydrolysis of cellulose using mixed cellulase species and ultrasonication pretreatment. *Biochemical Engineering Journal* 17 (2): 79-83.

Imran, M., Asad, M. J., Hadri, S. H., and Mehmood, M. 2012. Production and industrial applications of laccase enzyme. *Journal of Cell and Molecular Biology* 10 (1): 1-11.

Inderwildi, O., R. and King, D., A., 2009. Quo vadis biofuels?. *Energy and Environtal Science*. 2 (4): 343-346.

Ivanka, S., Albert, K., & Veselin, S. 2010. Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. *Advances in Bioscience and Biotechnology*.1:208-215.

Jackowiak, D., Bassard, D., Pauss, A. and Ribeiro, T. 2011 (a). Optimisation of a microwave pretreatment of wheat straw for methane production. *Bioresource Technology*. 102 (12). 6750-6756.

Jackowiak, D., Frigon, J.C., Ribeiro, T., Pauss, A. and Guiot, S., 2011 (b). Enhancing solubilisation and methane production kinetic of switchgrass by microwave pretreatment. *Bioresource Technology*. 102 (3): 3535-3540.

Kaijanen, L., Paakkunainen, M., Pietarinen, S., Jernström, E. and Reinikainen, S.P. 2015. Ultraviolet detection of monosaccharides: multiple wavelength strategy to evaluate results after capillary zone electrophoretic separation. *International Jounal of Electrochemical Science.* 10: 2950-2961.

Kalyani, D., Lee, K.M., Kim, T.S., Li, J., Dhiman, S.S., Kang, Y.C., Lee, J.K., 2013. Microbial consortia for saccharification of woody biomass and ethanol fermentation. *Fuel*. 107: 815–822.

Kang, H.K. and Kim, D., 2012. Efficient bioconversion of rice straw to ethanol with TiO_2/UV pretreatment. *Bioprocess and Biosystems Engineering*. 35(1-2): 43-48.

Kang, Q., Appels, L., Tan, T. and Dewil, R. 2014. Bioethanol from lignocellulosic biomass: current findings determine research priorities. *The Scientific World Journal*. 2014: 1-13.

Kansal, S. K., Singh, M., and Sud, D. 2008. Studies on TiO_2/ZnO photocatalysed degradation of lignin. *Journal of Hazardous Materials*. 153(1): 412-417.

Karimi, K. and Taherzadeh, M. J. 2016. A critical review of analytical methods in pretreatment of lignocelluloses: composition, imaging and crystallinity. *Bioresource Technology*. 200: 1008-1018.

Karimi, K., Kheradmandinia, S. and Taherzadeh, M.J. 2006. Conversion of rice straw to sugars by dilute-acid hydrolysis. *Biomass and Bioenergy*. 30 (3): 247-253.

Karp, S.G., Woiciechowski, A.L., Soccol, V.T. and Soccol, C.R. 2013. Pretreatment strategies for delignification of sugarcane bagasse: a review. *Brazilian Archives of Biology and Technology*. 56(4): 679-689.

Kaushik, P. and Malik, A. 2009. Fungal dye decolourization: Recent advances and future potential. *Environment International*. 35: 127– 141.

Kaya, F., Heitmann, J.A. and Joyce, T.W. 2000. Influence of lignin and its degradation products on enzymatic hydrolysis of xylan. *Journal of Biotechnology*. 80(3): 241-247.

Keyser, P., Kirk, T. K., and Zeikus, J. G. 1978. Ligninolytic enzyme system of *Phanaerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. *Journal of Bacteriology* 135(3): 790-797.

Khokhar, I., Haider, M.S., Mushtaq, S. and Mukhtar, I. 2012. Isolation and screening of highly cellulolytic filamentous fungi. *Journal of Applied Sciences and Environmental Management* 16(3): 223-226.

Kiiskinen, L. L., Ratto, M., and Kruus, K. 2004. Screening for novel laccase-producing microbes. *Journal of Applied Microbiology* 97:640–646.

Kiiskinen, L.L., Viikari, L., Kruus, K. 2002. Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*. *Applied Microbiology and Biotechnology*. 59: 198–204.

Kim, H. J., Chang, J.H., Jeong, B.Y. and Lee, J.H. 2013. Comparison of milling modes as a pretreatment method for cellulosic biofuel production. *Journal of Clean Energy Technologies* 1(1): 45-48.

Kim, J. K., Oh, B.R., Shin, H.J., Eom, C.Y. and Kim, S.W. 2008. Statistical optimization of enzymatic saccharification and ethanol fermentation using food waste. *Process Biochemistry*. 43 (11): 1308-1312.

Kim, Y., Mosier, N. and Ladisch, M. R. 2008. Process simulation of modified dry grind ethanol plant with recycle of pretreated and enzymatically hydrolyzed distillers' grains. *Bioresource Technology* 99 (12): 5177-5192.

Kline, L. M., Hayes, D. G., Womac, A. R. and Labbe, N. 2010. Simplified determination of lignin content in hard and soft woods via UV-spectrophotometric analysis of biomass dissolved in ionic liquids. *Bioresources*. 5(3): 1366-1383.

Knauf, M. and Moniruzzaman, M. 2004. Lignocellulosic biomass processing: a perspective. *International Sugar Journal*, 106(1263): 147-150.

Koroljova-Skorobogat'ko, O.V., Stepanova, E.V., Gavrilova, V.P., Morozova, O.V., Lubimova, N.V., Dzchafarova, A.N., Jaropolov, A.I. and Makower, A. 1998. Purification and characterization of the constitutive form of laccase from the basidiomycete *Coriolus hirsutus* and effect of inducers on laccase synthesis. *Biotechnology and Applied Biochemistry*. 28 (1): 47-54.

Kotake, T., Tsuchiya, K., Aohara, T., Konishi, T., Kaneko, S., Igarashi, K., Samejima, M. and Tsumuraya, Y. 2006. An α-L-arabinofuranosidase/β-D-xylosidase from immature seeds of radish (*Raphanus sativus* L.). *Journal of Experimental Botany*. 57(10): 2353-2362.

Kumagai, A., Kawamura, S., Lee, S. H., Endo, T., Rodriguez, M. and Mielenz, J. R. 2014. Simultaneous saccharification and fermentation and a consolidated

bioprocessing for Hinoki cypress and *Eucalyptus* after fibrillation by steam and subsequent wet-disk milling. *Bioresource Technology*. 162: 89-95.

Kumar, P., Barrett, D.M., Delwiche, M.J. and Stroeve, P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*. 48(8): 3713-3729.

Kumar, R., Wyman, C.E. 2009. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. *Biotechnology Progress*. 25: 302–314.

Kumar, S., Gupta, R., Lee, Y. Y. and Gupta, R. B. 2010. Cellulose pretreatment in subcritical water: effect of temperature on molecular structure and enzymatic reactivity. *Bioresource Technology*. 101 (4): 1337-1347.

Kumar, V., Pandey, P., Gupta, S. and Shukla, P. 2014. A reviving preliminary evoke on few xylanase producing fungal isolates from different ecological niche. *International Journal of Current Microbiology and Applied Sciences*. 3(4): 501-506.

Kunamneni, A., Ballesteros, A., Plou, F. J., & Alcalde, M. 2007. Fungal laccase—a versatile enzyme for biotechnological applications. *Communicating current research and educational topics and trends in applied microbiology*.1: 233-245.

Kuttiraja, M., Sindhu, R., Varghese, P. E., Sandhya, S. V., Binod, P., Vani, S., Pandey, A., Sukumaran, R., K. 2013. Bioethanol production from bamboo (*Dendrocalamus* sp.) process waste. *Biomass and Bioenergy*. 59: 142-150.

Larsen, J., Ostergaard Petersen, M., Thirup, L., Wen Li, H. and Krogh Iversen, F. 2008. The IBUS process–lignocellulosic bioethanol close to a commercial reality. *Chemical Engineering & Technology*. 31(5): 765-772.

Laureano-Perez, L., Teymouri, F., Alizadeh, H. and Dale, B.E. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. *Applied Biochemistry and Biotechnology*. 124 (1-3): 1081-1099.

Lavoie, J.M., Chornet, M., Beauchet, R. and Berberi, V. 2011. Biorefining lignocellulosic biomass via the feedstock impregnation rapid and sequential steam treatment. INTECH Open Access Publisher.

Lee, J. 1997. Biological conversion of lignocellulosic biomass to ethanol. *Journal of Biotechnology*. 56 (1):1-24.

Lee, J., Gwak, K., Park, J., Park, M., Choi, D., Kwon, M. and Choi, I. 2007. Biological pretreatment of softwood *Pinus densiflora* by three white rot fungi. *Journal of Microbiology*. *45* (6): 485-491

Leonowicz, A. and Malinowska, M. 1982. Purification of the constitutive and inducible forms of laccase of the fungus *Pholiota mutabilis* by affinity chromatography. *Acta Biochimica Polonica*. 29(3-4): 219-226.

Leonowicz, A., Trojanowski, J. and Orlicz, B., 1978. Induction of laccase in Basidiomycetes: apparent activity of the inducible and constitutive forms of the enzyme with phenolic substrates. *Acta Biochimica. Polonica*. *25* (4): 369-378.

Leštan, D., Leštan, M., & Perdih, A. 1994. Physiological aspects of biosynthesis of lignin peroxidases by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*. 60(2): 606-612.

Levin, L., Melignani, E. and Ramos, A.M. 2010. Effect of nitrogen sources and vitamins on ligninolytic enzyme production by some white-rot fungi. Dye decolorization by selected culture filtrates. *Bioresource Technology*. 101(12): 4554-4563.

Li, D., Alic, M., Brown, J. A., and Gold, M. H. 1995. Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. *Applied and Environmental Microbiology*. 61 (1): 341-5.

Li, L., Kong, X., Yang, F., Li, D., Yuan, Z. and Sun, Y. 2012. Biogas production potential and kinetics of microwave and conventional thermal pretreatment of grass. *Applied biochemistry and biotechnology*. 166(5): 1183-1191.

Liese, W. 1985. Anatomy and properties of bamboo. Recent research on bamboos. Proceedings of the International Bamboo Workshop. October 6-14, 1985, Hangzhou, China.

Liese, W. 1992. The structure of bamboo in relation to its properties and utilization. In S. Zhu, W. Li, X. Zhang, & Z. Wang (Eds.), Bamboo and its use (pp. 96-100). Proceedings of the International symposium on Industrial Use of Bamboo,

Beijing, China, 7-11 December 1992. International Tropical Timber Organization: Chinese Academy of Forestry, Beijing, China.

Limayem, A. and Ricke, S.C. 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. *Progress in Energy and Combustion Science*. 38(4): 449-467.

Lin, Q., S. 1977. The chemical composition of Chinese herbal medicine. Beijing: People's Medical Publishing House.

Lin, S. Y. 1992. Ultraviolet Spectroscopy. *In*: Methods in Lignin Chemistry, Lin, S.Y. and Dence, C.W.,Editors. New York: Springer-Verlag. p. 217-232.

Lin, Y. and Tanaka, S. 2006. Ethanol fermentation from biomass resources: current state and prospects. *Applied Microbiology and Biotechnology*. 69 (6): 627-642.

Littlewood, J., Wang, L., Turnbull, C. and Murphy, R.J. 2013. Techno-economic potential of bioethanol from bamboo in China. *Biotechnology for Biofuels*, 6 (1): 1.

Liu, X., Zhao, M. and Wang, Q. 2009. Biological characteristics of five wood-rotting fungi and wood-decaying ability to *Betula platyphylla*. *Frontiers of Forestry in China*. 4 (4): 508-515.

Lobovikov, M., Ball, L., Guardia, M. and Russo, L. 2007. World bamboo resources: a thematic study prepared in the framework of the global forest resources assessment 2005 (No. 18). Food & Agriculture Org.

Lobovikov, M., Paudel, S., Piazza, M., Ren, H., Wu, J. 2007. World bamboo resource: A thematic study parepared in the framework of the Global Forest Resources Assessment 2005. Food and Agriculture Organization of the United Nation: 1-80.

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J. and Wyman, C.E. 2008. How biotech can transform biofuels. *Nature Biotechnology*. 26(2): 169-172.

Ma, Y. S., Chang, C. N., Chiang, Y. P., Sung, H. F., and Chao, A. C. 2008. Photocatalytic degradation of lignin using Pt/TiO_2 as the catalyst. *Chemosphere*. 71(5): 998-1004.

Madhavi, V., & Lele, S. S. 2009. Laccase: properties and applications. *Bioresources*. 4(4): 1694-1717.

Mahalingam, P. U., and Maruthamalai, R.R.P. 2014. Screening and characterization lignin degrading fungi from decayed sawdust. *European Journal of Experimental Biology*, **4** (5): 90-94.

Maitan-Alfenas, G.P., Visser, E.M. and Guimarães, V.M. 2015. Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products. *Current Opinion in Food Science*. 1: 44-49.

Mason, W.H. 1926. Process and apparatus for disintegration of wood and the like. US Patent: 1578609.

Matsumoto, A., Tsubaki, S., Sakamoto, M., and Azuma, J. I. 2011. A novel saccharification method of starch using microwave irradiation with addition of activated carbon. *Bioresource Technology*. 102(4): 3985-3988.

Mayfield, M. B., Godfrey, B. J., and Gold, M. H. 1994. Characterization of the *mnp2* gene encoding manganese peroxidase isozyme 2 from the basidiomycete *Phanerochaete chrysosporium*. Gene. 142 (2): 231-5.

McAloon, A., Taylor, F., Yee, W., Ibsen, K. and Wooley, R. 2000. Determining the cost of producing ethanol from corn starch and lignocellulosic feedstocks. National Renewable Energy Laboratory Report.

Menon, V. and Rao, M. 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Scienc*e. 38(4): 522-550.

Monlau, F., Barakat, A., Steyer, J.P. and Carrere, H. 2012. Comparison of seven types of thermo-chemical pretreatments on the structural features and anaerobic digestion of sunflower stalks. *Bioresource Technology*. 120: 241-247.

Moo-Young, M., Moreira, A. R., and Tengerdy, R. P. 1983. *In*: The Filamentous Fungi, J.E. Smith, D.R. Berry and B. Kristiansen (eds.), Edward Arnold Publishers, London. pp. 117-144.

Moreira Neto, S.L., Matheus, D.R. and Machado, K.M.G., 2009. Influence of pH on the growth, laccase activity and RBBR decolorization by tropical basidiomycetes. *Brazilian Archives of Biology and Technology*. 52(5): 1075-1082.

Morjanoff, P.J. and Gray, P.P. 1987. Optimization of steam explosion as a method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification. *Biotechnology and Bioengineering*. 29 (6): 733-741.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*. 96(6): 673-686.

Mouille, G., Robin, S., Lecomte, M., Pagant, S. and Höfte, H. 2003. Classification and identification of Arabidopsis cell wall mutants using Fourier-Transform InfraRed (FT-IR) microspectroscopy. *The Plant Journal*. 35 (3): 393-404.

Murugesan, K., Arulmani, M., Nam, I. H., Kim, Y. M., Chang, Y. S., & Kalaichelvan, P. T. 2006. Purification and characterization of laccase produced by a white rot fungus *Pleurotus sajorcaju* under submerged culture condition and its potential in decolorization of azo dyes. *Applied Microbiology and Biotechnology*. 72 (5): 939-946.

Murugesan, K., Nam, I., Kim, Y. and Chang, Y. 2006. Decolourization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture. *Enzyme and Microbial Technology*. 40: 1662-1672.

Nam–Seok, C.H.O., Hee–Yeon, C.H.O., Soo–Jeong SHIN, Y.J.C., LEONOWICZ, A. and Shoji, O.H.G.A. 2008. Production of fungal laccase and its immobilization and stability. *Journal of the Faculty of Agriculture Kyushu University*. 53(1): 13-18.

Nandal, P., Ravella, S.R. and Kuhad, R.C., 2013. Laccase production by *Coriolopsis caperata* RCK2011: optimization under solid state fermentation by Taguchi DOE methodology. *Scientific Reports*. 3:1386.

Nazarpour, F., Abdullah, D.K., Abdullah, N., and Zamiri, R. 2013. Evaluation of biological pretreatment of rubberwood with white rot fungi for enzymatic hydrolysis. *Materials.* 6 (5): 2059–2073.

Nie, G., Reading, N. S., and Aust, S., D. 1999. Relative stability of recombinant versus native peroxidases from *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics*. 365: 328– 334.

Nigam, P. S. and Singh, A. 2011. Production of liquid biofuels from renewable resources. Progress in Energy and Combustion Science. 37(1): 52-68.

Nkemka, V. N. and Murto, M. 2013. Biogas production from wheat straw in batch and UASB reactors: the roles of pretreatment and seaweed hydrolysate as a co-substrate. *Bioresource Technology*. 128: 164-172.

Oberoi, H. S., Chavan, Y., Bansal, S. and Dhillon, G.S. 2010. Production of cellulases through solid state fermentation using kinnow pulp as a major substrate. *Food and Bioprocess Technology*. 3(4): 528-536.

Okano, K., Kitagawa, M., Sasaki, Y. and Watanabe, T. 2005. Conversion of Japanese red cedar (Cryptomeria japonica) into a feed for ruminants by white-rot basidiomycetes. Animal Feed Science and Technology. 120 (3): 235-243.

Okino, L.K., Machado, K.M.G., Fabris, C. and Bononi, V.L.R. 2000. Ligninolytic activity of tropical rainforest basidiomycetes. *World Journal of Microbiology and Biotechnology*. 16: 889-893.

Onuki, S., Koziel, J. A., van Leeuwen, J. H., Jenks, W. S., Grewell, D. and Cai, L., 2008. Ethanol production, purification, and analysis techniques: a review. *In* 2008 Providence. Rhode Island. 2008. (p. 1). American Society of Agricultural and Biological Engineers.

Palmeiri, G., Giardina, P., Marzullo, L., Desiderio, B., Nittii, G., Cannio, R., & Sannia, G. 1993. Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. *Applied Microbiology and Biotechnology*. 39 (4-5): 632-636.

Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G., Giardina, P. 2003. A typical isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. *Enzyme and Microbial Technology*. 33: 220–230.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A. and Sannia, G. 1997. A novel white laccase from *Pleurotus ostreatus*. *Journal of biological chemistry*. 272(50): 31301-31307.

Palonen, H., Thomsen, A.B., Tenkanen, M., Schmidt, A.S. and Viikari, L. 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. *Applied biochemistry and biotechnology*. 117 (1): 1-17.

Pandey, A., Azmi, W., Singh J., and Banerjee, U.C. 1999a. *In*: Biotechnology: Food Fermentation. V.K. Joshi and A. Pandey (eds.), Educational Publishers, New Delhi. pp.383-426.

Pandey, A., Selvakumar, P., Soccol, C.R. and Nigam, P. 1999b. Solid state fermentation for the production of industrial enzymes. *Current Science*. 77 (1): 149-162.

Pandiyan, K., Tiwari, R., Singh, S., Nain, P.K., Rana, S., Arora, A., Singh, S.B. and Nain, L. 2014. Optimization of enzymatic saccharification of alkali pretreated *Parthenium* sp. using response surface methodology. *Enzyme Research*. http://dx.doi.org/10.1155/2014/764898.

Pardo, A. G. 1996. Effect of surfactants on cellulase production by *Nectria catalinensis*. *Current Microbiology*. 33 (4): 275-278.

Park, K. M., & Park, S. S. 2008. Purification and characterization of laccase from basidiomycete *Fomitella fraxinea*. *Jounal of Microbiology and Biotechnology*. 18(6): 670-675.

Pasco, M. F., and Suckling, I. 1998. Chromophore changes during oxygen delignification of a Radiata Pine [*Pinus radiata*] Kraft Pulp. *Appita Journal*. 51 (2): 138-146.

Patel, H., Gupte, A., & Gupte, S. 2009. Effect of different culture conditions and inducers on production of laccase by a basidiomycete fungal isolate *Pleurotus ostreatus* HP-1 under solid state fermentation. *BioResources*. 4 (1): 268-284.

Patel, H., Gupte, S., Gahlout, M. and Gupte, M. 2014. Purification and characterization of an extracellular laccase from solid-state culture of *Pleurotus ostreatus* HP-1. *3 Biotech*. 4:77–84.

Pečiulytė, D. 2007. Isolation of cellulolytic fungi from waste paper gradual recycling materials. Ekologija. 53(4): 11-18.

Peng, H., Chen, H., Qu, Y., Li, H., and Xu, J. 2014. Bioconversion of different sizes of microcrystalline cellulose pretreated by microwave irradiation with/without NaOH. Appl. Energy 117, 142–148.

Periasamy, R., Rajadurai, M. and Palvannan, T. **2011**. Effect of different agro-wastes on laccase production by *Pleurotus ostreatus* IMI 395544. *International Journal of Environmental Science and Ecotechnology*. 1(1): 21-26.

Perlack, R., D., Eaton, L., M., Turhollow, J., A., F., Langholtz, M., H., Brandt, C., C., Downing, M., E., Graham, R., L., Wright, L., L., Kavkewitz, J., M., Shamey, A., M. and Nelson, R., G. 2011. US billion-ton update: biomass supply for a bioenergy and bioproducts industry.

Perlack, R.D., Wright, L., Turhollow, L.A., Graham, R.L., Stokes, B., Erbach, D.C. 2005. Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. Oak Ridge National Laboratory Report ORNL/TM-2005/66. Oak Ridge, TN: US Dept. of Energy.

Peterson, A.A., Vogel, F., Lachance, R.P., Fröling, M., Antal Jr, M.J. and Tester, J.W. 2008. Thermochemical biofuel production in hydrothermal media: a review of sub-and supercritical water technologies. *Energy & Environmental Science*. 1(1): 32-65.

Pinyou, P., Youngvises, N. and Jakmunee, J. 2011. Flow injection colorimetric method using acidic ceric nitrate as reagent for determination of ethanol. *Talanta*. 84 (3): 745-751.

Plácido, J., Chanagá, X., Ortiz-Monsalve, S., Yepes, M. and Mora, A. 2016. Degradation and detoxification of synthetic dyes and textile industry effluents by newly isolated *Leptosphaerulina* sp. from Colombia. *Bioresources and Bioprocessing*. 3(1): 1-14.

Planning Commission. 2003. Report of the Committee on Development of Biofuels. Planning Commission, Government of India. http://planningcommission.nic.in /reports /genrep/cmtt_bio.pdf>.

Pointing SB. 2001. Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology*. 57:20 –33.

Pointing, S.B. 1999. Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 2: 17-33.

Pointing, S.B., Jones, E.B.G. and Vrijmoed, L.L.P. 2000. Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. *Mycologia*:139-144.

Pontes, M. J. C., Santos, S. R. B., Araujo, M. C. U., Almeida, L. F., Lima, R. A. C., Gaiao, E. N. and Souto, U. T. C. P. 2006. Classification of distilled alcoholic beverages and verification of adulteration by near infrared spectrometry. *Food Research International*. 39 (2): 182-189.

Pooja, N.S. and Padmaja, G. 2014. Pretreatment techniques to enhance the enzymatic degradability of agricultural and processing residues of cassava. *Journal of Microbiology and Biotechnology Research*. 4 (1): 57-67.

Puri, V. P. 1984. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. *Biotechnology and Bioengineering*. 26 (10): 1219-1222.

Qi, J. and Xiuyang, L.Ü. 2007. Kinetics of non-catalyzed decomposition of D-xylose in high temperature liquid water. *Chinese Journal of Chemical Engineering*. 15 (5): 666-669.

Qiao, W., Yan, X., Ye, J., Sun, Y., Wang, W., and Zhang, Z. 2011. Evaluation of biogas production from different biomass wastes with/without hydrothermal pretreatment. *Renew Energy*. 36: 3313-338.

Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., Liotta, C.L. and Mielenz, J.R. 2006. The path forward for biofuels and biomaterials. *Science*. 311 (5760): 484-489.

Raghukumar, C., D'Souza-Ticlo, D. and Verma, A. 2008. Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. *Critical Reviews in Microbiology*. 34 (3-4): 189-206.

Rao, H., Z., & Sang, C., T. 2006. Microwave-assisted extraction technology of sapindus-saponin. *Journal of Liaoning University of Petroleum & Chemical Technology*. 26:70-72.

Rautela, G.S. and Cowling, E.B. 1966. Simple cultural test for relative cellulolytic activity of fungi. *Applied Microbiology*. 14(6): 892-898.

Rawat, R. and Tewari, L. 2012. Purification and characterization of an acidothermophilic cellulase enzyme produced by *Bacillus subtilis* strain LFS3. *Extremophiles*. 16 (4): 637-644.

Reid, I. D. 1985. Biological delignification of aspen wood by solid-state fermentation with the white-rot fungus *Merulius tremellosus*. *Applied and Environmental Microbiology*. 50 (1): 133-139.

Richardson, B. J., Lam, P. K., Zheng, G. J., McClellan, K. E., & De Luca-Abbott, S. B. 2002. Biofouling confounds the uptake of trace organic contaminants by semi-permeable membrane devices (SPMDs). *Marine Pollution Bulletin*. 44(12): 1372-1379.

Rocha, G. J. M., Gonçalves, A. R., Oliveira, B. R., Olivares, E. G. and Rossell, C.E.V. 2012. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. *Industrial Crops and Products*. 35(1): 274-279.

Romano, R.T., Zhang, R., Teter, S. and McGarvey, J.A. 2009. The effect of enzyme addition on anaerobic digestion of JoseTall Wheat Grass. *Bioresource Technology*. 100 (20): 4564-4571.

Romero, M.D., Aguado, J., González, L. and Ladero, M. 1999. Cellulase production by *Neurospora crassa* on wheat straw. *Enzyme and Microbial Technology*. 25(3): 244-250.

Sadasivam, S. and Manickam, A. 1991. Biochemical Methods. New Age International (P) Ltd., Publishers, New Delhi. Second Edition.

Sadhasivam, S., Savitha, S., Swaminathan, K. and Lin, F.H. 2008. Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. *Process Biochemistry*. 43: 736-742.

Saha, B.C. and Cotta, M.A. 2006. Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw. *Biotechnology Progress*. 22 (2): 449-453.

Saheed, O. K., Jamal, P., Karim, M. I. A., Alam, M. Z. and Muyibi, S. A., 2016. Utilization of fruit peels as carbon source for white rot fungi biomass production under submerged state bioconversion. *Journal of King Saud University-Science*. 28 (2): 143-151.

Saini, J.K. and Tewari, L. 2012. Simultaneous isolation and screening of cellulolytic bacteria: selection of efficient medium. *Journal of Pure and Applied Microbiology*. 6 (3): 1339-1344.

Saini, J.K., Anurag, R.K., Arya, A., Kumbhar, B.K. and Tewari, L. 2013. Optimization of saccharification of sweet sorghum bagasse using response surface methodology. *Industrial Crops and Products*. 44: 211-219.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S., Yokogawa, Y. 2003. Purification and characterization of an extracellular laccase of a fungus (family Chaetomiaceae) isolated from soil. *Enzyme and Microbial Technology*. 33: 520–526.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 2000. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press. ISBN 0-87969-309-6.

Sannigrahi, P., Pu, Y., Ragauskas, A. 2010. Cellulosic biorefineries--unleashing lignin opportunities. *Current Opinion in Environmental Sustainability*. 2(5): 383-393.

Saritha, M. and Arora, A. 2012. Biological pretreatment of lignocellulosic substrates for enhanced delignification and enzymatic digestibility. *Indian Journal of Microbiology*. 52(2): 122-130.

Sazci, A., Erenler, K. and Radford, A. 1986. Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. *Journal of Applied Bacteriology*. 61 (6): 559-562.

Scheel, T., Hofer, M., Ludwig, S. and Holker, U. 2000. Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of

manganese or aromatic compounds. *Applied Microbiology and Biotechnology*. 54 (5): 686-91.

Schell, D. J., and Harwood, C. 1994. Milling of lignocellulosic biomass e results of pilot scale testing. *Applied Biochemistry and Biotechnology*. (45-46):159-168.

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., Kruse, O. and Hankamer, B. 2008. Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy Research*. 1 (1): 20-43.

Schmid, F.X. 2001. Biological Macromolecules: UV-visible Spectrophotometry. eLS.

Schmidt, O., Magel, E., Frühwald, A., Glukhykh, L., Erdt, K. and Kaschuro, S. 2016. Influence of sugar and starch content of palm wood on fungal development and prevention of fungal colonization by acid treatment. *Holzforschung*. 70 (8): 783–791.

Scurlock, J.M.O., Dayton, D.C. and Hames, B. 2000. Bamboo: An overlooked biomass resource?. *Biomass and Bioenergy*, 19(4): 229-244.

Seo, G. S., Kirk, P. M., Flood, J., Bridge, P. D. and Holderness, M., 2000. Ganodermataceae: nomenclature and classification. *Ganoderma diseases of perennial crops*: 3-22.

Shafiei, M., Karimi, K., Zilouei, H. and Taherzadeh, M. J. 2014. Enhanced ethanol and biogas production from pinewood by NMMO pretreatment and detailed biomass analysis. *BioMed research international*. 2014. http://dx.doi.org/10.1155/2014/469378.

Shimokawa, T., Ishida, M., Yoshida, S., Nojiri, M. 2009. Effects of growth stage on enzymatic saccharification and simultaneous saccharification and fermentation of bamboo shoots for bioethanol production. *Bioresource Technology*. 100: 6651–6654.

Shrestha, P. 2008. Enhanced bioprocessing of lignocellulose: Wood-rot fungal saccharification and fermentation of corn fiber to ethanol. Graduate thesis and dissertations. Iowa State University. Paper 11492. ProQuest.

Silva, I. S., Menezes, C. R. D., Franciscon, E., Santos, E. D. C. D., & Durrant, L. R. 2010. Degradation of lignosulfonic and tannic acids by ligninolytic soil fungi cultivated under anaerobic conditions. *Brazilian Archives of Biology and Technology*. 53 (3): 693-699.

Sindhu, R., Binod, P. and Pandey, A. 2016. Biological pretreatment of lignocellulosic biomass–An overview. *Bioresource Technology*. 199: 76-82.

Singh A., Nigam P. S., Murphy J. D. 2011. Mechanism and challenges in commercialisation of algal biofuels. Bioresource Technology. 102(1): 26–34.

Singh, A. and Bishnoi, N.R., 2012. Enzymatic hydrolysis optimization of microwave alkali pretreated wheat straw and ethanol production by yeast. *Bioresource Technology*. 108: 94-101.

Singh, A., Bajar, S. and Bishnoi, N.R. 2014. Enzymatic hydrolysis of microwave alkali pretreated rice husk for ethanol production by *Saccharomyces cerevisiae*, *Scheffersomyces stipitis* and their co-culture.Fuel.116: 699-702.

Smith-Moritz, A. M., Chern, M., Lao, J., Sze-To, W.H., Heazlewood, J. L., Ronald, P. C. and Vega-Sánchez, M. E. 2011. Combining multivariate analysis and monosaccharide composition modeling to identify plant cell wall variations by Fourier transform near infrared spectroscopy. *Plant Methods*. 7(1): 1.

Soden, D., M., and Dobson, A. D. W. 2001. Differential regulation of laccase gene expression in *Pleurotus sajorcaju*. *Microbiology*. 147 (7): 1755-1763.

Söderström, J., Pilcher, L., Galbe, M. and Zacchi, G. 2002. Two-step steam pretreatment of softwood with SO_2 impregnation for ethanol production. *Applied Biochemistry and Biotechnology*. 98 (1-9): 5-21.

Solomon, B., D., Barnes, J., R. and Halvorsen, K., E. 2007. Grain and cellulosic ethanol: History, economics, and energy policy. *Biomass and Bioenergy*. 31(6): 416-425.

Song, Z., Yang, G., Guo, Y. and Zhang, T. 2012. Comparison of two chemical pretreatments of rice straw for biogas production by anaerobic digestion. *Bioresources*. 7(3): 3223-3236.

Sopajarn, A. and Sangwichien, C., 2015. Optimization of enzymatic saccharification of alkali pretreated *Typha angustifolia* for glucose production. *International Journal of Chemical Engineering and Applications*. 6 (4): 232-236.

Spadaro, J. T., Gold, M. H. and Renganathan, V. 1992. Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. *Applied Environmental Microbiology*. 58: 2397–2401.

Sree, N. K., Sridhar, M., Rao, L. V. and Pandey, A., 1999. Ethanol production in solid substrate fermentation using thermotolerant yeast. *Process Biochemistry*. 34 (2): 115-119.

Sritong, C., Kunavongkrit, A. and Piumsombun, C. 2012. Bamboo: An innovative alternative raw material for biomass power plants. *International Journal of Innovation, Management and Technology*. 3(6): 759.

Sukumaran, R. K., Surender, V. J., Sindhu, R., Binod, P., Janu, K. U., Sajna, K. V., Rajasree, K. P. & Pandey, A. 2010. Lignocellulosic ethanol in India: prospects, challenges and feedstock availability. *Bioresource Technology*.101 (13): 4826-4833.

Sukumaran, R.K., Singhania, R.R., Mathew, G.M. and Pandey, A. 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. *Renewable Energy*. 34 (2): 421-424.

Sun, F., and Chen, H. 2008. Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw. *Bioresour Technol*. 99:5474–5479.

Sun, Y. and Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*. 83(1): 1-11.

Taherzadeh, M. J. and Karimi, K. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *International Journal of Molecular Sciences.* 9 (9): 1621-1651.

Talavera, G. and Castresana, J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology*. 56(4): 564-577.

Tanaka, K., Calanag, R. C. R., and Hisanaga, T. 1999. Photocatalyzed degradation of lignin on TiO_2. *Journal of Molecular Catalysis A: Chemical*. 138(2): 287-294.

Tarkow, H. and Feist, W.C. 1969. A mechanism for improving the digestibility of lignocellulosic materials with dilute alkali and liquid ammonia. *Advances in Chemistry Series*. 95: 197-218.

Teghammar, A., Yngvesson, J., Lundin, M., Taherzadeh, M.J. and Horváth, I.S. 2010. Pretreatment of paper tube residuals for improved biogas production. *Bioresource Technology*. 101 (4): 1206-1212.

Thiyagarajan, A., Saravanakumar, K., and Kaviyarasan, V. 2008. Optimization of extracellular peroxidsae production from *Coprinus* sp. *Indian Journal of Science and Technology*. 1:1-5.

Thomas, R. J. 1977. Wood: Structure and Chemical Composition in Wood Technology Chemical Aspects. American Chemical Society (ACS) Symposium (Series 43, p. 21).

Thompson, D.N., Chen, H.C. and Grethlein, H.E. 1992. Comparison of pretreatment methods on the basis of available surface area. *Bioresource Technology*. 39 (2): 155-163.

Thostenson, E.T. and Chou, T.W. 1999. Microwave processing: fundamentals and applications. Composites Part A: *Applied Science and Manufacturing*. 30 (9): 1055-1071.

Tien, M. and Kirk, K. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods in Enzymology. 161: 238-249.

Todero-Ritter, C.E., Camassola, M., Zampieri, D., Silveira, M.M. and Dillon, A.J.P. 2013. Cellulase and xylanase production by *Penicillium echinulatum* in submerged media containing cellulose amended with sorbitol. *Enzyme Research*. 2013: 1-9.

Tomalang, F. N., Lopez, A. R., Semara, J. A., Casin, R. F., & Espiloy, Z. B. 1980. Properties and utilization of Philippine erect bamboo. In Lessard, G., & Chouinard, A. (Eds), International Seminar on Bamboo Research in Asia held in Singapore, May 28-30, 1980: proceedings (pp. 266-275). Singapore:

International Development 44 Research Center and the International Union of Forestry Research Organization.

Torget, R., Himmel, M.E. and Grohmann, K. 1991. Dilute sulfuric acid pretreatment of hardwood bark. *Bioresource Technology*. 35 (3): 239-246.

Tortella, G.R., Rubilar, O., Gianfreda, L., Valenzuela, E. and Diez, M.C. 2008. Enzymatic characterization of Chilean native wood-rotting fungi for potential use in the bioremediation of polluted environments with chlorophenols. *World Journal of Microbiology and Biotechnology*. 24(12): 2805-2818.

Tripathy, S. S. and Raichur, A. M. 2008. Enhanced adsorption capacity of activated alumina by impregnation with alum for removal of As (V) from water. *Chemical Engineering Journal*. 138 (1): 179-186.

Tucker, M. P., Nguyen, Q. A., Eddy, F. P., Kadam, K. L., Gedvilas, L. M. and Webb, J. D. 2001. Fourier transform infrared quantitative analysis of sugars and lignin in pretreated softwood solid residues. *Applied Biochemistry and Biotechnology*. 91(1-9): 51-61.

Tutt, M., Kikas, T., Kahr, H., Pointner, M., Kuttner, P. and Olt, J. 2014. Using steam explosion pretreatment method for bioethanol production from floodplain meadow hay. *Agronomy Research*. 12(2): 417-424.

Ueda, M., Shintani, K., Nakanishi-Anjyuin, A., Nakazawa, M., Kusuda, M., Nakatani, F., Kawaguchi, T., Tsujiyama, S.I., Kawanishi, M., Yagi, T. and Miyatake, K. 2012. A protein from *Pleurotus eryngii* var. tuoliensis CJ Mou with strong removal activity against the natural steroid hormone, estriol: purification, characterization, and identification as a laccase. *Enzyme and Microbial Technology*. 51 (6): 402-407.

United States Census Bureau (USCB). 2008. World population clock. United States Census Bureau. http://www.census.gov.

Us, E. and Perendeci, N.A. 2012. Improvement of methane production from greenhouse residues: optimization of thermal and H_2SO_4 pretreatment process by experimental design. *Chemical Engineering Journal*. 181: 20-131.

Vallejos, M.E., Zambon, M.D., Area, M.C. and da Silva Curvelo, A.A. 2015. Low liquid-solid ratio fractionation of sugarcane bagasse by hot water autohydrolysis and organosolv delignification. *Industrial Crops and Products*. 65: 349-353.

Van den Brink, J. and de Vries, R. P. 2011. Fungal enzyme sets for plant polysaccharide degradation. *Applied Microbiology and Biotechnology*. 91(6):1477-1492.

Van Der Westhuizen, G.C.A. 1958. Studies of wood-rotting fungi: 1. cultural characteristics of some common species. Bothalia.7 (1): 83-99.

Van Walsum, G.P., Allen, S.G., Spencer, M.J., Laser, M.S., Antal Jr, M.J. and Lynd, L.R. 1996. Conversion of lignocellulosics pretreated with liquid hot water to ethanol. *In*: Seventeenth Symposium on Biotechnology for Fuels and Chemicals. Humana Press. pp157-170.

Vasconcelos, A., F., D., Barbosa, A., M., Dekker, R., F., Scarminio, I. S., & Rezende, M., I. 2000. Optimization of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response-surface method.*Process Biochemistry*. 35 (10): 1131-1138.

Viikari, L., Vehmaanperä, J., & Koivula, A. 2012. Lignocellulosic ethanol: from science to industry. *Biomass and Bioenergy*. 46: 13-24.

Vinzant, T.B., Ehrman, C.I., Adney, W.S., Thomas, S.R. and Himmel, M.E. 1997. Simultaneous saccharification and fermentation of pretreated hardwoods. *Applied Biochemistry and Biotechnology*. 62 (1): 99-104.

Wahab, R., Mohamed, A., Mustafa, M.T. and Hassan, A. 2009. Physical characteristics and anatomical properties of cultivated bamboo (*Bambusa vulgaris* Schrad.) culms. *Journal of Biological Sciences*. 9(7): 753-759.

Wang, G.S., Pan, X.J., Zhu, J.Y., Gleisner, R. and Rockwood, D. 2009. Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) for robust enzymatic saccharification of hardwoods. *Biotechnology Progress*. 25(4): 1086-1093.

Wang, Z., Keshwani, D.R., Redding, A.P. and Cheng, J.J. 2010. Sodium hydroxide pretreatment and enzymatic hydrolysis of coastal Bermuda grass. *Bioresource Technology*. 101(10): 3583-3585.

Weil, J., Sarikaya, A., Rau, S.L., Goetz, J., Ladisch, C.M., Brewer, M., Hendrickson, R. and Ladisch, M.R. 1997. Pretreatment of yellow poplar sawdust by pressure cooking in water. *Applied Biochemistry and Biotechnology*. 68 (1-2): 21-40.

Wesenberg, D., Kyriakides, I. and Agathos, S.N. 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances*. 22 (1): 161-187.

Wi, S. G., Cho, E. J., Lee, D. S., Lee, S. J., Lee, Y. J. and Bae, H. J. 2015. Lignocellulose conversion for biofuel: a new pretreatment greatly improves downstream biocatalytic hydrolysis of various lignocellulosic materials. *Biotechnology for Biofuels*. 8(1): 1-11.

Wilkie, A.C., Riedesel, K.J. and Owens, J.M. 2000. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass and Bioenergy*. 19(2): 63-102.

Woo, H. L., Hazen, T. C., Simmons, B. A., and DeAngelis, K. M. 2014. Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils. *Systematic and Applied Microbiology*, 37 (1): 60-67.

Wyman, C. 1996. Handbook on bioethanol: production and utilization. CRC Press.

Wyman, C., E. 2007.What is (and is not) vital to advancing cellulosic ethanol. *Trends in Biotechnology*. 25:153–157.

Xu, F., Yu, J., Tesso, T., Dowell, F. and Wang, D. 2013. Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: a mini-review. *Applied Energy*. 104: 801-809.

Xu, J. Z., Zhang, J. L., Hu, K. H. and Zhang, W. G. 2013. The relationship between lignin peroxidase and manganese peroxidase production capacities and cultivation periods of mushrooms. *Microbial Biotechnology*. 6(3): 241-247.

Yang, B., Wyman, C. 2008. Pre-treatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts and Biorefining*. 2: 26-40.

Yaropolov, A. I., Skorobogatko, O. V., Vartanov, S. S., and Varfolomeyev, S. D. 1994. Laccase: properties, catalytic mechanism, applicability. *Applied Biochemistry and Biotechnology*. 49: 257–279.

Yu, H., Guo, G., Zhang, X., Yan, K. and Xu, C. 2009. The effect of biological pretreatment with the selective white-rot fungus *Echinodontium taxodii* on enzymatic hydrolysis of softwoods and hardwoods. *Bioresource Technology*. 100 (21): 5170-5175.

Yu, Z. and Zhang, H., 2003. Ethanol fermentation of acid-hydrolyzed cellulosic pyrolysate with *Saccharomyces cerevisiae*. *Bioresource Technology*. 90(1): 95-100.

Zadrazil, F., and Isikhuemhen, O. 1997. Solid state fermentation of lignocellulosics into animal feed with white rot fungi. In Advances in Solid State Fermentation: 273-287). Springer Netherlands.

Zhang, M. J., Liu, P. R., Zhao, J. Z., et al....1993. Study on the com-prehensive utilization of *Sapindus mukorssi*. *Natural Product Research and Development.* 5: 76-78.

Zhang, X. Y., Xu, C. Y., Wang, H. X. 2007. Pretreatment of bamboo residues with Coriolus versicolor for enzymatic hydrolysis, *Journal of Bioscience and Bioengineering*, 104, 149-151.

Zhang, X., Yu, H., Huang, H., & Liu, Y. 2007. Evaluation of biological pretreatment with white rot fungi for the enzymatic hydrolysis of bamboo culms. *International Biodeterioration & Biodegradation*. 60 (3): 159-164.

Zhang, Y., Li, Q., Su, J., Lin, Y., Huang, Z., Lu, Y., Sun, G., Yang, M., Huang, A., Hu, H. and Zhu, Y. 2015. A green and efficient technology for the degradation of cellulosic materials: structure changes and enhanced enzymatic hydrolysis of natural cellulose pretreated by synergistic interaction of mechanical activation and metal salt. *Bioresource Technology*. 177: 176-181.

Zhang, Y., Zeng, Z., Zeng, G., Liu, X., Liu, Z., Chen, M., and Xie, G. 2012. Effect of Triton X-100 on the removal of aqueous phenol by laccase analyzed with a combined approach of experiments and molecular docking. *Colloids and Surfaces B: Biointerfaces*. 97: 7-12.

Zhang, Z., Zhou, G. and Lin, Q. 2007. Research progress and some problems on cluster Bamboo in China [J]. *Tropical Forestry*. 2: 004.

Zhao, X., Zhang, L. and Liu, D. 2012. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels. Bioproducts and Biorefining*. 6 (4): 465-482.

Zhao, X., Zi, L., Bai, F., Lin, H., Hao, X., Yue, G. and Ho, N., W., Y.2012. Bioethanol from lignocellulosic biomass. *Advances in Biochemical Engineering/Biotechnology*. 128: 25–51.

Zheng, Y., Zhao, J., Xu, F. and Li, Y. 2014. Pretreatment of lignocellulosic biomass for enhanced biogas production. *Progress in Energy and Combustion Science*. 42: 35-53.

Zhuang, X., Wang, W., Yu, Q., Qi, W., Wang, Q., Tan, X., Zhou, G. and Yuan, Z. 2016. Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products. *Bioresource Technology*. 199: 68-75.

Zouari-Mechichi, H., Mechichi, T., Dhouib, A., Sayadi, S., Mart´ınez, A., T. and Mart´ınez, M., J. 2006. Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: decolorization of textile dyes by the purified enzyme. *Enzyme and Microbial Technology*. 39: 141–148.

Appendices

APPENDIX-I

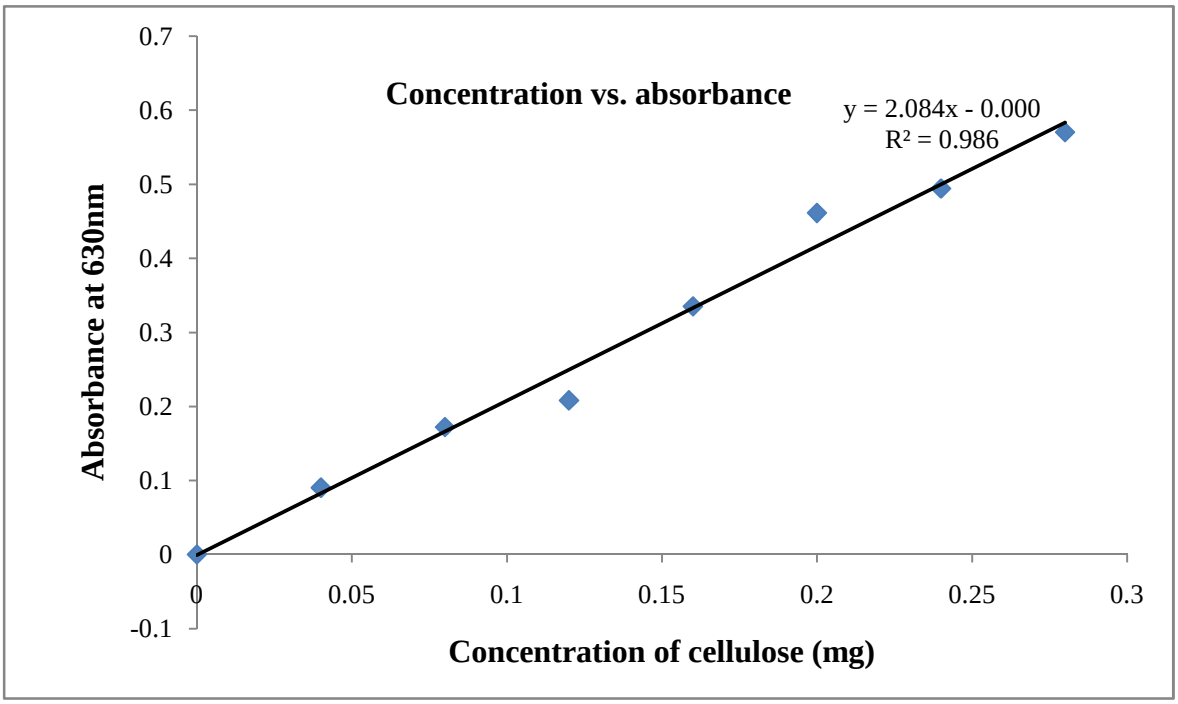


Fig 1: Standard curve of cellulose

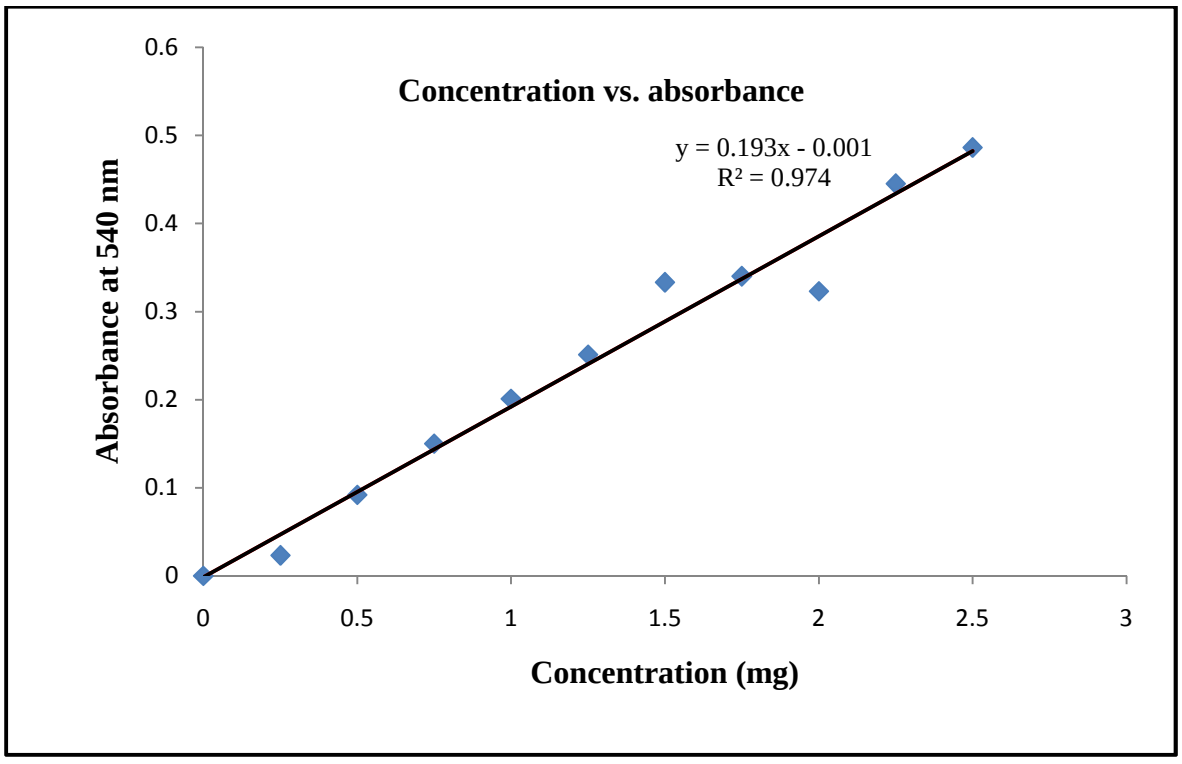


Fig 2: Standard curve of glucose for FPase and CMCase activity

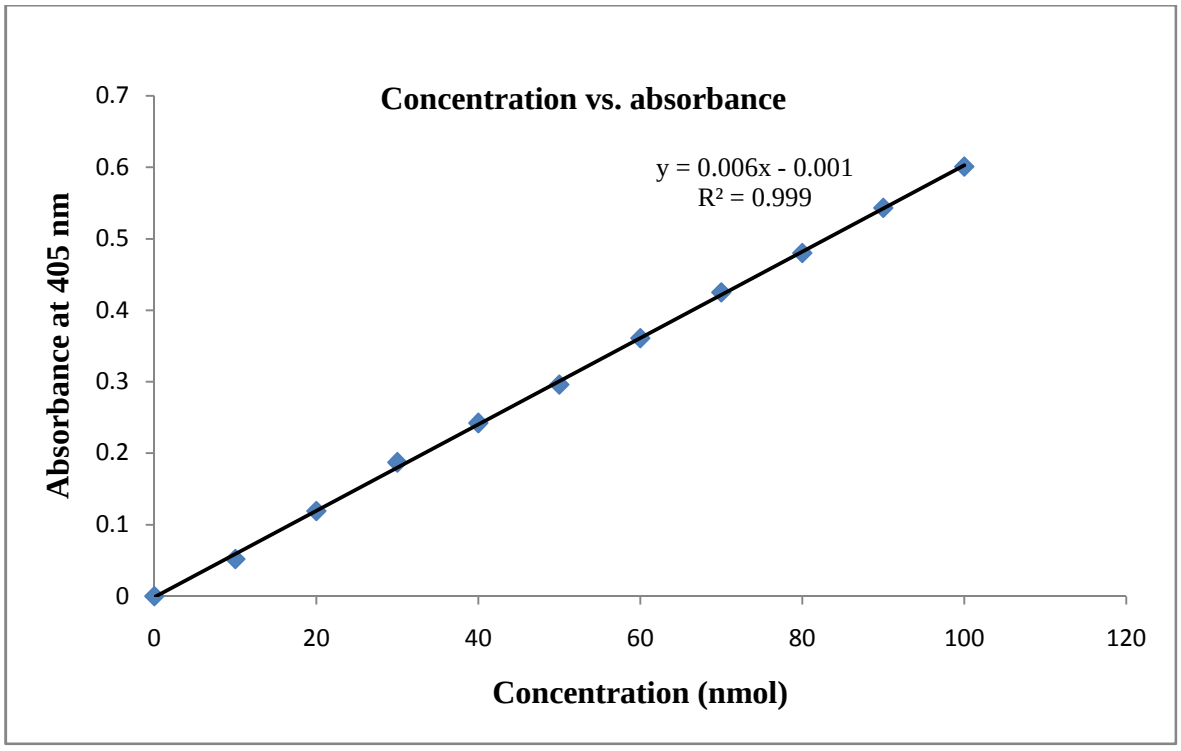


Fig 3: Standard curve of p-Nitrophenol for β-glucosidase assay

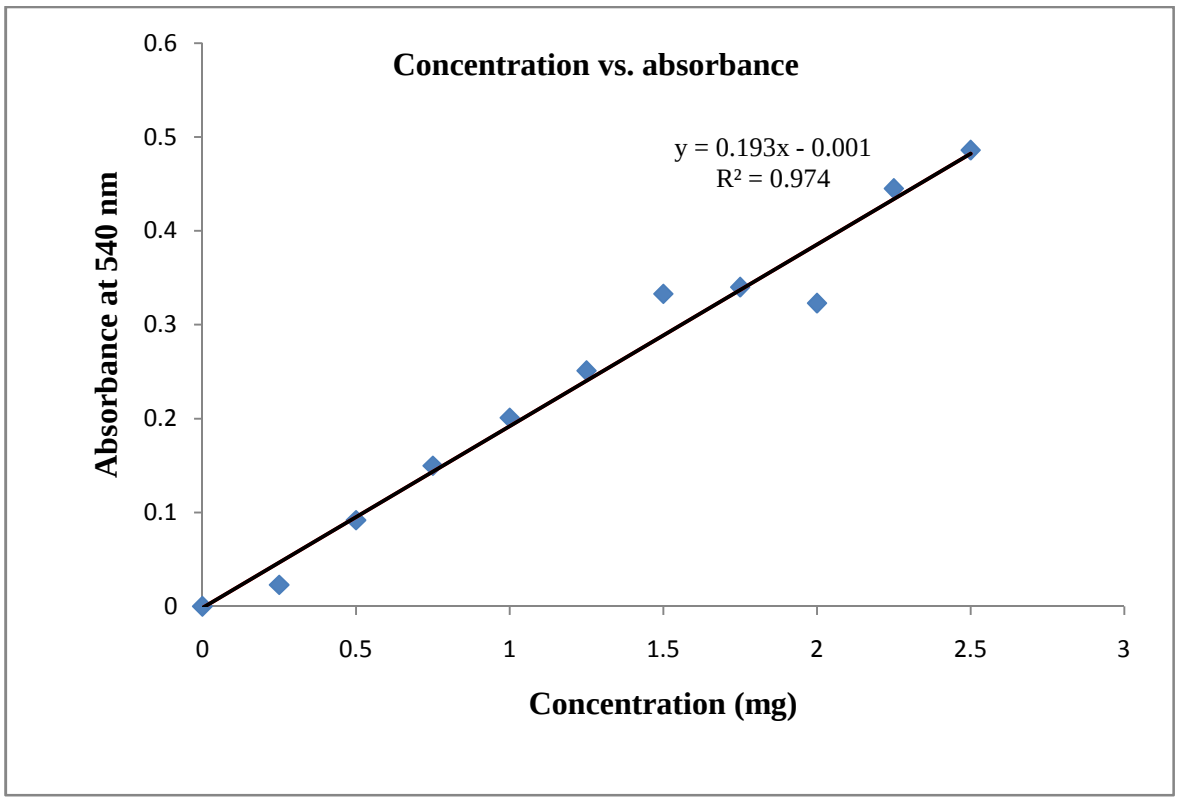


Fig 4: Standard curve of glucose for reducing sugar estimation

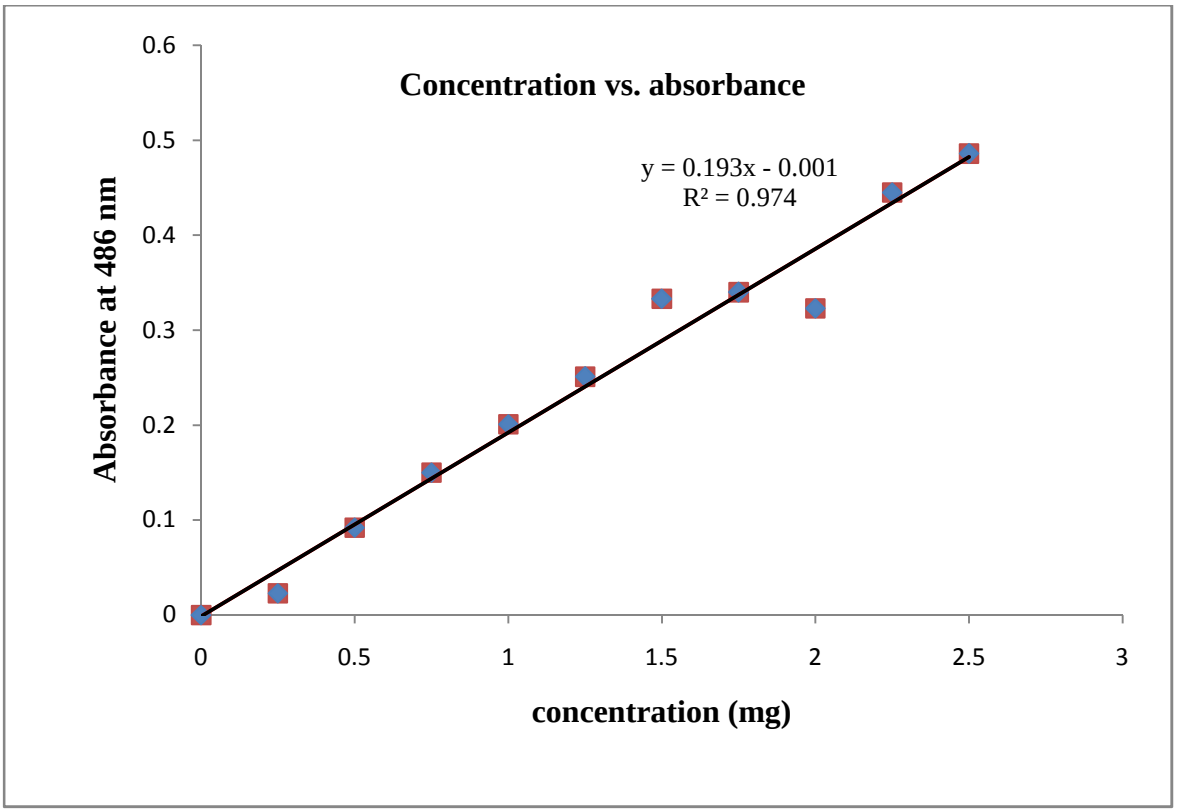
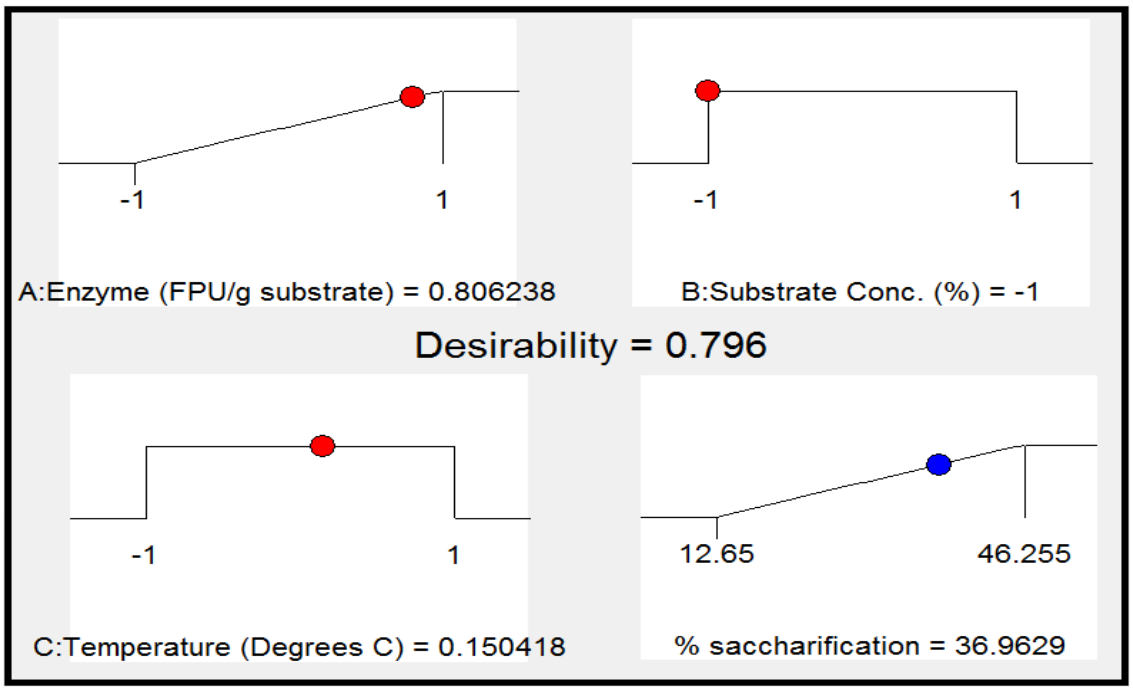


Fig 5: Standard curve of ethanol for ethanol estimation

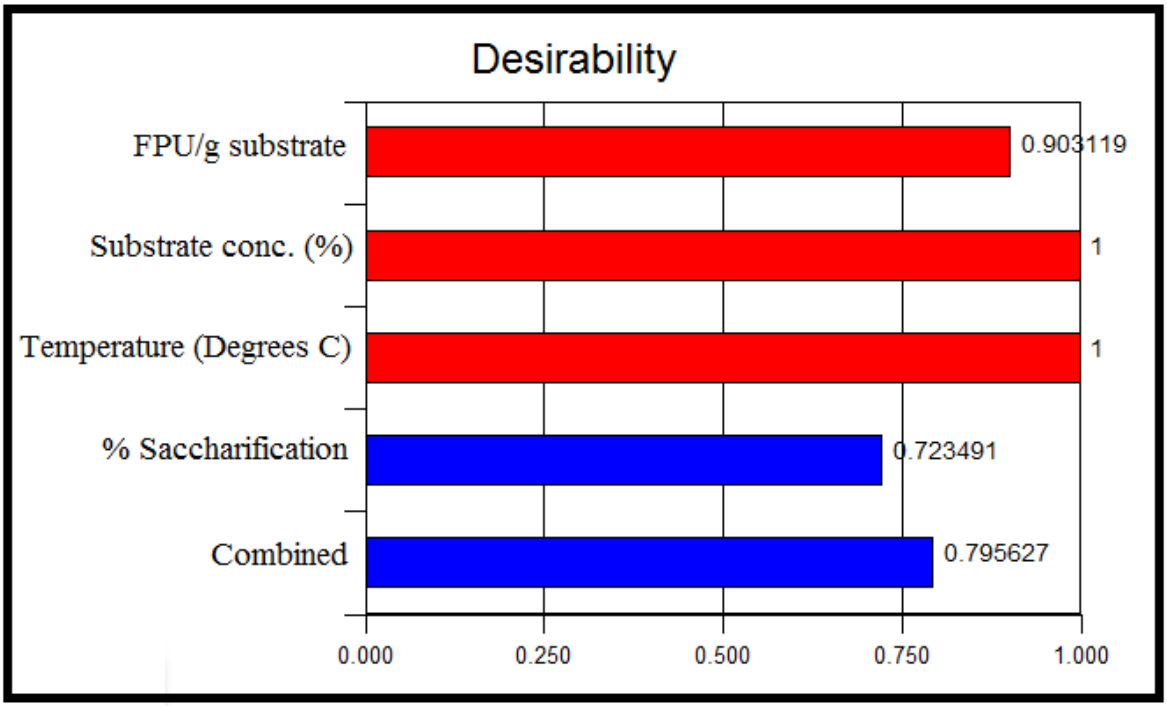
APPENDIX-II

Table 1: ANOVA for response surface quadratic model for alkaline hydrogen peroxide pretreatment of *bambus balcooa* powder

Analysis of variance table [Partial sum of squares - Type III]						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	136.92	9	15.21	1.93	0.1995	**not significant**
A-Solid:Liquid	3.38	1	3.38	0.43	0.5337	
B-NaOH Conc. (%)	1.13	1	1.13	0.14	0.7169	
C-Hydrogen Peroxide Conc. (%)	17.41	1	17.41	2.21	0.1811	
AB	50.41	1	50.41	6.39	0.0394	
AC	2.89	1	2.89	0.37	0.5642	
BC	10.24	1	10.24	1.30	0.2921	
A^2	25.75	1	25.75	3.26	0.1138	
B^2	5.31	1	5.31	0.67	0.4391	
C^2	15.57	1	15.57	1.97	0.2029	
Residual	55.24	7	7.89			
Lack of Fit	54.49	3	18.16	97.35	0.0003	**significant**
Pure Error	0.75	4	0.19			
Cor Total	192.15	16				



(a)



(b)

Fig 5: Ramps (a) and bar graph (b) obtained after numerical optimization of enzymatic saccharification of biologically pretreated *Bambusa balcooa* wood

VITA

The author Raj Kumar Pandey, son of Mr. Vijay Kumar Pandey, and Mrs. Nirmala Pandey, was born on December 14, 1989 in Pithoragarh district, Uttaranchal. He passed his high school examination from Govt. Intermediate College Tanakpur, Champawat in 2004, and intermediate examination from Govt. Intermediate College Sailanigoth, Tanakpur, Champawat in 2006. He earned Bachelor of Science degree (Zoology, Botany, Chemistry) in 2009 from Kumaun University Nainital. He completed his M. Sc. degree program in Microbiology from H. N. B. Garhwal University, Srinagar Garhwal, in 2011. He took admission for the degree of Doctor of Philosophy (Microbiology), in the College of Basic Sciences and Humanities, G. B. Pant University of Agriculture and Technology Pantnagar in I semester, 2012-13 and awarded by University scholarship from 2012-13 to 2014-15 and qualified NET exam (2017) conducted by ICAR in Agriculture Microbiology. He received Young Scientist Award (2014) and Environmental Microbiologist of the Year Award (2017).

Permanent Address:

Raj Kumar Pandey
S/O Mr. Vijay Kumar Pandey
Nayakgoth, City: Tanakpur
Distt. Champawat
Pin Code- 262309
Uttarakhand, India
☎ *+91 8979600498, 7088536989*
E Mail Id: *pandeyrajkumar227@gmail.com*

Name	**:**	Raj Kumar Pandey	**Id. No.**	**:** 44125
Sem. & year of admission	**:**	Ist Sem., 2012-13	**Degree**	**:** Ph.D.
			Department	**:** Microbiology
Major	**:**	Microbiology		
Minor	**:**	Molecular Biology and Biotechnology		
Thesis title	**:**	Bioprocess Optimization for Enzymatic Delignification and Bioconversion of *Bambusa balcooa* Biomass into Fermentable Sugars for Bioethanol Production		
Advisor	**:**	Dr. Lakshmi Tewari		

ABSTRACT

In India, lignocellulosic biomass from *Bambusa sp.* can serve as a potential feed stock for bioethanol production. Lignin, the polyphenolic polymer of the biomass, hinders the enzymatic digestibility of cellulose, thus the goal of any pre-treatment technology is to remove this structural component to improve the cellulose accessibility for enzymatic saccharification. During present study, the biomass from four different bamboo genotypes was screened for chemical composition and *Bambusa balcooa* was selected as potential substrate owing its high biomass yield (45.87 Kg.), high cellulose (37.93%) and relatively low lignin (29.5%) content. The biomass was subjected to various physical, chemical and biological pre-treatments for decreasing the lignin content and enhancing accessibility of cellulose to enzymatic saccharification. For biological pretreatement, 114 fungal and bacterial isolates were examined for their abilities to produce lignocellulose degrading laccase enzyme. Maximum extracellular laccase activity (156.793 Uml^{-1}) was observed within 15 to 18 d of incubation using potential fungal isolate (WDP2). However, optimization of culture conditions further enhanced the enzyme activity to 189.26 Uml^{-1} and lowered the incubation period from 10-12 days. For biological delignification, whole cell pretreatment was given using potential fungus WDP2. The significant decrease in lignin content from an initial value of 29.5% to 8.4% and increase in cellulose content from 37.93% to 61.59% with 42.5 Uml^{-1} laccase activity in optimised SSF medium was achieved at 48days. Optimization of bioprocess for enzymatic saccharification of biologically (fungal) pretreated *B. balcooa* wood powder was done using Box Behnken design (BBD) in RSM. Maximum percent saccharification (46.255%) was observed with enzyme concentration @ 15FPU/g substrate, substrate (Bamboo) concentration @ 1%, w/v, at 40^0C. The final optimum conditions for maximum % saccharification were: cellulase enzyme 18.4 Uml^{-1} FPU/g substrate, substrate concentration 1.0%, and temperature 39.49^0C. Spectral analysis revealed clearly the chemical changes and porosity development in the wood after pretreatment and saccharification polyphasic approach using phenotype and molecular (5.8S ITS r-RNA gene sequencing) markers used for characterization and identification of cultures. The potential lignolytic isolate WDP2 was identified as *Lengites elegans*, while the isolate WFPn1 was identified as *Ganoderma lucidum*.

(Lakshmi Tewari)
Advisor

(Raj Kumar Pandey)
Author

विद्यार्थी का नाम	:	राज कुमार पाण्डेय	**परिचयांक संख्या**	:४४१२५
षठमास व प्रवेश वर्ष	:	प्रथम, २०१२-१३	**उपाधि**	:पी० एच० डी०
मुख्य विषय	:	सूक्ष्मजीव विज्ञान	**विभाग**	:सूक्ष्मजीव विज्ञान
सहायक विषय	:	आण्विक जीवविज्ञान एवम् जैव प्रौद्योगिकी		

शोध शीर्षक : **जैव ईथेनॉल उत्पादन हेतु जैवविकर आधारित लिग्निन विलगन व *बैम्बूसा बालकोआ* जैवभार के किण्वन योग्य शर्कराओं में जैव रूपान्तरण हेतु जैव पद्धति अनुकूलन**

सलाहकार : डा० लक्ष्मी तिवारी

सारांश

भारतवर्ष में जैव ईथेनॉल उत्पादन के लिए, बाँस जैव पदार्थ किण्वन योग्य शर्कराओं का एक प्रतिनिधि स्रोत है। लिग्निन, जो कि कई फिनॉल इकाइयों का बहुलक है, विकर आधारित सैलुलोज विघटन में सर्वाधिक बाधा डालता है। अतः किसी भी पूर्वापचार तकनीक का मुख्य लक्ष्य विकर आधारित शर्करीकरण के लिए सैलुलोज अभिगम्यता में सुधार हेतु इस संरचनात्मक घटक का विघटन है। वर्तमान अध्ययन में बाँस के चार विभिन्न जीनरूपों को जैवभार प्राप्ति, व लिग्निन व सैलुलोज मात्रा के आधार पर जैव इथेनॉल उत्पादन के लिये परखा गया। सभी चार बाँस जैवभारों में से उच्चतम जैवभार प्राप्ति (४५.८७ किग्रा०), उच्च सैलुलोज मात्रा (३७.६३%), व अपेक्षाकृत कम लिग्निन (२६.५%) के आाधार पर *बैम्बूसा बालकोआ* जैवभार को चुना गया। इसके जैवभार को विभिन्न भौतिक, रासायनिक व जैविक पूर्वोपचारों के अधीन करके लिग्निन मात्रा में कमी व विकार आधारित शर्करीकरण हेतु सेलुलोज मात्रा में वृद्धि के लिये देखा गया। जैविक पूर्वोपचार हेतु कुल ११४ कवक व जीवाणु संवर्द्धन लिग्नोसैलुलोज विघटनकारी लैक्केज विकर उत्पादन हेतु परीक्षित किए गए। कवक विलग WDP2 के संवर्द्धन के द्रव में अधिकतम वाह्यकोशिकीय लैक्केज क्रियाशीलता (१५६.७६३ यू/मिली), १५ से १८ दिन के ऊष्मायन अवधि में पाई गई। तथापि, उत्पादन परिस्थितियों के अनुकूलन के बाद यह क्रियाशीलता १८६.२६ यू/मिली, घटे हुई ऊष्मायन अवधि (१२ दिन) में पाई गयी। जैविक लिग्निन विलगन हेतु सशक्त कवक विलग WDP2 द्वारा पूर्ण कोशिका पूर्वोपचार दिया गया। अनुकूलित माध्यम से ठोस स्थिति किण्वन विधि द्वारा महत्वपूर्ण लिग्निन विघटन (२६.५% से ८.४%) व सैलुलोज बढ़त (३७.६५% से ६१.५६%), ४२.५ यू/मिली लैक्केस एन्जाइम क्रियाशीलता के साथ पाई गयी। आर एस एम के बॉक्स बैहन्कन रूपरेखा द्वारा जैविक पूर्वोपचारित *बै० बालकोआ* के विकर आधारित शर्करीकरण जैव प्रक्रम का अनूकूलन किया गया। १५ FPU/ग्रा० क्रियाधार, सैलूलोज विकर सान्द्रता, १% क्रियाधार (कवक पूर्वोपचारित *बै० बालकोआ* चूर्ण) सान्द्रता, व ४०°से० ऊष्मायन ताप पर अधिकतम प्रतिशत शर्करीकरण (४६.२५५%) पाया गया। १८.४ यू/मिली FPU/ ग्रा० क्रियाधार, १% क्रियाधार सान्द्रता व ३६.४६°से० ऊष्मायन तापमान, अधिकतम प्रतिशत शर्करीकरण के लिये अंतिम व निर्णायक अनुकूलित परिस्थितियाँ पायी गयीं। पूर्वोपचारित व शर्करीकृत बांस चूर्ण में रासायनिक व संरचनात्मक बदलाव का अध्ययन पराबैंगनी-दृश्य स्पेक्ट्रोफोटोमीटरी, एफटीआईआर स्पेक्ट्रोस्कोपी व इलैक्ट्रॉन सूक्ष्मदर्शी विश्लेषण द्वारा किया गया। कवक विलग संरचनात्मक, जैव रासायनिक व आण्विक गुणों के आधार पर चारित्रिक किये गये। ५.८ S ITS r-RNA जीन अनुक्रम विश्लेषण के आधार पर कवक विलग WDP2 *लैन्जाइटस एलेगेन्स* पहचाना गया व कवक विलग WFPn1 *गैनोडर्मा ल्यूसिडम* के साथ सर्वाधिक पहचान दिखाया हुआ पाया गया।

डा० लक्ष्मी तिवारी
(सलाहकार)

राज कुमार पाण्डेय
(लेखक)

Int.J.Curr.Microbiol.App.Sci (2016) *5*(5): 581-592

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 5 (2016) pp. 581-592**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.505.059

Exploration of Plant-Biomass Degrading Fungi for In Vitro Mycoremediation of Toxic Synthetic Dyes

Raj Kumar Pandey[1*], Babita Rana[1], Salil Tewari[2], Anwesa Sarkar[3], Ashutosh Dubey[4], Dinesh Chandra[5] and Lakshmi Tewari[1]

[1]Department of Microbiology, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India
[2]Department of Genetics and Plant Breeding, College of Agriculture, G.B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India
[3]Department of Post Harvest Process and Food Engineering, College of Technology, G.B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India
[4]Department of Biochemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India
[5]Department of Biological Sciences, G. B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India
**Corresponding author*

Keywords

Plant-Biomass, Mycoremediation, Toxic Synthetic Dyes, *Ganoderma* sps, congo red and brilliant green.

Article Info

Accepted:
18 April 2016
Available Online:
10 May 2016

A B S T R A C T

The use of extracellular enzyme systems from wood decaying fungi are now growing very fast for bioremediation of synthetic and toxic dyes from environment. In view of this, present study was undertaken for decolorization of two synthetic industrial viz. congo red and brilliant green using wood rot fungal cultures. Fifty five wood rotting fungal cultures were tested qualitatively for production of extracellular lignolytic enzymes. Selected cultures were used for testing the in vitro dye removal potential in dye containing broth. Samples were withdrawn periodically and percent decolorization was calculated. The fungal cultures removed the dye from the media either by accumulating it in mycelia (bio-sorption) or by metabolizing it to some non-coloured components. The cultures varied in their dye decolorizing potential, showing 50.21-97.37% decolorization of brilliant green within 24 d. All the selected cultures showed complete bio-sorption of congo red dye within one month. The efficient strains were further selected for the production of various enzymes involved in the dye decolorization and crude enzyme activities in culture supernatantes were calculated. In all the cases, maximum extracellular laccase, lignin peroxidases and Mn dependent peroxidase activities were observed within 15 to 18 d of incubation in culture supernatant. Light microscopy and phase contrast microscopy clearly revealed bio-sorption of the dye by fungal cultures in the photomicrographs. Among various cultures tested, the potential isolate showing maximum dye decolorizing/ bioabsorbing ability identified as *Ganoderma* sp.

Introduction

Various synthetic and toxic dyes and pigments are used in many industries such as textile, cosmetic, printing, drug, and food-processing (Mohan *et al.*, 2002). These synthetic compounds are very harmful due to their mutagenic/ carcinogenic nature and thus belong to the most dangerous pollutants. These dyes can be released into our environment as effluents from many synthesis plants and factories/ industries (Elizabeth *et al.*, 1998). Most of these pollutants are very stable, even at extreme conditions as high temperatures, as well as to microbial attack, making them recalcitrant and tough to degrade (Pagga and Brown, 1986). The major drawback is also that sometime these compounds can be transformed to more carcinogenic compounds under anaeobic conditions (Kulkarni *et al.*, 1985; Bumpus & Brock 1988).

Various physicochemical methods, such as adsorption, electrocoagulation, precipitation, flocculation, ion exchange, membrane filtration, ozonation, etc. have been used for decolorization of these harmful carcinogens, however, these methods possess some limitations such as high cost, formation of hazardous by-products, and intensive energy requirements (Brown *et al.*, 1981). On the other hand, biological processes provide a low-cost, environmentally benign, and efficient alternative for the treatment of dye wastewater (Ali *et al.*, 2009). Currently, a lot of studies have focused on wood rot fungi that seem to be more prospective organisms because of their unique lignolytic, oxidoreductive enzyme systems. These fungi are capable of degrading many xenobiotic compounds including various types of dye such as azo, anthraquinone, reactive, and triphenylmethane dyes (Dey *et al.*, 1994). The two methods of the bioremediation of these pollutants are biosorption, involves the entrapment of dyes in the matrix of the adsorbent (microbial biomass) without destruction of the pollutant; whereas other is biodegradation i.e. original dye structure is fragmented into smaller compounds resulting in the decolorization or many times detoxification of synthetic dyes (Singh & Arora, 2011). Over the past few decades, numerous microorganisms have been isolated and characterized for degradation of various synthetic dyes, but there is a dearth of information regarding the complete and proper degradation and detoxification of these dyes by microbial systems despite their increased use by the textile industry. Hence, the selection of potent microbial system that have the capability for degradation and detoxification of these dyes of interest is very important from biotechnological aspect of dye effluent treatment. In view of above, present study was undertaken for the selection of potential wood rot fungal gene pool for decolorization of synthetic and toxic dyes such as congo red, and brilliant green using wood rot fungal cultures. Various wood rot fungi were isolated and checked for their dye decolorization ability on the basis of the presence of dye decolorizing unique enzymes namely laccase (Lac), lignin peroxidise (LiP) and Mn dependent peroxidase (MnP). The laccase enzyme that was shown to be involved in decolorization reaction was quantified along with the LiP and MnP.

Material and Methods

Chemicals

Veratryl alcohol, and 2,2-azinobis(3-ethylbenzthiazoline-6- sulfonic acid) (ABTS), were purchaged from HiMedia Mumbai, India. The triarylmethane dye

(brilliant green) and di-azo dye congo red used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). The chemical structures and characteristics of the dyes used are depicted in Table 1. The stock solutions of each dye were prepared by membrane filtration. All other chemicals used were of analytical grade.

Cultures Used

Various lignocellulolytic fungal cultures used during present investigation, were isolated using fruiting bodies from diverse sources (decaying woods, infected wood and infected trees) collected from different locations of Uttarakhand, India. The standard cultures of plant pathogenic fungi viz. *Ganoderma* sp., *Fusarium oxysporium*, *Rhizoctonia solani*, *Helminthosporium maydis*, and *Alterneria* spp. were obtained from Department of Microbiology, C. B. S. H. and department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture and Technology Pantnagar, U.S. Nagar Uttarakhand.

Isolation and Conservation of Microbial Gene Pool

All the fungal cultures were isolated and maintained on Potato Dextrose Agar (PDA) medium (gL^{-1}; potato peeled 200.0, dextrose 20.0 g, and agar 15.0, pH 5.6±0.2). For isolation of fungi, the small piece from the fruiting body surface sterilized using 70% ethanol, then washed with sterile distilled water and inoculated in triplicates at centre of the potato dextrose agar plates. Pure mycelial growth appeared on the plates were further purified. All the microscopic analyses were done based on LPCB (Lacto phenol cotton blue) staining.

Screening for Production of Lignin Degrading Enzymes

Screening of the cultures for overall lignin modifying activity was done using Lignin Modifying Enzyme Basal Medium (LBM) contained (g/L) KH_2PO_4, 1.0; $C_4H_{12}N_2O_6$, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.01; Yeast Extract, 0.01; $CuSO_4.5H_2O$, 0.001; $Fe_2(SO_4)3$, 0.001; $MnSO_4.H_2O$, 0.001; Agar, 16.0 (Pointing, 1999). LBM was supplemented with 1 ml of separately sterilized 20% glucose solution and 1 ml of aqueous tannic acid solution to each 100 ml of growth medium prepared. The cultures were inoculated on plates containing LBM and observed for growth and zone formation. The qualitative measure of extracellular lignin modifying activity is the presence of brown oxidation zone around the fungal colony. It is reported as the index of relative enzyme activity (I_{LIG}). The following formula was used for calculating the ILIG index.

Screening of the cultures for extracellular laccase activity was done using assay plates contained 15 ml of Potato Dextrose Agar (PDA) media, amended with 0.01% guaiacol (Kiiskinen *et al.*, 2004). Active fungal culture disc was inoculated on agar medium in triplicates. The qualitative measure of extracellular laccase activity observed as presence of brick red zone of oxidized guaiacol around the fungal colony. It is reported as the index of relative enzyme activity (I_{LAC}) and calculated using same formula as mentioned above.

For detection of lignin peroxidase enzyme the fungal mycelial disc inoculated on glucose malt extract salt agar medium contained glucose 2% (w/v); malt extract 2% (w/v); $NaNO_3$ 0.2% (w/v); KH_2PO_4 0.2% (w/v); KCl 0.2% (w/v); $MgSO_4.7H_2O$ 0.1% (w/v); $FeSO_4.7H_2O$ 0.002% (w/v); pH 6.5 (Thiyagarajan *et al.*, 2008). Plate was incubated at 28°C for 3 days and thereafter, 3ml of 1.7mM and 2.5 mM of ABTS and hydrogen peroxide respectively were overlapped on the plate and were kept in dark at 25°C for 5 minutes. Appearance of

clear bluish green zone around the fungus gave an indication of peroxidase production by the fungus. It is reported as the index of relative enzyme activity (I_{PER}) and calculated using same formula as mentioned above.

Dye Decolorization Study

Dye Decolorization on Agar Plate

Dye degradation ability of selected fungal cultures was assayed in low nitrogen basal medium containing (g/L) glucose, 1.0; $CaCl_2$, 1.5; $MgSO_4$, 2.0; KH_2PO_4, 1.5; NH4Cl, 0.15, and 1.6% agar as described by Murugesan *et al.,* 2006. The media were supplemented with textile dyes at the concentration of 100 mg/l. The above medium was poured on petri-dishes and inoculated with mycelial disc and incubated at 300C under dark. Plates were regularly monitored at every 24 h for growth and decolorization activities.

Percent Decolourization of Dyes Using Fungal Cultures

The cultures screened out from the above experiments were used to quantify dye decolorizing potential in vitro. The five active fungal discs were grown in the broth medium containing (g/L) glucose, 1.0; $CaCl_2$, 1.5; $MgSO_4$, 2.0; KH_2PO_4, 1.5; NH_4Cl, 0.15, supplemented with different dyes at the concentration of 100 mg/l. Samples were withdrawn periodically at an interval of 72 h and observed for colour change by measuring optical densities, using BioMate 3S UV-Visible Spectrophotometer (Thermo Scientific). The cultures showing the bio-sorption of the dye were also checked visually and by microscopically. The percent decolorization (%) was calculated using the following formula:

Dye Decolorizing Enzyme Production from Selected Cultures

The selected isolates were further screened for extracellular enzymes- lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) in carbon limited liquid medium which contained (g %): glucose, 0.3; KH_2P0_4, 0.5; NH_4N0_3, 12.5 mM; $MgS0_4.7H_20$, 0.1; tween 20, 0.02; veratryl a1cohol, 1mM; trace metal solution, 0.1% (Packiyam, 2013), at pH 5.0. For the determination of MnP activity, the basal medium was supplemented with MnS04 (0.05%). Growth medium (100 ml) was taken in 500 ml Erlenmeyer flasks and inoculated with the 5 mycellial discs. Samples were removed at regular intervals and crude enzyme collected after centrifugation at 10,000 rpm for 10 min, at 4°C. This cell free supernatant was used as the source for crude enzymes. The crude enzyme from fungal culture PAF5 was prepared from optimized broth medium and subjected to the one dimensional SDS-PAGE for extracellular protein banding patterns.

Enzyme Assays

Culture supernatants were used for the assay of the various lignolytic ernzymes enzymes. Lignin peroxidase (LiP) activity was assayed according to Tien and Kirk, 1988 with some modifications. Briefly it was estimated by measuring the rate of H202-dependent oxidation of veratryl alcohol to veratraldehyde, spectrophotometrically. The standard reaction mixture (2.05 ml) contained 0.8 mM veratryl alcohol in 0.1 M citrate buffer (pH 3.0) and 1 ml of culture supernatant. The reaction was started by the addition of 150 mM H202 and the linear increase in absorbance at 310 nm was monitored for one minute at 30°C. One unit of LiP was defined as 1 umol of

veratraldehyde formed per minute and was expressed as U/ml. MnP activity was by the method of Paszczynski *et al.,* 1988 with some modifications, and measured by monitoring the oxidation of Mn2 + to Mn3 +. The assay solution (3.06 ml) contained 0.1 mM guaiacol and 0.1 mM MnS04 in 0.1 M citrate buffer (pH 5.0) with 1ml of culture filtrate. The reaction was started by 0.1 mM H202 addition. One unit of enzyme activity was defined as the increase in absorbance at 465 nm per minute. The laccase activity was determined according to Niku-Paavola *et al.,* 1990 with some modifications, by monitoring the oxidation of 500 µM ABTS buffered with 50 mM citrate buffer (pH 4.5) at 436 nm. The reaction mixture (3 ml) contained 1 ml of culture filtrate. One unit was defined as 1 µM of ABTS oxidized per minute.

The completely decolorized broth cultures of selected isolates were also checked for the extracellular laccase enzyme activity as described above.

Statistical Analysis

Analysis of variance (ANOVA) was done with Statistical software using the program SPSS and OP Stat. All the statistical experiments were conducted in triplicates, and the results have been reported in terms of critical difference (CD).

Results and Discussion

Selection of Lignolytic Fungal Cultures Based on Relative Enzyme Activity Indices

A number of fungal strains were isolated from fruiting bodies (Figure 1) and other decaying wood samples. Out of fifty five isolates, six isolates showed overall lignin modifying activities. Out of these six isolates, five cultures showed laccase activities while lignin peroxidase activities were observed in only two isolates (Table 2). Thus a total of six isolates were selected on the basis of zone formation (Figure 2) and relative enzyme activity indices (Table 3). The values of relative enzyme activity indices varied from 1.1 to 2.0 for I_{LIG}, from 1.3 to 3.0 for I_{LAC} and from 1.5 to 2.0 for I_{PER}. The maximum value for I_{LIG} (2.0) was shown by the fungal isolate PAF7, whereas maximum relative laccase activity indices (3.0) were found for the isolate PAF5. Only two fungal cultures PAF5 (2.0) and *Ganoderma* sp. (1.6) were found positive for peroxidase relative activity indices (Table 3).

Dye Decolorization Study

Several wood rotting fungi have been reported to possess lignin degrading (ligninolytic) enzymes and hence play an important role in the degradation of Lignocellulosic waste in the ecosystems. These lignin-degrading enzymes have been reported to be not only directly involved in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various synthetic xenobiotic compounds, including dyes (Okino *et al.,* 2000). Therefore, to confirm the fungal dye decolourizing capacity, two synthetic dyes were incubated with the five selected fungal isolates for 21 d on dye containing agar and liquid medium at 28°C during the present study. Out of the two dyes tested, all the fungal cultures were poor to grow very efficiently on both dyes containing agar media (figure 3), thereby showing more resistance towards both dyes. The similar agar plate screening method for determining dye decolourizing potential of wood rot fungi *Ganoderma* sp. has also been performed in previous study (Arulmani *et al.,* 2005). Broth cultures were found better

as compared to solid cultures for decolourization studies because in all the cases although fungal growth was observed in the presence of dyes but decolourization began with the formation of very less intense or negligible decolorized zones. During the liquid cultivation experiments, the batch cultures turned from an initial deep coloration to a lighter colour, eventually becoming colourless in most of the cases, indicating either the dye decolorization or dye adsorption into the fungal mycelia (Figure 4). The extent of dye decolourization by broth cultures was monitored spectrophotometrically. The spectrophotometric quantification results revealed clearly the high decolourization potential of fungal cultures towards brilliant green, and congo red dyes (Table 4).

The degree of maximum percent decolourization of brilliant green dye using various fungal isolates varied from 50.21 (PAF7) to 97.37 (PAF5). Congo red dye was removed efficiently by all selected cultures and percent decolourization of the broth varied from 94.00 (PAF7) to 98.58 (PAF5) within 15 d of incubation. Previously only 40% decolourization of anthraquinone dye, Remazol Brilliant Blue R (RBBR) by *Ganoderma* sp. was reported which could be increased up to 92.4% upon addition of HBT as redox mediator (Murugesan *et al.*, 2006).

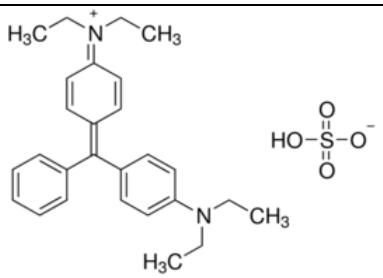
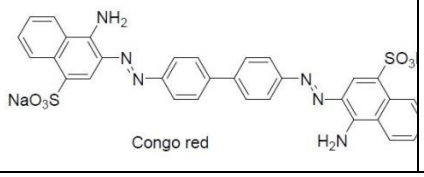
However, contrary to previous findings, we could get much higher decolourization rate for the two dyes congo red, and brilliant green by the fungal isolates used. Thus from the above data, the culture PAF5, showing maximum percent decolourization of brilliant green and congo red was selected as a potential wood rotting fungal culture for further experiments.

Thus on the basis of relative enzyme activity indices and dye decolourization potential, the four fungal isolates viz. PSB1, PAF5, PAF7 and *Ganoderma* sp., were selected for enzyme production and further studies.

Enzyme Assay

The selected cultures were grown in enzyme production liquid medium amended with varatryl alcohol as inducer. Only extracellular laccase enzyme was synthesized maximally with peak laccase activity of 164.63 U ml^{-1} by fungal isolate PAF5 on 18th d of incubation (Figure 5). The LiP and MnP were synthesized in very less amount with peak lignin peroxidase activity of 0.34 U ml^{-1}, was observed with fungal isolate PSB1 within 21 d of incubation; and manganese dependent peroxidase activity of 0.0022 U ml^{-1} also with the fungal culture PAF5 on the 21 d of incubation (data not shown). In this way, the fungal cultures were able to secrete all the three extracellular oxidative and reductive enzymes. The cultures showed maximum ability for synthesizing laccase enzyme while all the cultures had very low LiP and MnP biosynthetic abilities. The enzymatic decolourization by potential fungal isolate PAF5 was also confirmed by checking laccase enzyme activities in completely decolorized (40 d old) fungal culture filtrates grown in presence of Congo red, and brilliant green dyes. The enzyme activity was determined in completely decolourized culture filtrates because due to the intense colour of the dye in medium one could not calculate the activity in dye containing broth. The very low laccase activity in Congo red containing culture filtrate of PAF5 (1U/ml) may be partially correlated with the maximum bio-sorption of the dye by fungal mycelium and partially due to ageing of the culture that might have had lead to the degradation of the enzyme protein. Maximum laccase activity of 28.9 U/ml was observed in completely decolourized culture filtrates (40 d old) of brilliant green dye (figure 6, a).

Table.1 The Structural Formulae, Industrial uses and hazardous effects of the synthetic Dyes decolourized during Present Study*

S.No.	Dye	Chemical Structure	Uses and Hazards
3	Brilliant Green		Related to malachite green, genotoxic and carcinogenic properties induces vomiting when swallowed and is toxic when ingested
2	Congo red		As stain biological samples, in textile industry, carcinogenic in nature.

*Source: Wikipedia Free Encyclopedia

Table.2 Diversification of Fungal Isolates On the Basis of Qualitative Enzyme Assay

S.No.	Enzyme Activity	Total Isolates	Isolates showing positive enzyme activity	Isolates do not showing enzyme activity	Selected Isolates
1	Overall lignin modifying activity	55	6	49	PSB1, PAF1, PAF3, PAF5, PAF7, *Ganoderma* sp.
2	Laccase activity	55	5	50	PSB1, PAF3, PAF5, PAF7, *Ganoderma* sp.
3	Overall peroxidase activity	55	2	53	*Ganoderma* sp., PAF5

Table.3 Relative Overall Lignin Modifying Activity Indices of Selected Fungal Cultures

S.N.	Culture	Relative index (I_{LIG})	Relative index (I_{LAC})	Relative index (I_{PER})
1	PSB1	1.1	2.3	-
2	PAF1	1.2	-	-
3	PAF3	1.4	1.3	-
4	PAF5	1.8	3.0	2.0
5	PAF7	2.0	2.3	-
6	*Ganoderma* sp.	1.4	2.4	1.5
		SEm± 0.041 CD at 5% = 0.137	SEm± 0.029 CD at 5% = 0.10	SEm± 0.01 CD at 5% = 0.15

Table.4 Percent Decolorization of Malachite Green and congo red using selected Lignolytic Fungal Cultures

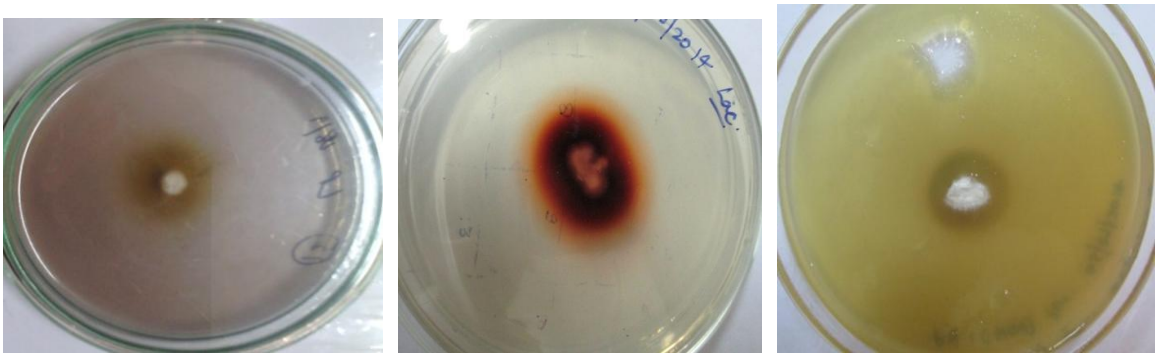
S.No.	Fungal Culture	Percent Decolourization (%)	
		Brilliant Green	Congo Red
1	PSB1	92.66	95.85
2	PAF3	96.35	96.84
3	PAF5	**97.37**	**98.58**
4	PAF7	50.21	94.00
5	*Ganoderma* sp.	96.59	96.89
		SEm± 0.155 CD at 5%= 0.505	SEm± 0.142 CD at 5% = 0.464

Fig.5 Fruiting bodies of few wood rot fungi selected for the study PAF3 (a), PAF5 (b), and PSB1 (c).



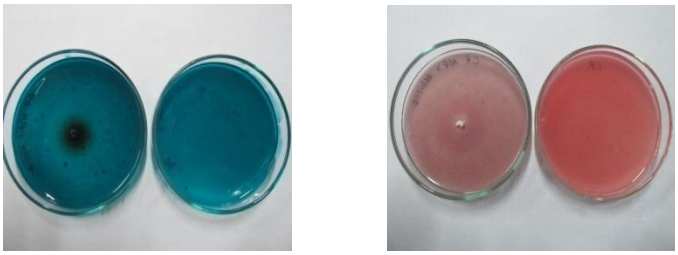
A B C

Fig.6 Zone formation around the colony by selected isolate for various enzyme activities viz., overall lignin modifying activity by (a), laccase activity (b) and lignin peroxidase acitivity (c) using fungal culture PAF5.



A B C

Fig.7 Qualitative detection of the dye decolorizing potential of the fungal isolates on agar plate. Brilliant green decolorization by *Ganoderma* sp. (a), and congo red decolorization by PAF5 (b).



A B

Fig.8 Dye decolorization and bio-absorption by fungal cultures. Bio-absorption of congo red by PAF5 (a), decolorization of brilliant green by *Ganoderma* sp (b), decolorization of brilliant green by PAF5 (c), phase contrast microscopic view of the fungal mycelia showing the attachment/ accumulation of the dye congo red by fungal culture PAF5 (d).



A B C D

Fig.9 Laccase production during growth of fungi. The fungal cultures were grown in standard conditions in defined medium (pH 6) at 30°C. Maximum enzyme activity was observed in culture filtrate of fungal culture PAF5.

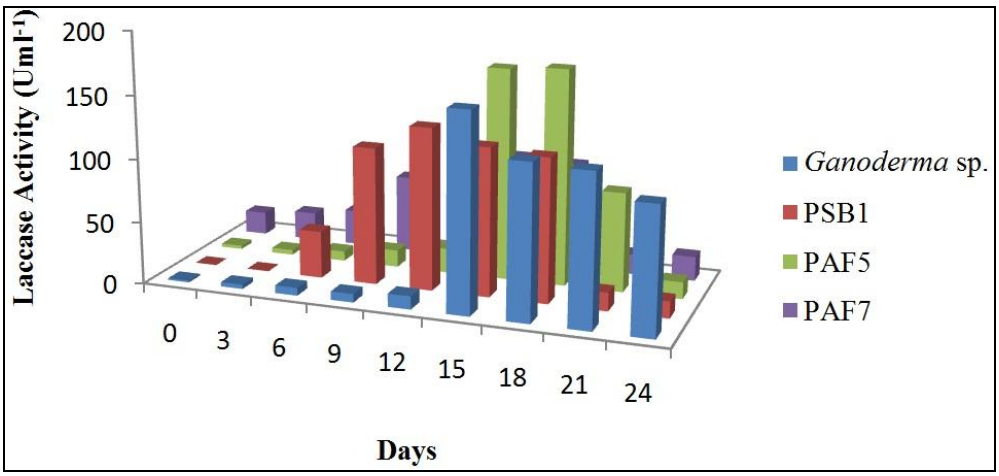
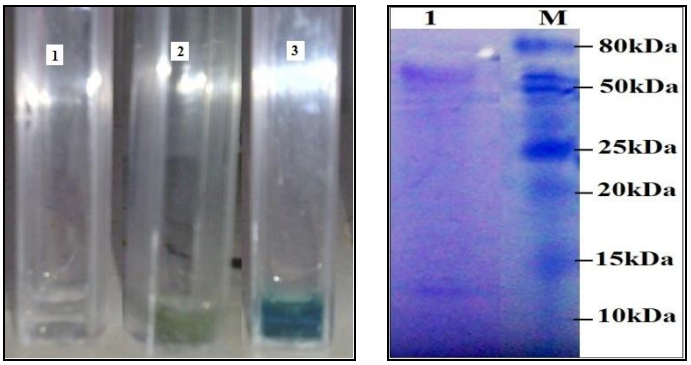


Fig.10 Laccase enzyme activity in brilliant green containing decolourized broth by fungal culture PAF5 (a), colour produced after addition of 500 µM ABTS within 0 min. (a1) within 1 min. (a2) and within 2 min. (a3); Extracellular protein profiling of potential culture PAF5 (b1). M is broad range (10-230 kDa) pre-stained protein ladder (New England BioLabs).



A **B**

This induction of enzymes may be correlated with their involvement in dye decolourization process. Significant roles of Lignin peroxidase, Mn-dependent peroxidase, and laccase in dye degradation by wood rotting fungi have been well documented (McMullan *et al.,* 2001). Further, among these three enzymes, direct involvement of laccase in decolourization of synthetic dyes has also demonstrated by previous workers (Novotny *et al.,* 2004).

Thus, our results are in agreement with the previous findings that have also revealed the vital role shown by laccase enzyme in bioremediation of synthetic toxic dyes. In SDS-PAGE, Variable banding pattern of extracellular proteins were observed for PAF5 using laccase production medium. The molecular weight of extracellular proteins varied from 10 kDa to 80kDa and a total of five Coomassie Brilliant Blue G (Hi Media) stained bands were observed (Figure 6, b). The results showed the production of laccase and other related enzymes, as the same size range is also reported for laccase in various studies (Edens *et al.,* 1999; Imran *et al.,* 2012).

The most potential fungal isolate, PAF5, was selected for further studies based on its maximum ability to synthesize laccase enzyme and dye decolourizing ability.

Identification of Potent Isolate

The most potential isolate, PAF5 was selected for further study based on its maximum zone of laccase enzyme production, maximum enzyme production in broth media, and maximum ability to decolorize the industrial dyes. The culture was identified as *Ganoderma* sp. based on phenotypic characteristics and the molecular characterization of the isolate is under process.

It may be concluded from the present investigation that mycoremediation employing wood rotting fungi has a vast potential for decolourization or removal of toxic carcinogenic synthetic dyes from the industrial effluents. Moreover, biological removal of dye from industrial effluents may be more economic and ecofriendly approach. It is also apparent from the study that most of the lignolytic fungi are having very high biosorption potential towards the synthetic dyes such as congo red, indicating its efficiency for utilization in effluent treatment processes in future.

References

Ali, H., Ahmad, W., Haq, T. 2009. Decolorization and degradation of malachite green by *Aspergillus flavus* and *Alternaria solani*, *African J. Biotechnol.,* 8: 1574–1576.

Arulmani, M., Murugesan, K., Arumugam, P., Dhandapani, R., Kalaichelvan P.T. 2005. Decolorization of basic dyes methyl violet and emerald green by Pleurotus sajorcaju and its effluent decolorization activity, *Indian J. Appl. Microbiol.,* 1: 47–52.

Brown, D.H., Hitz, H.R., Schafer, L. 1981. The assessment of the possible inhibitory effect of dyestuffs on aerobic wastewater bacteria. Experience with a screening test. *Chemosphere*, 10:245– 261.

Bumpus, J.A., Brock, B.J. 1988. Biodegradation of crystal violet by white rot fungus *Phanerochaete chrysosporium*, *Appl. Environ. Microbiol.*, 54: 1143–1150.

Dey, S., Maiti, T.K., Bhattacharyya, B.C. 1994. Production of some extracellular enzymes by a lignin peroxidase-producing brown rot fungus, *Pleurotus ostreiformis*, and its comparative abilities for lignin degradation and dye decolorization, *Appl. Environ. Microbiol.,* 60: 4216–4218.

Edens, W.A., Goins, T.Q., Dooley, D., Henson, J.M. 1999. Purification and characterization of a secreted laccase of *Gaeumannomyces gramminis* var. tritici. *Appl. Environ. Microbiol.,* 65: 3071–3074.

Elizabeth, R., Michael A.P., Rafael, V-D. 1998. Industrial Dye Decolorization by Laccases from Ligninolytic Fungi, *Curr. Microbiol.*, 38: 27–32.

Imran, M., Asad, M.J., Hadri, S.H., Mehmood, M. 2012. Production and industrial applications of laccase enzyme. *J. Cell and Mol. Biol.,* 10(1): 1-11.

Kiiskinen, L.L., Ratto, M., Kruus, K. 2004. Screening for novel laccase-producing microbes, *J. Appl. Microbiol.,* 97: 640–646.

Kulkarni S.V., Blackwell, C.D., Blackard, A.L., Stackhocese, C.W., Alexander, M.W. 1985. Textile dyes and dyeing equipment, classifi- cation, properties and environmental aspects. U.S. Environmental Protection Agency, Research Triangle Park, NC. EPA-600/2-85/ 010.

McMullan, G., Meehan, C., Conneely, A., Kirby, N., Robinson, T., Nigam, P., Banat, I.M., Marchant, R., Smyth, W.F. 2001. Microbial decolourization and degradation of textile dyes, *Appl. Microbiol. Biotechnol.,* 56: 81-87.

Mohan, S.V., Rao, N.C., Srinivas, S., Prasad, K.K., Karthikeyan, J. 2002. Treatment of simulated Reactive Yellow 22 (azo) dye effluents using Spirogyra species, *Waste Management*, 22: 575-582.

Murugesan, K., Nam, I., Kim, Y., Chang, Y. 2006. Decolourization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture, *Enzyme and Microbial Technol.,* 40: 1662-1672.

Niku-Paavola, M.L., Karhunen, E., Kantelinen, A., Viikari, L., Lundell, T., Hatakka, A. 1990. The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus Phlebia radiate, *J. Biotechnol.*, 13: 211–221.

Novotny, C., Svobodova, K., Erbanova, P., Cajthaml, T., Kasinath, A., Lang, E., Sasek, V. 2004. Lignolytic fungi in bioremediation: extracellular enzyme production and degradation rate, *Soil biol. Biochem.,* 36: 1545-1551.

Okino, L.K., Machado, K.M.G., Fabris, C., Bononi, V.L.R. 2000. Ligninolytic activity of tropical rainforest basidiomycetes. *World J. Microbiol. Biotechnol.*, 16: 889-893.

Packiyam, E.J.E. 2013. Production, Purification, and Partial Characterization of laccase produced from *Pleurotus florida* and its potential applications. Thesis. Doctor of Phylosophy. Bharathiar University Coimbatore.

Pagga, U., Brown, D.H. 1986. The degradation of dyestuffs, part II: Behaviour of the dyestuffs in aerobic biodegradation test, *Chemosphere*, 15: 479–491.

Paszczynski, A., Crawford, R.L., Huynh, V. 1988. Manganese peroxidase of *Phanerochaete chrysosporium*: purification, *Methods in Enzymol.*, 161: 264-270.

Pointing, S.B. 1999. Qualitative methods for the determination of ligno-cellulolytic enzyme production by tropical fungi, *Fungal Diversity*, 2: 17-33.

Singh, K., Arora, S. 2011. Removal of synthetic textile dyes from wastewaters: a critical review on present treatment technologies, *Critical Rev. Environ. Sci. Technol.*, 41: 807–878.

Thiyagarajan, A., Saravanakumar, K., Kaviyarasan, V. 2008. Optimization of extracellular peroxidsae production from Coprinus sp., *Indian J. Sci. Technol.*, 1: 1-5.

Tien, M., Kirk, K. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*, *Methods in Enzymol.*, 161: 238-249.

How to cite this article:

Raj Kumar Pandey, Babita Rana, Salil Tewari, Anwesa Sarkar, Ashutosh Dubey, Dinesh Chandra and Lakshmi Tewari. 2016. Exploration of Plant-Biomass Degrading Fungi for In Vitro Mycoremediation of Toxic Synthetic Dyes. *Int.J.Curr.Microbiol.App.Sci.* 5(5): 581-592. doi: http://dx.doi.org/10.20546/ijcmas.2016.505.059

Other Achievements:

A. Research papers/ short communications in national/international journals

1. **Pandey, R. K.,** Rana, B., Tewari, S., Sarkar, A., Dubey, A., Chandra, D., & Tewari, L. 2016. Exploration of Plant-Biomass Degrading Fungi for In Vitro Mycoremediation of Toxic Synthetic Dyes. *International Journal of Current Microbiology and Applied Sciences*, *5*(5), 581-592.
2. Sakia, S., **Pandey, R. K**., & Tewari, L.. 2015. Phosphate solubilising potential of fungal isolates from bamboo rhizosphere. *International Journal of Basic and Applied Agricultural Research*, *13*(3), 492-496.
3. **Pandey, R. K**., Bisht, S., Agrawal, S., Tewari, S., & Tewari, L. 2017. Role of lignolytic fungal enzymes in removal of industrially important carcinogenic dyes. *International Journal of Basic and Applied Agricultural Research*. [Paper accepted].

B. Review articles in national/international journals

1. **Pandey, R. K**., Barh, A., Chandra, D., Chandra, S. Pandey, V., Pankaj & Tewari, L. 2016. Biotechnological Applications of Hyperthermophilic Enzymes. *International Journal of Current Research and Academic Reviews*. 4(3): 39-47.
2. **Pandey, R. K**. & Tewari, L. 2015. Microbial Enzymes involved in starch Processing Industries. *Scholarly Journal of Biological Science*. 4(1), pp. 1-3.
3. Chandra, S., Chandra, D., Barh, A., **Pandey, R. K**., & Sharma, I. P. 2017. Bryophytes: Hoard of remedies, an ethno-medicinal review. *Journal of traditional and complementary medicine*, *7*(1), 94-98.
4. Barh, A., Singh, S., Chandra, D., Pankaj, **Pandey, R. K**., Chandra, S., & Singh, N. K.. 2015. Enhanced Bioremediation Techniques for Agricultural Soils. *International Journal of Current Research and Academic Reviews*. 3 (7): 166-173.
5. **Pandey, R. K.,** Rana, A., Sharma, P., Pathak, R. K., Rana, M., & Tewari, L. 2016. Thriving at High Temperatures: Molecular Adaptations in Hyperthermophic Microorganisms. *International Journal of Current Research and Academic Reviews*. 2016; 4(8): 45-51.
6. Kumar, S., Srivastva, R. K., **Pandey, R. K.,** Jadhav, R, Kumar, A. 2017. Technological Intervension in Waste-Water Treatment by using Nanomaterials. *Research Journal of Chemical and Environmental Sciences*. 5 (4).

C. Research papers in conference proceedings

1. **Pandey, R. K**., Taj, G., Tewari, L. 2015. Docking Analysis of Ligninases With Monolignols to Find Most Effective Enzyme System For Degradation of Lignin.

Proceeding of the 1st National Conference on Recent Advances in Sciences & Technology. Seemant Institute of Technology, Pithoragarh. p227-235.

2. **Pandey, R. K**., Tewari, S., Dubey, A., Tewari, L. 2016. Optimization of Laccase Production for Biological Delignification of Bamboo Biomass for Sustainable Bioethanol Production. Symposium on Topical developments in Biochemistry and allied sciences. PE-7.
3. **Pandey, R. K**., Kothyari, Y. Singh, S. & Tewari, L. 2016. Use of Molecular Docking Analysis for Lignin Biodegradation by using Peroxidase Enzyme System. Proceeding of the 2nd National Conference on Recent Advances in Sciences & Technology. Seemant Institute of Technology , Pithoragarh. p170.

D. Awards/Prizes Received

1. **Young Scientist award** for best poster presentation in three days National Conference on Science of OMICS for Agricultural Productivity: Future Perspectives, held at GBPUAT Pantnagar, Uttarakhand from March 4-6, 2014.
2. **First Prize for Best poster** in Workshop on Advances in Biotechnology and its Applications in Conservation Biology, organized by Dolphin (P.G.) Institute of Biomedical and Natural Sciences Dehradun, Uttarakhand during Oct 16-17, 2014.
3. **First Prize for Best poster** entitled in National Seminar on "Holistic Development of Agroforestry: Potential and Policy Issues" at GBPUAT Pantnagar, Uttarakhand, during Feb. 13-14, 2015.
4. **First Prize for the Paper Presentation** in Symposium on Topical developments in Biochemistry and Allied Sciences, held during 7th May, 2016, at GBPUAT Pantnagar, Uttarakhand.
5. **Environmental Microbiologist of the Year award 2017** In World Clean Environment Congress Organised by Scientific and Environment Research Institute Kolkata at India International Centre, New Delhi on June 05, 2017.

Other

1. Workshops /Lectures Attended: 15
2. Presentations 09
3. lectures 02
4. Trainings 05
5. Extra-Curricular Activities
6. Life membership of All India Agricultural Students Association (AIASA)