OPTIMIZATION OF LIGNOCELLULOLYTIC ENZYME COCKTAIL PRODUCTION FROM *COTYLIDIA PANNOSA* AND ITS APPLICATION IN BIOETHANOL PRODUCTION AND DYE DECOLORIZATION

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By

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CERTIFICATE

This is to certify that the thesis entitled, **"Optimization of lignocellulolytic enzyme cocktail production from *Cotylidia pannosa* and its application in bioethanol production and dye decolorization"** which is being submitted by **Deepika Sharma (Enrollment No. 106553)** in fulfillment for the award of degree of **Doctor of Philosophy** in **Biotechnology** at **Jaypee University of Information Technology, Waknaghat, India** is the record of candidate's own work carried out by her under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Title: Optimization of lignocellulolytic enzyme cocktail production from *Cotylidia pannosa* and its application in bioethanol production and dye decolorization

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ABSTRACT

A successful biomass-to-biofuel bioprocessing requires the efficient hydrolysis and saccharification of biomass followed by efficient conversion of hydrolysates into biofuel. The bioprocessing largely depends on the inherent recalcitrant characteristics of biomass and the repositories of the enzymes used. The low efficiencies and higher cost of existing enzymes for this bioconversion is still a bottleneck in the technology. In this context, the bioprospecting activities can aid in tapping vast sources of microorganisms with superior and efficient key enzymes. Therefore, the present investigation was aimed at evaluation of different fungal cultures for their lignocellulolytic potential. Subjecting to qualitative and quantitative screening assays for nineteen different fungal strains, the fungal strain F6, identified as *Cotylidia pannosa* (*C. pannosa*), was recognized as the most efficient producer of all three lignocellulolytic enzymes. Followed by initial screening, the lignocellulolytic enzyme activities were further optimized to identify process parameters that supported maximum enzyme activities. Using one factor at a time approach, cellulase, xylanase and laccase activity of 8.44 U/mL, 6.74 U/mL and 10.0 U/mL, respectively were obtained using wheat bran (2%) as substrate when the fermentation was conducted at a pH of 5.0, for 56 to 72 hrs at a temperature of 30°C under submerged fermentation. The optimization of enzyme activities was also conducted using solid state fermentation which resulted in cellulase, xylanase and laccase activity of 4.33 U/mL, 5.23 U/mL and 7.1 U/mL respectively using the parameters as described for submerged fermentation. Based on the results of one factor at a time approach, the lignocellulolytic enzyme production by *C. pannosa* was further optimized under submerged fermentation by a multi-factorial approach involving Response

surface methodology (RSM). As compared to one factor at a time approach, optimization via RSM further enhanced the cellulase, xylanase and laccase activities by 2.3-fold, 2.1-fold, and 1.4-fold respectively. The optimization was followed by preparation and evaluation of crude enzyme cocktail from *C. pannosa*. The characterization of the crude enzyme cocktail revealed the moderately thermostable nature of the enzyme cocktail as it remained stable up to 50°C at an optimum pH of 5.0. The zymogram analysis of the crude enzyme cocktail resulted in detection of multiple cellulase and xylanase isozymes along with a single laccase. The saccharification of wheat bran with *C. pannosa* and precipitated crude enzyme cocktail revealed the higher efficiency of crude enzyme cocktail in terms of shortening the duration of saccharification time by 20 hrs as compared to *C. pannosa*. The fermentation of wheat bran hydrolysate with *Saccharomyces cerevisiae* MTCC 174 resulted in similar levels of ethanol. The fungus and the crude cocktail were also able to decolourize Congo red (CR) dye readily to a maximum extent of 94% within 96 hrs of incubation. The decolorization studies indicated that the mechanism for decolorization by *C. pannosa* consists of a combination of biosorption by fungal mycelia and biodegradation by extracellular laccase with the former being the most efficient mode.

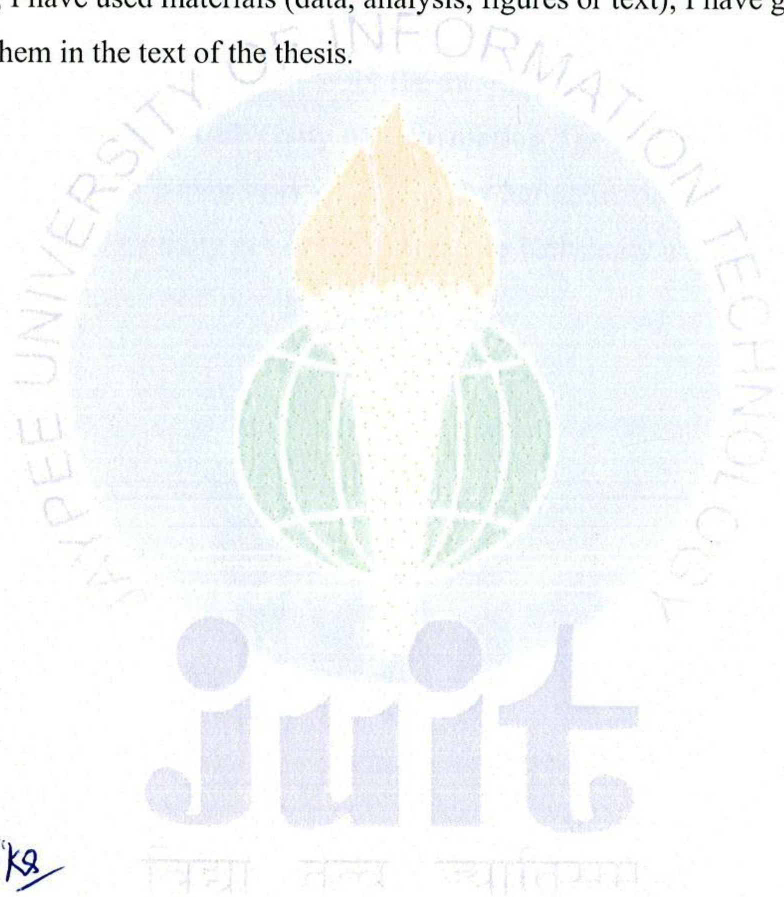
DECLARATION

I certify that:

a. The work contained in this thesis is original and has been done by me under the guidance of my supervisor.

b. The work has not been submitted to any other organization for any degree or diploma.

c. Wherever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.



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LIST OF ABBREVIATIONS

μM	Micro Molar
AAO	Aryl Alcohol Oxidase
ANOVA	Analysis of Variance
ADF	Acid Detergent Fibres
ABTS	2,20´ -Azinobis (3-Ethylbenzothiazoline-6-Sulfonic Acid)
BB	Bromophenol Blue
BGL	β-Glucosidase
BLAST	Basic Local Alignment Search Tool
CBB R-250	Coomassie Brilliant Blue R-250
CBD	Cellulose Binding Domain
CBH	Cellobiohydrolases
CBM	Cellulose Binding Module
CCD	Central Composite Design
CMC	Carboxymethyl Cellulose
CR	Congo Red
CSKHPKV	Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya
CTAB	Cetyl Trimethyl Ammonium Bromide
DE	Decolorization Efficiency
DOE	Design of Experiment
DP	Degree of Polymerization
EC	Enzyme Classification
EDTA	Ethylenediaminetetraacetic Acid
EG	Endo 1,4-β-Glucanases
FPA	Filter Paper Assay
GH	Glycoside Hydrolase
GLOX	Glyoxal Oxidase
IHBT	Institute of Himalayan Bioresource Technology
IMTECH	Institute of Microbial Technology
ITS	Internal Transcribed Spacer
IUPAC	International Union of Pure and Applied Chemists
KDa	Kilo Dalton

L	Litre
LCC	Lignin-carbohydrate complex
LiP	Lignin peroxidase
LPOM	Lytic polysaccharide mono-oxygenases
mg	Milligram
mM	Millimolar
MnP	Manganese Peroxidase
MSW	Municipal Solid Waste
NDF	Neutral Detergent Fibre
NDS	Neutral Detergent Solution
OD	Optical Density
OG	Orange G
OVAT	One Variable at A Time
PAGE	Polyacrylamide Gel Electrophoresis
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PCR	Polymerase Chain Reaction
pNPG	P-Nitrophenyl-β-D-Glucopyranoside
RPM	Revolution Per Minute
RSM	Response Surface Methodology
SETS	Sorbent Energy Transfer System
SDS	Sodium Dodecyl Sulphate
SHF	Separate Hydrolysis and Fermentation
SmF	Submerged Fermentation
SSF	Solid State Fermentation
U/mL	Unit Per Milliliter
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WRF	White-Rot Fungi
YEP	Yeast Extract Peptone

CHAPTER 1

INTRODUCTION

The sustained utilization of fossil fuels to meet the majority of the world's energy demand is endangered by increasing concentrations of CO_2 in the atmosphere and concerns over global warming [1]. Plummeting use of fossil fuels would significantly cut the amount of CO_2 produced along with other pollutants [2]. To reduce the world's dependence on petroleum-derived fuels alternatives are being sought after [3]. The only justifiable alternate to address this issue is to employ carbon based sources that are everlastingly available in enormous amounts, and which can be used without the problem of greenhouse gas emission. Plant biomass is by far the lone carbon source that can fulfill these requirements: it arises by carbon dioxide fixation during photosynthesis, and its dry weight consists mainly of three polymers (cellulose, hemicelluloses, and lignin) whose monomer constituents (hexose and pentose sugars and phenylpropan compounds) can be transformed to useful starting materials for industry by fermentation or biotransformation (the so-called biorefinery concept) [4].

However, an imperative step in the notion of employing plant biomass as "biofuels/biorefineries" is the production of the monomeric components such as hexose and pentose sugars in a sufficiently high concentration by means of technologies that do not release harmful by-products. The only process that can meet this requirement, in theory, is enzymatic hydrolysis which has been studied since the early 1960s. These studies have shown that cellulolytic, hemicellulolytic, and ligninolytic enzymes are predominantly produced by fungi, and some of these fungi have been successfully used for the production of enzymes utilized in the hydrolysis of plant cell wall material [4].

One of the biggest concerns with enzymatic hydrolysis is the price associated with the production of enzymes. The impact of enzyme costs to the economics of lignocellulosic biofuel production continues to be a hot topic for debate [5]. The cost and success of the biomass to bioethanol progression depend mainly on the inherent recalcitrance of biomass and the repertoire of enzymes involved in depolymerization of the constituent polysaccharides [6]. A solution to this problem is to find alternate ways for the production of enzymes which may be achieved by finding more potent microbial strains or by creating genetically modified strains that can excrete greater amounts of enzymes, or both [7].

For the production of lignocellulolytic enzymes, polysaccharides present in lignocellulosic materials (such as agro and forestry residues, herbaceous grasses and woody plants), including cellulose and hemicellulose are of immense importance [6]. Using lignocellulosic raw materials for enzyme production is advantageous. Another additional advantage of using lignocellulosic raw material is that it allows bioethanol production in countries with climatic conditions unsuitable for crops such as sugarcane or corn [8]. This raw material is less expensive as compared to conventional media components for the production of the enzyme. Lignocellulosic complex, the most abundant biopolymer on the earth comprises about 50% of world biomass [9]. Lignocellulose feedstocks which include agricultural and forest residues, industrial and municipal wastes, and dedicated energy crops, on account of their high carbohydrate content, hold remarkable potential for large-scale bioethanol production [10]. Lignocellulosic biomass comprises of cellulose $(C_6H_{10}O_5)_x$, a homopolymer of glucose, in bound form along with hemicelluloses $(C_5H_8O_4)_m$ and lignin $[C_9H_{10}O_{3.}(OCH3)_{0.9-1.7}]_n$. Generally, lignocellulosic biomass contains about 40−60% cellulose, 20−40% hemicelluloses, and 10−25% lignin [11]. The cell wall polysaccharides can be hydrolyzed into monomeric sugars which are used for biorefining to produce a range of biomaterials.

The majority of plant biomass is available in the form pentose and hexose sugars, comprising mainly of cellulose (a glucose homopolymer); followed by hemicelluloses (a sugar hetero-polymer); and least of all lignin (a complex aromatic polymer). Both the cellulose and hemicellulose can be broken down enzymatically into the component sugars which may be then fermented to ethanol [11]. The classical model for degradation of cellulose to glucose involves the cooperative action of endocellulases (EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and beta-glucosidases (EC 3.2.1.21) [12]. Hydrolysis of hemicelluloses is brought about by enzymes like glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, endo-hemicellulases and others, the concerted action of which hydrolyze glycosidic bonds, ester bonds and remove the chain's substituents or side chains. These include endo-1, 4-β-xylanase, β-xylosidase, β-mannanase, β-mannosidase α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase and other enzymes [13].

Cellulose, hemicellulose, and lignin are not just individual units in a plant cell wall but are intimately interlocked making it tough to deconstruct enzymatically [14]. Lignin and carbohydrates (e.g., cellulose and hemicellulose) together form the lignin–carbohydrate complexes [15]. Anchoring of lignin to plant-wall polysaccharides contributes to

recalcitrance [16, 17] by reducing the accessibility of cellulose to enzymes [18]. For a complete deconstruction of these heterogeneous structures in the plant cell wall synergistic reactions of enzymes, such as cellulases, hemicellulases, accessory enzymes and lignin-modifying enzymes is required [19].

Fungi and bacteria both have been profoundly exploited for their ability to hydrolyze lignocellulosic materials by producing a wide variety of cellulases and hemicellulases [20]. To date, the majority of enzymes developed and being tested for lignocellulose degradation are from fungi [21] because of their ability to produce profuse amounts of cellulases and hemicellulases secreted directly into the medium for easy extraction and purification [6]. *Trichoderma reesei* was one of the first cellulolytic organisms isolated in the 1950s. By 1976, a remarkable collection of more than 14,000 fungi showing activity against cellulose and other insoluble fibers had been collected [22]. Various wood-rot fungi like white- and brown-rot, have been reported to effectively degrade lignin, cellulose, and hemicellulose. They produce extracellular enzymes like ligninase, cellulase, and hemicellulase to degrade the lignocellulosic complex [23]. The lignin-degrading enzymes secreted by white-rot fungi enable them to completely mineralize lignin to carbon dioxide and water, in turn exposing the hemicellulose and cellulose in the wood matrix [24] which are hydrolyzed by conglomerates of hemicellulase and cellulase. In contrast to the white-rot delignification process, brown-rot fungi modify the lignin structure in the wood matrix [25] enabling the access of enzymes for holocellulose degradation. Nevertheless, filamentous higher fungi primarily the basidiomycetes, which cause white rot are the major degraders [26]. Lignin modification by soft rot fungi and brown rot fungi is limited, as their growth is generally limited to the outer surfaces of wood. White rot fungi are considered as the major lignin degrader [27]. White-rot basidiomycetes such as *Pleurotus ostreatus, Trametes versicolor, Phanerochaete chrysosporium*, *Ganoderma lucidum*, *Coriolus versicolor* and *Polyporus brumalis,* constitute a crucial source of organisms with lignocellulosic machinery for the production of extracellular ligninolytic (laccase) and hydrolytic (cellulases and hemicellulases) enzymes, which are responsible for the degradation of major substrate components of lignocellulosic biomass into value-added products [28, 29].

The fauna of North-Western Himalayan region of India still needs to be explored extensively and as such, the prospective for discovering novel strains capable of producing hydrolytic enzymes of industrial potential exists [30, 31]. Isolation and screening of microbial strains for the existence of an efficient lignocellulosic enzyme machinery is a routine research being done by bioethanol industries. Research has shown that some white

rot fungi possess cellulase and xylanase activities but are devoid of laccase activity, which plays a crucial role in the pre-treatment step. Dhiman *et al.* (2013) utilized rice straw as a substrate for production of an endoglucanase, cellobiohydrolase, and β-glucosidase in addition to xylanase, laccase, mannanase, and lignin peroxidase from a white rot fungus, *Armillaria gemina* SKU2114 [32]. For the production of lignocellulolytic enzymes, industries generally focus towards the production of engineered commercial enzymes such as Accellerase® Trio™ from Genencor and Cellic CTec3 from Novozymes which act in a synergistic manner to unlock and saccharify polysaccharides contained within the lignocellulose complex to fermentable sugars. The Accellerase® Trio™ is an amalgamation of multiple enzyme activities including exoglucanase, endoglucanase, hemicellulases (including xylanases), and β-glucosidase but lacks ligninolytic activity whereas Cellic CTec3 activity is limited to only cellulosic substrates [33, 34]. Therefore, the need for developing more potent efficient enzyme preparations arises for the enzymatic saccharification process to be more economical. This necessitates the isolation and screening of novel fungi capable of efficient degradation of lignocellulosic biomass by employing a proficient lignocellulolytic enzyme system.

Apart from the role of white rot fungi in bioconversion of lignocellulosic material to bioenergy, this fungal group is an effective bioremediator for toxic textile industrial pollutants because of their dye adsorption and contaminant degradation capabilities due to their efficient enzymatic machinery. Therefore, the biodegradation abilities of fungi are given particular emphasis in management of environment through green route.

The bioconversion of lignocelluloses relies heavily on significant technological innovations focussing on efficient and low-cost enzymes, feedstocks and efficient process design. For attaining these goals, understanding the role of individual, square and interaction effects of process variables on the ultimate output will have a crucial part. Response surface methodology (RSM), which lends the principles from statistical and mathematical fundamentals is a well-practiced approach in biological sectors for getting the effect of individual, square and interaction terms of process variables on the output through developing a non-linear regression equation [35-37]. Furthermore, the response optimizer function of RSM will prove to be helpful in predicting the process variables for getting an optimum output. Several researchers have acknowledged the modeling efficiency of RSM for industrially relevant lignocellulolytic enzyme production by basidiomycetes such as *Phanerochaete chrysosporium* [38], *Lentinula edodes* [39], *Agaricus arvensis* [40]**.** Application of RSM in the development of non-linear regression models and optimization

for multiple enzymes from a cocktail is scarce. Most of the studies have targeted the optimization of production of individual enzymes and/or optimization of degradation of lignocellulosic substrates with commercial enzyme preparations [41].

Understanding the importance and the necessity of identifying novel fungal strains capable of efficient lignocellulosic biomass degradation by engaging their proficient lignocellulolytic enzyme system, the following objectives were laid down for the present study:

Objective 1:

Screening and identification of fungal cultures for lignocellulolytic enzyme activities

Objective 2:

Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged and solid-state fermentation using one factor at a time approach

Objective 3:

Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged fermentation using Response Surface Methodology (Multifactorial approach)

Objective 4:

Characterization of crude enzyme cocktail for lignocellulolytic enzyme activities and its application in saccharification of wheat bran for bioethanol production and dye decolorization

CHAPTER 2

REVIEW OF LITERATURE

2.1 Lignocellulose: A treasured resource

Lignocellulose, a renewable organic material is the major structural component of all plants [42]. It encompasses around half of the plant matter produced by photosynthesis. Approximately 200 billion tons of lignocellulosic biomass per year is generated by terrestrial plants [43]. A minuscule quantity of lignocellulosic material is brought to some use, the majority of it reportedly goes to waste [44] which is disposed of chiefly via combustion [45]. In the last few decades, researchers throughout the globe have investigated the role of biomass and associated waste in energy production [46]. Lignocellulose biomass predominantly consists of three polymers, cellulose, hemicellulose and lignin along with minor amounts of inorganic material [47]. These three polymers are sturdily intermeshed and chemically bonded by noncovalent forces in addition to covalent cross-linkages [48]. Lignocellulosic biomass which includes agricultural and forestry residues along with herbaceous energy crops can act as low-cost feedstocks for production of bioethanol and other value-added commodity chemicals [49]. Therefore, these are referred to as "the second generation" feedstock for fuel and chemical production to distinguish it from "first generation" feedstocks which are edible [50].

2.1.1 Lignocellulosic biomass for fuel and chemical production

It is estimated that by 2035 the global energy demand would shoot up more than one-third, primarily owing to an increase in standard of living in China, India, and the Middle East [51]. At present, 80–88% of the world's energy supplies is met by fossil fuels, which are limited and exhaustible resources and at their current rate of consumption cannot be relied upon as the sole source of energy [52]. The transportation sector has been the leading consumer of fossil fuels, utilizing around 60-65% of it. This amount is undoubtedly going to increase as the world population increases [53]. Increase in demand of fossil fuels with a simultaneous decrease in their abundance along with the increasing environmental hazards associated with them has shifted the focus of energy generation towards biofuel use [42]. The only way to reduce the atmospheric carbon dioxide concentration is by reducing the use

of fossil fuels. The urge to discover suitable, renewable, less polluting and cheap sources of bioenergy [54], identified lignocellulosic biomass as the potential candidate for meeting the world's future energy demands. Lignocellulosic biomass is produced as a waste in large amounts by diverse industries associated with forestry, pulp and paper, agriculture, and food, in addition to municipal solid waste (MSW), and animal wastes [42].

Substantial fruitful efforts have been made for converting these lignocellulosic residues to valuable products such as biofuels, chemicals and animal feed [55]. These waste materials, frequently available at reduced costs, act as second substrates for commercial biofuel production [56] and an ideal replacement for fossil fuels. Ethanol from biomass is obtained by fermentation of carbohydrates present in biomass [6]. First generation biofuels use carbohydrates such as sucrose or starch which can be easily converted to simpler fermentable sugars. In contrast to first generation biofuels, second generation biofuels employ the use of complex lignin-cellulose-hemicelluloses matrix of the biomass to disintegrate carbohydrate polymers to fermentable sugars by hydrolysis. This hydrolysis step is characteristically catalyzed by cocktails of enzymes including cellulases, hemicellulases and other accessory enzymes that target and degrade specific constituents of the cell wall [57].

India, a country with an optimistic viewpoint towards renewable energy technologies is dedicated to the utilization of renewable sources for supplementing its power requirements. It is one of the few nations that has a dedicated ministry for renewable energy which oversee the growth of biofuels along with other renewable energy sources [58]. In the year 2003, Indian government's Planning Commission presented an exhaustive report on biofuel development [59]. The report identified bioethanol and biodiesel as the primary biofuels needed to be developed for the nation. Intricate strategies for endorsing both bio-ethanol and biodiesel were articulated and the time required for enacting the development of biofuels and application of policies were defined. Blending of 10% ethanol and 20% biodiesel in gasoline and petroleum diesel, respectively by 2011-2012 was proposed by the committee [59]. Additionally, a blend of 5% ethanol in petrol was made obligatory in 11 states and three union territories of the nation. The government's decision for mandatory blending caused an increase in demand for fuel-grade ethanol. This necessitates the utilization of other raw materials such as grains or lignocellulosic biomass for fuel ethanol production. With an enormous population to feed and limited availability of land, developing bioethanol technologies based on biomass feedstock not associated with food or feed value is the need of the hour [58].

The primary challenge in converting biomass to bioethanol is attaining yields that make it cost-competitive with the current fossil-based fuels. The initial conversion of biomass into fermentable sugars is a key bottleneck in the process of biofuel production, and innovative biotechnological solutions are required to improve their efficiency, which would lower the overall cost of bioethanol production.

2.2 Structural features of lignocellulose

Lignocellulosic materials are primarily made up of cellulose, hemicellulose, and lignin. In addition to these polymers, plants also contain additional structural polymers like proteins, waxes, etc. [60]. Wood from different species is typically composed of 35–50% cellulose, 20–35% hemicellulose and 10–25% lignin [61]. The Chemical composition of some common lignocellulosic materials is given in Table 2.1.

Table 2.1 Chemical composition of some traditional lignocellulosic biomass

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Corn stover	15	35	8
Rice straw	35	25	12
Wheat straw	30	50	20
Hard wood stem	40-45	24-40	18-25
Softwood stems	45-50	25-35	25-35
Leaves	15-20	80-85	0
Switch grass	45	30	12
Sugarcane bagasse	40	24	25

Source: [20, 55, 62]

Cellulose and hemicellulose are polysaccharides, while lignin is an aromatic polymer synthesized from phenylpropanoid precursors [47]. Plant biomass used for biofuel and other biorefineries generally refers to the use of plant cell wall which constitutes more than 50% of the plant's dry weight [4].

Three layers make up the plant cell wall (a) the “primary cell wall” which is a somewhat thin but unceasingly extending layer produced by growing cells, the “secondary cell wall” which is a dense layer, formed inside the primary cell wall after cell growth termination,

and finally the "middle lamella" which is the outmost layer forming an interface between the secondary walls of adjacent plant cells and holds them together) (Figure 2.1).

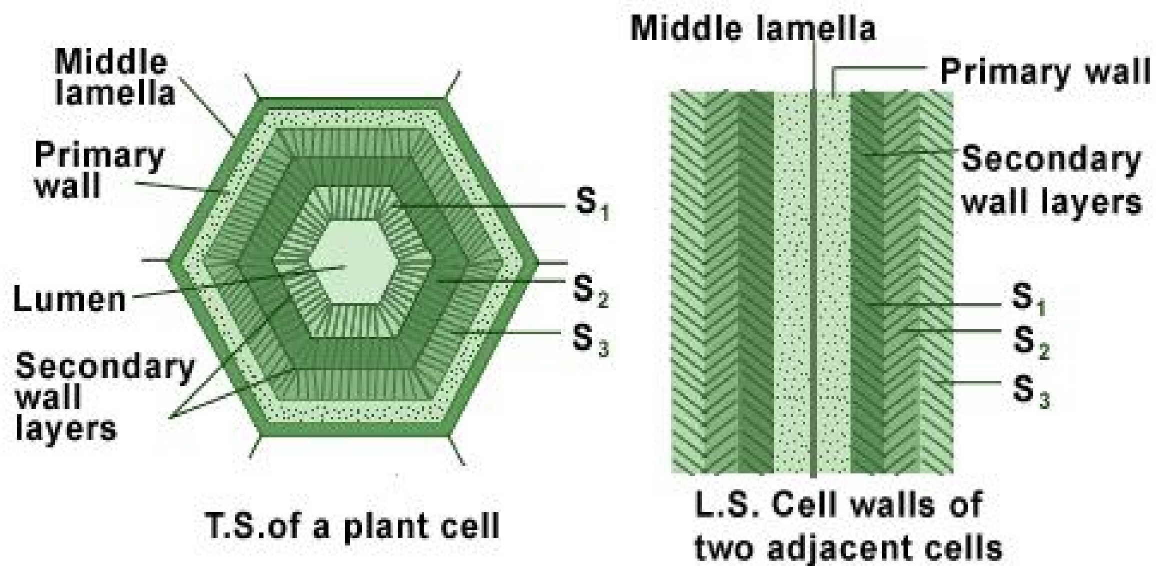


Figure 2.1 Organization of the different layers of the plant cell wall

The polysaccharides, cellulose, hemicellulose, and pectin make up the primary cell wall [63]. Here, the aggregated cellulose (microfibrils) gets covalently linked to hemicellulosic chains, forming a cellulose-hemicellulose network which is entrenched in the pectin matrix. The secondary wall is comprised of cellulose, hemicelluloses (mostly xylan), and lignin. Lignin fills up the spaces between cellulose, hemicellulose, and pectin components of the cell wall.

2.2.1 Cellulose

Cellulose, one of the major components of primary as well as secondary plant cell walls reaches its highest concentration (40%) in the secondary cell walls. Cellulose consists of unbranched, unsubstituted 1,4-β-D-glucan chains (Figure 2.2) that can reach degrees of polymerization of 2,000–6,000 and 2,000–10,000 residues in primary and secondary walls, respectively [4].

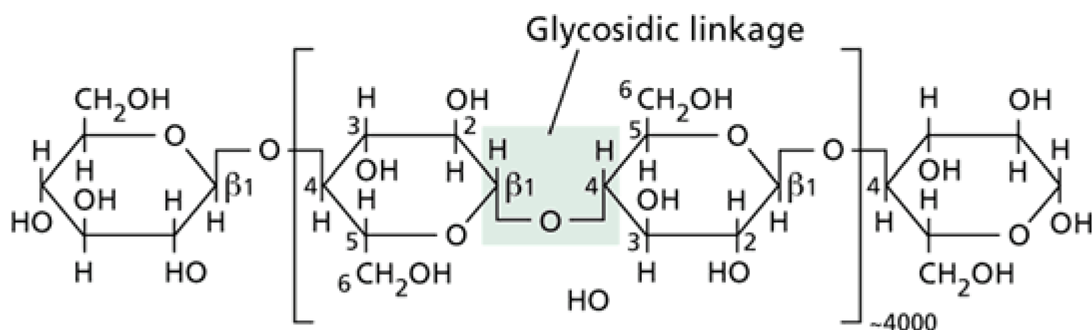


Figure 2.2 The chemical structure of cellulose

The numerous hydroxyl groups on the glucose moieties from one chain form hydrogen bonds with oxygen molecules on the same (intra-molecular) or neighboring chain (inter-molecular), so as to firmly grasp the strings side by side forming microfibrils possessing high tensile strength [64].

2.2.2 Hemicelluloses

Xyloglucan

Primary cell walls of dicots and nongraminaceous monocots predominantly contain hemicellulose in the form of xyloglucan. The Xyloglucan backbone comprises of 1,4-linked β-D-glucose residues, 75% of which are substituted at O6 with either mono-, di-, or tri-glycosyl side chains (Figure 2.3). Xyloglucans, sturdily associated with cellulose enhance the structural integrity of the cell wall [65].

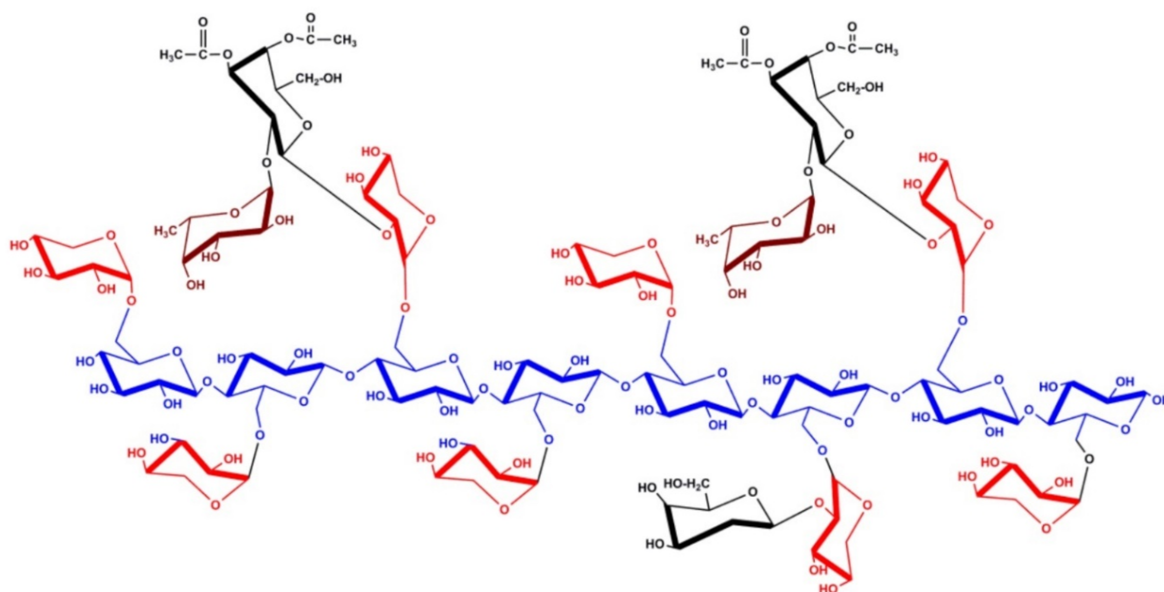


Figure 2.3 Structure of xyloglucan; In blue backbone β-D-glucans; in red α-D-xylose; in black α-D-galactose and in brown α-L-fucose residues

The cell walls of dicotyledons contain xyloglucans partially replaced by glucuronoarabinoxylan, which has a linear β-1,4-linked D-xylopyranosyl backbone with neutral as well as acidic side chains attached at intervals throughout its length. The acidic side chains terminate with glucuronosyl or 4-*O*-methyl glucuronosyl residues, as compared to neutral side chains which terminate with xylosyl and/or arabinosyl residues [66].

Xylan

The predominant hemicellulose polymer in cereals and hardwood is xylan which contains a β-1,4-linked D-xylose backbone, to which several side chains can be attached. This results in the formation of xylan with distinct structures. Both branched as well as linear xylans have been isolated. The xylans of cereals are characterized by the presence of large quantities of L-arabinose and are therefore termed as arabinoxylans. On the other hand, hardwood xylans contain scores of D-glucuronic acid linked to the backbone and are hence named as glucuronoxylans (Figure 2.4) [67].

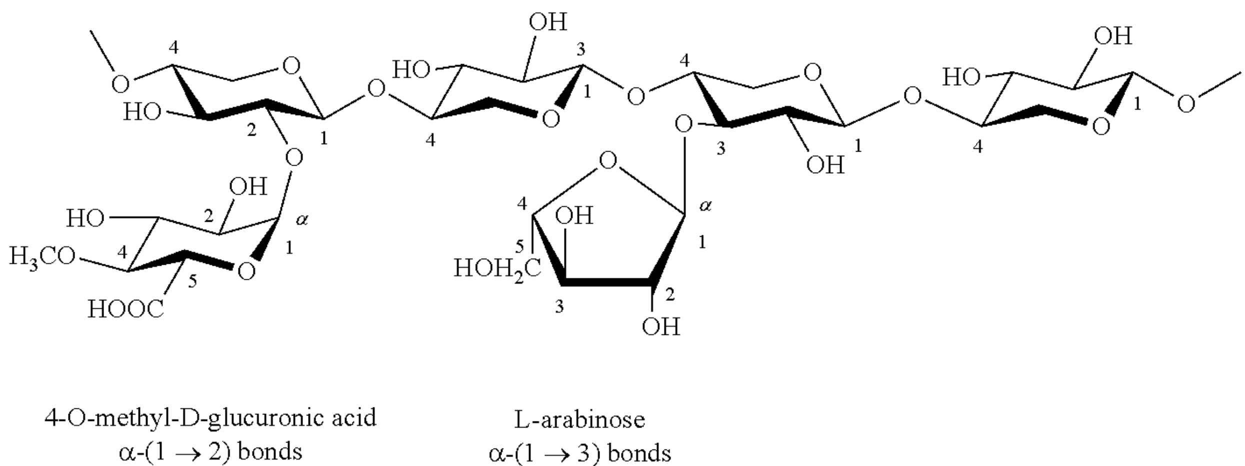


Figure 2.4 Chemical structure of xylan

Galactomannans

A second group of hemicellulolytic structures known as the galactomannans and galactoglucomannans constitutes the main hemicellulosic fraction of the gymnosperms cell walls (12–15%). The backbone consists of β-1,4-linked D-mannose residues, to which D-galactose residues can be attached via α-1,6-linkage (Figure 2.5) in ratios varying from 1:1 to 5:1 depending on the source [68].

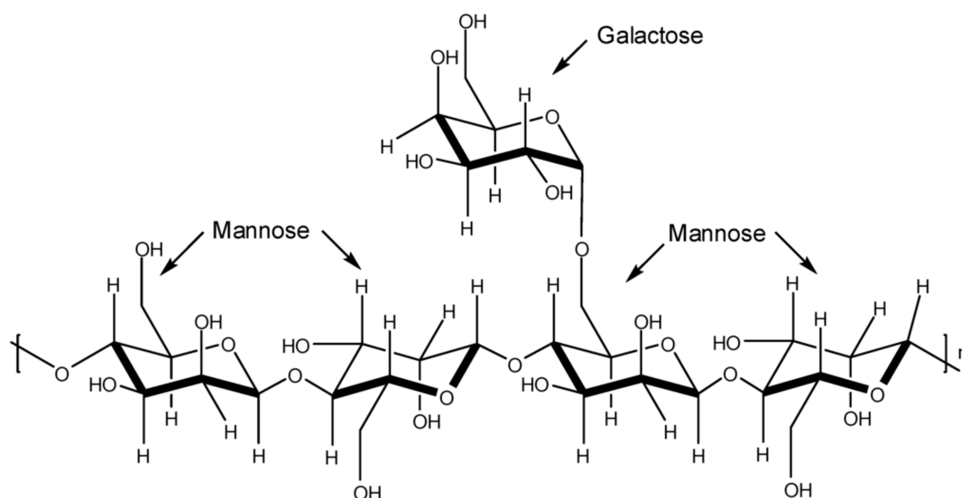


Figure 2.5 Chemical structure of galactomannans

2.2.3 Lignin

Lignin, the third primary heterogeneous polymer in lignocellulosic residues forms a tightly knitted network with both cellulose and hemicellulose polymers providing structural support and rigidity to cell wall [42]. After cellulose, the most abundant carbon source present on earth is lignin with a production of 60 billion metric tons per year [69]. Lignin, present in all vascular plants is characterized by a complex structure derived from the oxidative coupling of three primary hydroxycinnamyl alcohols i.e. *p*-coumaryl, coniferyl, and sinapyl alcohol (Figure 2.6), which makes it extremely resistant to depolymerization.

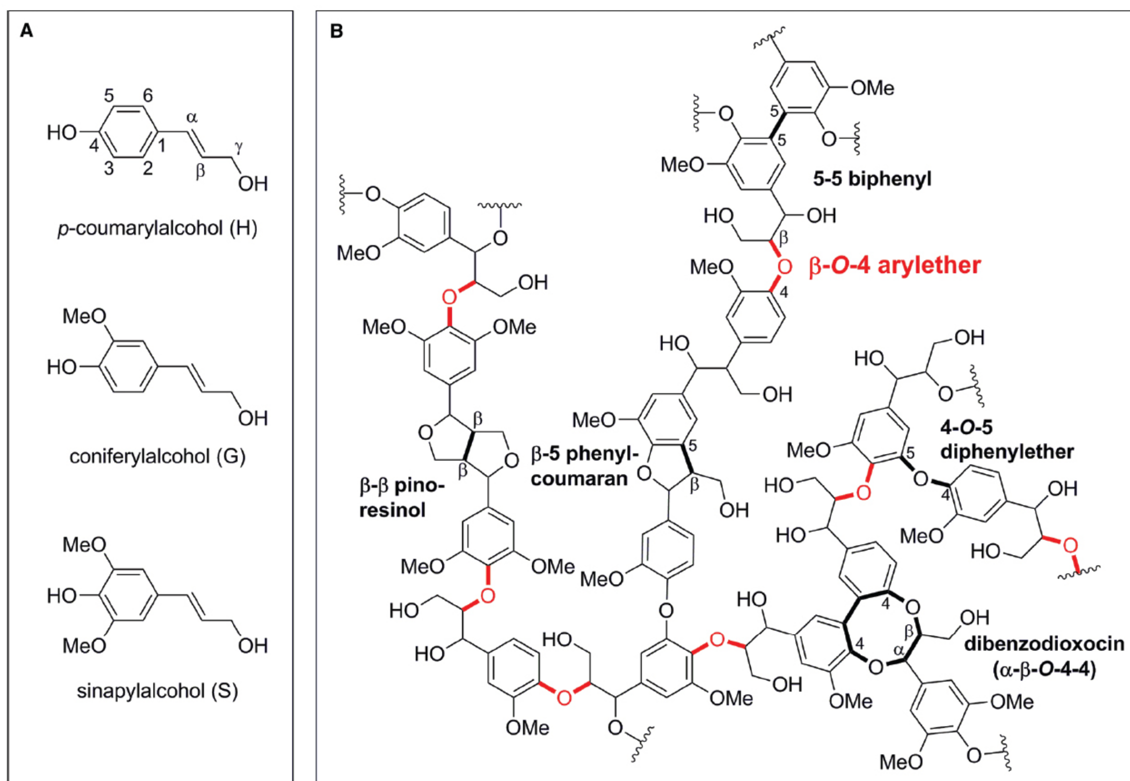


Figure 2.6 Schematic representation of lignin precursors and lignin structure. **(A)** Monolignols used in natural lignin synthesis, **(B)** Representative lignin structure with β-*O*-4 arylether bonds highlighted in red

Interaction of lignin with the cellulose fibrils creates a rigid structure which strengthens the plant cell wall. Lignins are also known to form numerous types of covalent cross-links with hemicelluloses [70]: (a) formation of diferulic acid bridges between lignin and arabinoxylans, pectin polymers, or xylan and lignin, (b) formation of ester linkage between lignin and the glucuronic acid residues in xylan and (c) a protein- and pH-dependent binding of pectin and glucuronoarabinoxylan to xyloglucan in the presence of fucose on the xyloglucan [71]. Unlike cellulose or hemicellulose, the chains present don't contain any repeating subunits, which renders this polymer desperate to enzymatic hydrolysis.

2.3 Lignocellulose pretreatment

Owing to the affordable price and high polysaccharide content, lignocellulosic biomass has emerged as an attractive feedstock. However, lignocellulosic biomass is not directly amenable to the production of bioethanol and other chemicals production [72]. The recalcitrance of lignocellulose plant cell wall to enzymatic attack is a very well established fact. Factors that contribute to this recalcitrance have been identified as the presence of

lignin, hemicellulose, the crystallinity of cellulose, lignin-carbohydrate complex, degree of polymerization, ash content, pore size and surface area. In order to overcome this recalcitrance, the plant biomass is subjected to a technique called as pretreatment. The primary objective of pretreatment is to enhance the substrate's surface area and porosity in addition to reducing the crystallinity of cellulose [73], and disruption of the hydrogen bonds responsible for the heterogeneous structure of cellulosic materials [49]. This renders the lignocellulosic biomass more accessible to enzymatic attack. Investigations pertaining to pretreatment of biomass such as wood, grasses, and corn have reported an upsurge in sugar yield by more than 90% of the theoretical yield [74]. The pretreatment of any lignocellulosic biomass is crucial before it can be subjected to enzymatic hydrolysis. No single method of pretreatment is sufficient enough to remove completely all of the factors contributing to the recalcitrance of various lignocellulosic materials, such as grasses, softwoods, and hardwoods. This is because different lignocellulosic biomasses differ in their physical and chemical properties and, hence, require different approaches to render them accessible to enzymes.

As the enzymatic hydrolysis processes epitomize the key economic costs in lignocellulose bioconversion [5, 75], investigative studies on pretreatment methods for lignocellulosic biomass have been primarily engrossed in the identification, evaluation, development and demonstration of efficient approaches that can sustain the succeeding enzymatic hydrolysis of the treated biomass by utilising lower enzyme dosages and smaller bioconversion time [76].

Over the time, numerous pretreatment technologies have been established and applied to various sources of lignocellulosic plant biomass which (Figure 2.7) can be broadly classified as:

1. Physical pretreatment.
2. Chemical pretreatment.
3. Biological pretreatment

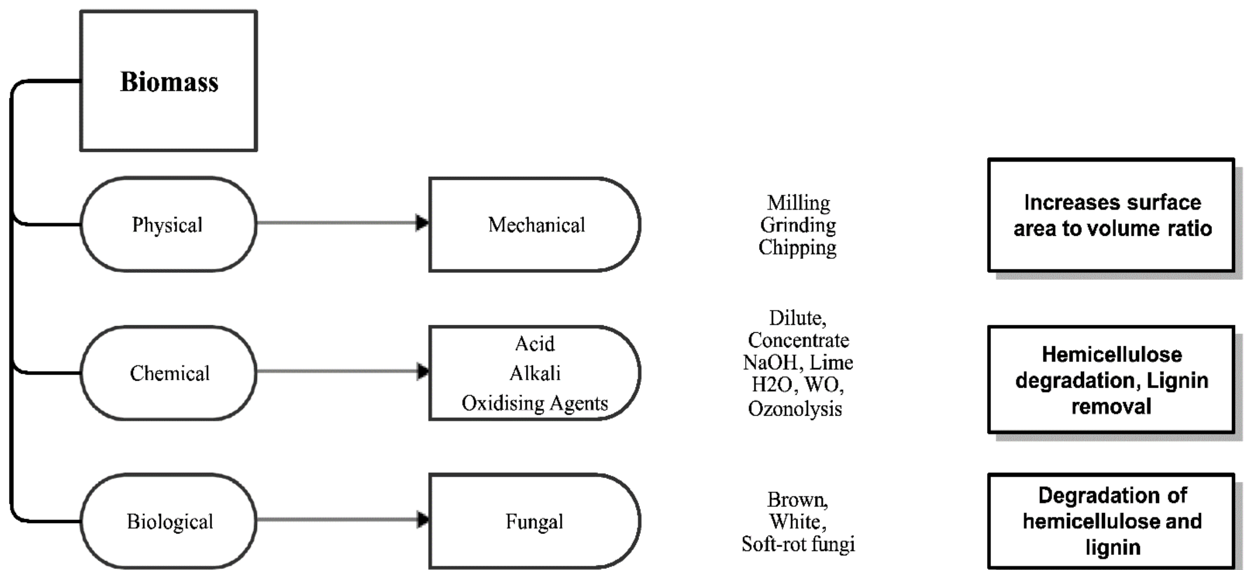


Figure 2.7 The most common pretreatment methods used and their possible effects

Physical pretreatment methods

Physical pretreatment of lignocellulosic biomass is a necessary prerequisite to increasing the surface area to volume ratio, which enhances the mass and heat transfer and renders many other pretreatment methods more efficient. It also increases the vulnerability of the untreated substrate towards enzymatic hydrolysis by decreasing both the degree of polymerization and the crystallinity of cellulose [77].

One of the most frequently used forms of physical pretreatment is mechanical comminution often achieved by a combination of chipping, grinding and milling of the biomass [76]. The particle size of biomass can be reduced to a maximum of 0.2 mm using grinding and milling, although it has been shown that reducing the particle size below 0.4 mm does not have a significant effect on sugar yields from biomass [78].

Another physical pretreatment technique known as the extrusion process is employed for the production of gaseous products and residual char [79]. During extrusion, the lignocellulosic biomass is subjected to a temperature of 300°C trailed by mixing and shearing which causes physical as well as chemical modifications of cellulose.

Chemical pretreatment methods

Chemical methods for biomass pretreatment comprises acidic, alkaline, and oxidative-based pretreatments which increase the accessibility of lignocellulosic biomass for subsequent enzymatic hydrolysis. Although, the highly proficient and less time-consuming nature of

these methods favor their application in lignocellulosic biomass pretreatment, the incurring costs associated with high chemical demand, equipment, and processing have hindered their actual wide-scale use.

Biological pretreatment

Biological pretreatment being an efficient, eco-friendly and cheap technique [80] is a “soft” alternative to the other methods discussed above which require significant inputs of energy and also cause pollution. It uses lignocellulolytic microorganisms for selective degradation of lignin and hemicelluloses. The most efficient microorganisms include filamentous fungi (white-rot basidiomycetes and actinomycetes) which owing to their global presence can be isolated from various sources such as soil, living plants or lignocellulosic waste material [81]. Their higher affinity for lignin allows preferential degradation of the lignin component of lignocellulosic biomass prior to the carbohydrate components [82-84]. Several white-rot fungi such as *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Ceriporia lacerata, Pleurotus ostreaus*, *Cyathus stercolerus,* and *Pycnoporus cinnarbarinus* have been extensively studied for their high delignification efficiency on many lignocellulosic biomasses (Figure 2.8).

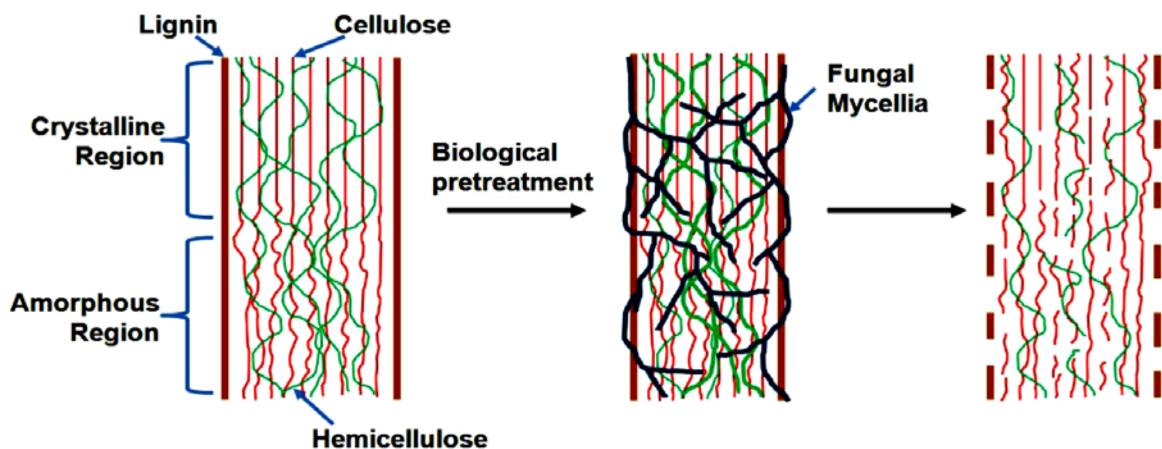


Figure 2.8 Schematic diagram of biological pretreatment of lignocelluloses. White-rot fungi decrease lignin content and alter chemical and physical structures of lignocelluloses that make biodegradation of lignocelluloses more efficient

A pretreatment process can be considered as an ideal process if it circumvents the needs for reducing biomass size, renders the lignocellulosic biomass prone to quick hydrolysis with the subsequent increase in monomeric sugars yields, minimizes the formation of inhibitory

compounds and has highly truncated energy demands with minimum capital and operational cost requirement [85].

One such efficient and economical method for acquiring fermentable sugars is enzymatic hydrolysis which is performed under mild and eco-friendly reaction conditions biomass [86]. The enzymatic hydrolysis is dependent on some crucial factors such as temperature, pH, time, enzyme activities, and substrates, etc. Enzyme-mediated saccharification has generally been achieved separately from fermentation in a process known as separate hydrolysis and fermentation (SHF), compared to the occurrence of saccharification and hydrolysis simultaneously, a process known as simultaneous saccharification and fermentation (SSF). Nowadays simultaneous saccharification and fermentation of both cellulose and hemicellulose is extensively explored by co-fermentation of both hexoses and pentoses sugars by employing genetically engineered microbial strains capable of fermenting xylose and glucose in the same medium where both enzymes for degradation of cellulose and hemicelluloses exist. The major advantage of this technology is that both saccharification and fermentation can be performed in the same chamber making the entire process cheap, feasible and cost-effective.

Efficient delignification of various feedstocks by fungus *Ceriporiopsis subvermispora* via the collective action of manganese peroxidase and laccase has been reported [87]. Since last few years, research has changed its course towards biological delignification. Moreover, recent advances in the characterization of ligninolytic enzymes participating in lignin degradation have taken the studies in this area, a step forward making them prone to biotechnological exploitation [88].

The advantage and disadvantages of different pretreatment method of lignocellulosic biomass have been discussed in Table 2.2

Table 2.2 Advantages and disadvantages of lignocellulose pretreatment methods

Pretreatment methods	**Advantages**	**Disadvantages**
Biological	Degradation of lignin and hemicellulose Low energy cost	Low rate of hydrolysis
Milling	Reduction in cellulose crystallinity	High power and energy consumption

Steam explosion	Causes lignin transformation and hemicellulose solubilization, Cost-effective, Higher yield of glucose and hemicellulose in the two-step process	Generation of toxic compounds and partial hemicellulose degradation
Ammonia fiber expansion (AFEX)	Increases accessible surface area	Not efficient for materials with high lignin content
CO_2 explosion	Low formation of inhibitors, increases accessible surface area	High cost of ammonia, does not affect lignin-hemicellulose interactions
Wet oxidation	Cost-effective, does not generate toxic compounds, efficient lignin removal	High cost of oxygen and alkali catalysts
Ozonolysis	Low formation of inhibitors minimizes energy demand reduces lignin content	High cost of the large amount of ozone needed
Organosolv	No generation of toxic compounds causes lignin and hemicellulose hydrolysis	Pollution concerns, High cost
Concentrated acid	High glucose yields, ambient temperatures	Solvents need to be drained and recycled, High cost of acid and the need for it to be recovered, reactor corrosion issues, formation of inhibitors
Diluted acid	Less corrosion than concentrated acid and less formation of inhibitors	Generation of degradation products, low sugar concentrations in exit stream

Source: [74, 76, 89, 90]

2.4 Lignocellulosic biomass-degrading microorganisms

Biodegradation of a compound substrate like lignocellulose relies not only on ecological conditions but also on the degradation efficiency of different microbial populations [91]. The deterioration of photosynthetically fixed carbon by microorganisms is a crucial process. By releasing 10^{11} tons of monosaccharides annually, it has become an indispensable part of one of the foremost geochemical cycles occurring in the biosphere [92]. This is reasonable when the half‑life of cellulose is taken into consideration which in the absence of microorganisms and at neutral pH is projected to be several million years [93]. Hence, the chief responsibility for the majority of carbon turnover is on the biological degradation of cellulose and hemicelluloses.

Cellulose in its native form is generally recalcitrant and very few microorganisms are capable of degrading this complex. Even though some cellulolytic and hemicellulolytic microorganisms are known, their relevance in the ecosystem as efficient degraders of lignocellulose biomass remains unclear. While fungi are associated with degradation of biomass present above ground, the importance of bacteria increases as we go deeper underground [94].

Fungi, as well as bacteria, have been extensively explored for their aptitude to produce diverse cellulases and hemicellulases for hydrolysing lignocellulosic materials [20]. Fungi are usually the organisms of choice for the degradation of lignocellulosic biomass as they have the ability to produce large amounts of cellulases and hemicellulases directly into the medium allowing easy extraction and purification.

2.4.1 Lignocellulose-degrading Bacteria

Extensive studies pertaining to cellulase production have been performed in bacteria such as *Cellulomonas fimi*, *Acidothermus cellulolyticus*, *Rhodospirillum rubrum*, *Clostridium stercorarium*, *Pyrococcus furiosus*, *Saccharophagus degradans* and *Bacillus polymyxa* [12, 95-97]. Moreover, numerous other bacteria belonging to diverse classes such as *Clostridium*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora,* and *Streptomyces* have also been reported to produce cellulases [98].

Similarly, various bacterial species such as *Bacillus subtilis*, *Clostridium thermocellum*, *Bacillus circulans*, *Thermobacillus xylanolyticus*, *Streptomyces halstedi*, *Bacillus amyloliquefaciens* and *Dictyoglomus thermophillus*, have been investigated for their ability to produce hemicellulose-degrading enzymes [99]. From *Clostridium thermocellum*, a

glycoside hydrolase family 43 (GH43) enzyme exhibiting hemicellulase activity, along with its potential application in consolidated bioprocessing has been described [100]. Lynd *et al*. (2002) also reported an extensive assessment of various bacterial species for their ability to promote consolidated bioprocessing [101].

Bacteria are usually thought of as secondary lignocellulose degraders, having the capacity to depolymerise cellulose and hemicellulose aerobically as well as anaerobically [102]. C*ellulomonas composti* and *Thermobifida fusca* are two examples of aerobic degraders [103]. Under aerobic conditions, free extracellular and cell-associated cellulases and hemicellulases are responsible for degradation [102]. *C. cellulolyticum* and *C. thermocellum* are typical examples of anaerobic degraders which cause degradation via a cellulosome complex to which relevant hydrolases are anchored [104].

Very limited studies have explored the ability of bacteria in degrading lignin. Crawford (1978) and Watanabe *et al*. (2003) observed low levels of lignin degradation by Streptomycete species [105, 106]. Researchers have also reported the isolation of multicopper oxidases with laccase activity from bacteria [60, 107].

2.4.2 Lignocellulose-degrading Fungi

One of the first cellulolytic organisms to be identified was *Trichoderma reesei* which was isolated in the 1950s. A remarkable collection of >14,000 fungi, active against cellulose and other insoluble fibers was available by 1976 [108]. Table 2.3 shows different fungi along with the various lignocellulolytic enzymes they produce.

Compared to bacteria, fungi are regarded as the primary degraders of lignocellulosic biomass [109, 110]. The highly proficient enzymatic system of fungi allows efficient degradation of lignocellulosic biomass. Moreover, the hyphal organization of fungi, which allows active penetration into the lignocellulosic biomass also enhances the degradative efficiency of fungi [111]. The hyphal structure of filamentous fungi assists them in transporting nutrients, including nitrogen and iron, from the carbon-rich lignocellulosic substrate [112]. Another distinguishing feature of fungi which aids them in efficient degradation of lignocellulosic biomass is their resistance towards wood derived biocides (tannins and various phenolic compounds like terpenes, stilbenes, flavonoids, and tropolones) which are known to inhibit bacterial growth. The heartwood of fallen trees contain these compounds in abundance.

Table 2.3 Examples of various fungi producing different lignocellulolytic enzymes and their substrates

	Group	Fungal strain	Enzymes	Substrate
Aerobic fungi **(Extracellular lignocellulolytic enzymes)**	Ascomycetes	*T. reesei*	Cellulases (CMCase, CBH, BGL), Hemicellulase (xylanase)	Wheat straw
		T. harzianum	Cellulases (CMCase, CBH), β-1,3-glucanases	Wheat bran,
		A. niger	Cellulases, Xylanases	Sugar cane
		Pestalotiopsis sp.	Cellulases (CMCase, CBH), Laccase	Forest litter of *Quercus*
	Basidiomycetes	*P. chrysosporium*	Cellulases (CMCase, CBH, BGL), CDH, LiP, MnP, Hemicellulase (xylanases)	Red oak, grape seeds, barley bran,
		F. palustris	Cellulases (CMCase, CBH, BGL)	Microcrystalline
Anaerobic rumen fungi (Chytridiomycetes) **Cell-wall-associated lignocellulolytic enzymes, "cellulosome")**	*Anaeromyces*	*A. mucronatus 543*	Cellulase (CMCase), Hemicellulase (xylanase)	Orchard grass hay
	Caecomyces	*C. communis*	Cellulases, Hemicellulases (xylanase, β-D xylosidase)	Maize stem
	Cyllamyces	*C. aberensis*	Cellulases, Xylanases	Grass silage
	Neocallimastix	*N. frontalis*	Cellulases, Hemicellulase (xylanase, β-galactosidase)	Cotton fibre, wheat straw
	Orpinomyces	*Orpinomyces sp.*	Cellulase (CMCase, CBH, β-glucosidase), Hemicellulases (xylanase, mannanases)	Wheat straw
	Piromyces	*Piromyces sp.*	Cellulases (CMCase, CBH, β-glucosidase) Hemicellulases (xylanase, mannanases)	Maize stem

CMCase: Carboxymethylcellulases (endoglucanase), CBH: Cellobiohydrolases, BGL: β-glucosidases, CDH: Cellobiose dehydrogenase, MnP: Manganese peroxidises, LiP: Lignin peroxidises.

Fungi produce two forms of extracellular enzymatic systems: the hydrolytic system, which comprises of cellulose and hemicellulose degrading enzymes; and an exclusive oxidative and extracellular ligninolytic system, which degrades lignin [12]. Multicellular fungi employ different mechanisms for degrading lignocellulosic biomass. These mechanisms differ regarding how these organisms make celluloses and hemicelluloses available for degradation by the corresponding enzymes and their behavior during attack and degradation of wood. Depending upon their mode of attack, the fungi are classified into three broad categories: soft-rot, brown-rot, and white-rot.

2.4.2.1 Soft-rot

Soft-rot type of degradation is a characteristic feature of the ascomycetes and deuteromycetes like *Trichoderma reesei, Chaetomium sp, Ceratocystis sp,* etc. [113]. Soft-rot fungi have the ability of efficiently manufacturing only cellulases and hemicellulases, not ligninases. This causes extensive loss of the carbohydrate polymers and a subsequent reduction in the strength of the decayed wood. *Trichoderma reesei*, a soft-rot fungus, has been exhaustively explored for the production, characterization, and application of cellulose and hemicellulose degrading enzymes. Chahal (1985) has described the production of cellulose and hemicellulose degrading enzymes (e.g. cellulase, β-glucosidase and xylanase) by *T. reesei QMY -1* during solid-state fermentation of wheat straw [114]. Similarly, Li *et al*. (2005) also reported the cellulolytic enzyme profiles of two strains of *T. reesei* (*T. reesei QM9414 and T. reesei Rut C-30)* when cultivated on crude or fractionated corn fiber [115]. Contrary to the earlier beliefs that soft-rot fungi only attacks wood with high moisture levels [116], it has now been established that it can also occur in dry environments. In the latter case, it resembles brown-rot fungi in terms of appearance when observed with naked eyes. Lignin and guaiacyl components of softwood influence the rate of wood decay by soft-rot fungi with lignin being the biggest impediment [117].
Compared to syringyl lignin which is readily oxidized and mineralized by ligninolytic peroxidases or laccases of soft-rot fungi, these enzymes lack the oxidative potential to attack the recalcitrant guaiacyl lignin [118].

2.4.2.2 Brown-rot

Brown-rot fungi, acting primarily on coniferous softwoods, can efficiently degrade only the cellulose and hemicellulose components of woody biomass. They are incapable of

depolymerizing lignin although some species can modify it by demethylation [117]. The fungi initially disrupt the cellulose chain, trailed by the subsequent removal of first the side chain followed by the main chain hemicelluloses (arabinan and galactan, and xylan and mannan, respectively). The decay process concludes with the complete hydrolysis of cellulose and demethylation of lignin [119]. The molecular mechanisms underlying brown-rot are only partially understood. Available data suggest that it involves both an oxidative and a hydrolytic attack. The participation of an oxidative, non-enzymatic attack during the initial stages of decay enables the brown-rot fungi to directly attack the cellulose in wood without any need of first removing lignin or hemicelluloses [120, 121]

Numerous brown-rot fungi such as *Gloeophyllum trabeum*, *Coniophora puteana*, *Serpula lacrymans and Meruliporia incrassata*, are known to cause the destruction of wood used in the construction of various structures and buildings [122]. The two most destructive fungi, *C. puteana* and *S. lacrymans*, commonly found in temperate regions, favor softwood as the substrate in comparison to the hardwood.

A unique mechanism for degrading wood polysaccharides is employed by brown-rot fungi. Compared to white-rot fungi that sequentially depolymerize cell wall carbohydrates to an extent to which they can utilize the hydrolysis products in fungal metabolism, brown-rot fungi cause rapid depolymerization of cellulose and hemicellulose, resulting in accumulation of products as the fungi are incapable of utilizing all the products in metabolism [24, 123].

2.4.2.3 White-rot

White-rot is the most frequently occurring wood-rotting process acting on both hard as well as softwoods. White-rot is the characteristic feature of basidiomycetes and has been explored in detail. White-rot fungi employ two distinct mechanisms for degrading lignin (a) simultaneous (nonselective) delignification and (ii) selective delignification (sequential decay) [122, 124]. However, these two patterns are not essentially mutually exclusive but possess a comprehensive range of overlap. This means that many white-rot fungi can cause rot employing both of these mechanisms, and the relative degree of the mechanism employed varies among different species of the same taxon [124]. The mechanism of lignin degradation that a white-rot fungus employs depends on the lignocellulose species [125], cultivation time, and other factors [126].

Simultaneous delignification, which acts primarily on hardwood, degrades cellulose, lignin, and hemicellulose at the same time. Typical examples of white-rot fungi exhibiting this mode are *Trametes versicolor, Irpex lacteus, Phanerochaete chrysosporium, Heterobasidion annosum,* and *Phlebia radiata*, [124].

In comparison, selective delignification involves the degradation of lignin and hemicelluloses prior to cellulose. In selective delignification, the proportion of lignin, hemicellulose, and cellulose decayed by a particular fungus may differ immensely, and even different strains of the same species, e.g., of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, might behave in a different way on the same kind of wood [127].

This mechanism is explicitly found in certain basidiomycetous species such as *Phlebia tremellosa*, *Ganoderma australe, Phellinus pini, Ceriporiopsis subvermispora and* several *Pleurotus* sp. [124].

For delignification process, white-rot fungi produce an array of enzymes such as laccases and peroxidases like lignin peroxidase and manganese peroxidase [128]. These ligninolytic enzymes are additionally supplemented by a consortium of enzymes involved in cellulose and hemicellulose degradation. The collective action of all these enzymes permits white-rot fungi to efficiently hydrolyze woody material and absorb simple sugars required for their growth and metabolism.

Enzymes associated with lignin degradation are lignin peroxidase (LiP, EC 1.11.1.14), laccase (Lac, EC 1.10.3.2, benzenediol:oxygen oxidoreductase), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16), and H_2O_2-forming enzymes such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO, EC 1.1.3.7) [127, 129]. White-rot fungi in addition to producing a battery of enzymes involved in lignin degradation, also produce cellulases, xylanases, and other hemicellulases. Manganese peroxidase (MnP) and laccase (Lac) is reported to be produced by almost all white-rot fungi, while only a few of them produce lignin peroxidase (LiP) [127]. The powerful lignin-degrading enzymes of white-rot fungi have allowed them to bridge the lignin barrier and, hence, overcome the rate-limiting step in the carbon cycle [130]. One of the extensively studied fungi is *Phanerochaete chrysosporium.* Phenol oxidases are essential enzymes participating in the oxidative degradation of lignin by white-rot fungi. Lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases from white-rot fungi *P. chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* have been best studied.

A comparison between white-rot, brown-rot, and soft-rot fungi has been listed in Table 2.4

Table 2.4 Comparison of white rot, brown rot, and soft rot fungi

		White Rot	**Brown Rot**	**Soft Rot**
Appearance		Bleached, spongy; loss of strength	Brown, brittle, powdery; loss of strength	Soft in wet, brown and crumbly in dry environments
Wood type and condition	SR SD	Hardwood Hard- and softwood	Mostly softwood Forest ecosystems, wood in service	Mostly hardwood Forest ecosystems, wet wood
Cell wall polymer concerned		Cellulose and hemicellulose lignin	Cellulose and hemicellulose lignin slightly modified	Cellulose and hemicellulose, lignin slightly modified
Anatomy	SR SD	Starting from the lumen From the middle lamella and secondary wall	Degradation in distance from hyphae	Degradation in proximity of hyphae Starting from the lumen
Fungi	SR SD	*Trametes versicolor Phanerochaete chrysosporium Heterobasidium annosum Ganoderma australe* *Phlebia tremelosa*, *P. pini Ceriporiopsis subvermispora Pleurotus spp.*	*Gloeophyllum trabeum Laetiporus sulfureus Piptoporus betulinos Postia placenta Serpula lacrymans*	*Chaetomium globosum Thielavia terrestris Paecilomyces spp. Trichoderma reesei*

SR, selective rot; SD, simultaneous delignification.
Data in table adapted from [124].

2.5 Enzymes responsible for degradation of lignocellulosic biomass

Fungi associated with lignocellulolytic enzyme production are widespread and include species from the ascomycetes (e.g. *T. reesei*), basidiomycetes including white-rot fungi (e.g. *P. chrysosporium),* brown-rot fungi (e.g. *Fomitopsis palustris*) and finally a few anaerobic species (e.g. *Orpinomyces sp.*) which degrade cellulose in the gastrointestinal tracts of ruminant animals [131, 132]. These fungi degrade biomass by deploying a battery of extracellular cellulases [133], hemicellulases [131] and ligninases [110, 134], reflecting the

complexity of the lignocellulosic substrate and enzyme system. For biomass conversion, lignocellulose-active enzymes produced by white-rot fungi are predominantly valuable since they selectively transform both lignin and polysaccharides [135]. Table 2.5 shows the various enzymes involved in the degradation of lignocellulosic biomass. These enzymes can be broadly classified as Carbohydrate-Active enzymes (CAZymes) and Fungal Oxidative Lignin enzymes (FOLymes) [136, 137]. The following is a summary of the key enzymes involved in the degradation of lignocellulosic biomass.

Table 2.5 Enzymes involved in lignocellulose degradation

Enzyme	**Systematic name**	**EC number**	**Mode of action**
Endo-1,4-β glucanase	1,4-β-D-Glucan-4-glucanohydrolase	3.2.1.4	Endo-hydrolysis of 1,4-β-D-glucosidic linkages
Exo-1,4-β-glucanase	1,4-β-D-Glucan cellobiohydrolase	3.2.1.91	Hydrolysis of 1,4-β-D-glucosidic linkages releasing cellobiose
β-Glucosidase	β-D-Glucoside glucohydrolase	3.2.1.21	Hydrolyzes cellobiose and short chain cello-oligosaccharides to glucose
Endo-1,4-β- xylanase	1,4-β-D-Xylan xylanohydrolase	3.2.1.8	Hydrolyzes mainly interior β-l,4-xylose linkages of the xylan backbone
α-L-Arabinofuranosidase	α-L-Arabinofuranoside arabinofuranohydrolase	3.2.1.55	Hydrolyzes terminal nonreducing α-arabinofuranose fromarabinoxylans
α-Glucuronidase	α-Glucuronoside glucanohydrolase	3.2.1.31	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Acetyl-ester acetylhydrolase	3.1.1.6	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Carboxylic ester hydrolase	3.1.1.1	Hydrolyzes fruloylester bonds in xylans
Lignin peroxidase		1.11.1.7	Oxidation of benzilic alcohols, cleavage of C-C bonds, cleavage of C-O bonds.

Manganese peroxidase		1.11.1.7	Catalytically dependent on H_2O_2 and Mn^{2+} ions
Laccase	Donor: hydrogen peroxide oxidoreductase	1.10.3.2	Oxidizes phenolic subunits of lignin

2.5.1 Cellulases – the cellulose degrading enzymes

Extensive investigation of enzyme mechanisms involved in cellulose degradation has been conducted [23, 138]. Active hydrolysis of the β-1,4-glycosidic bonds in cellulose is achieved by a group of enzymes known as cellulases which employ the retaining and the inverting mechanisms for catalyzing degradation. Both of these mechanisms involve the participation of two catalytic carboxylate residues and acid-base catalysis for catalyzing the reaction [42]. Cellulases belonging to glycoside hydrolase family 12 (GH 12) use the retaining mechanism while those belonging to GH family 6 use the inverting mechanism for hydrolysis of glycosidic bonds [133, 139]. Fungi known to produce cellulases include *Sclerotium rolfsii, Phanerochaete chrysosporium* and various species of *Trichoderma, Aspergillus* and *Penicillium* [140-142].

Cellulases of most organisms are modular enzymes which contain a catalytic core associated with a cellulose binding domain (CBD) via a flexible and heavily glycosylated linker region [143]. For cellulose degradation, the catalytic domain is brought into the appropriate orientation by CBD. During the hydrolysis of insoluble cellulose, binding of cellulases along with the formation of cellulose-cellulase complexes is considered as a critical step [144]. The classical model for complete degradation of cellulose to glucose involves the cooperative action of endocellulases (EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91), and beta-glucosidase (EC 3.2.1.21) (Figure 2.9) [145, 146].

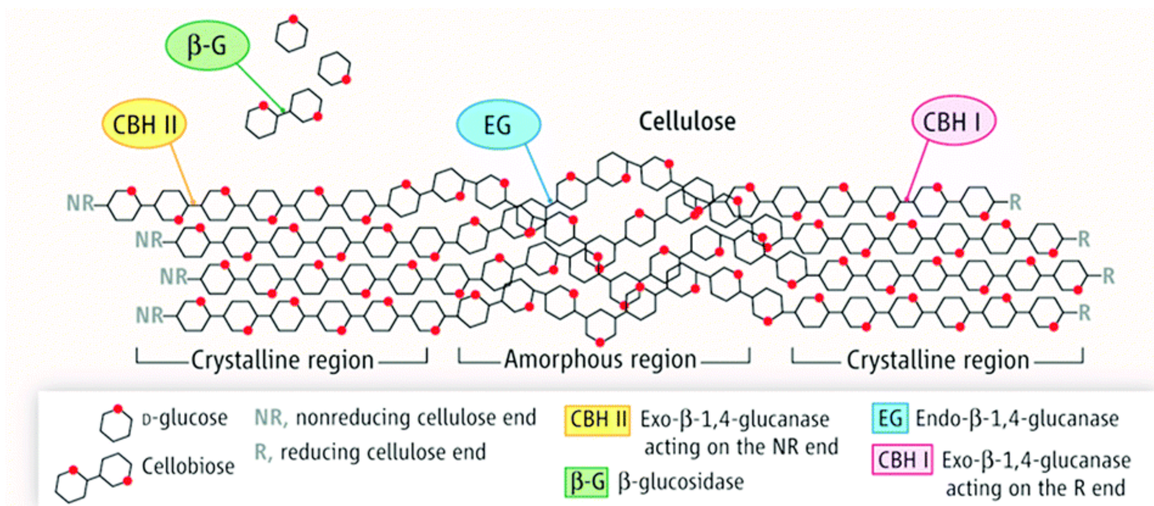


Figure 2.9 Classical model of enzymatic cellulose degradation

2.5.1.1 Endo 1,4-β-glucanases (EG) (EC 3.2.1.4, endocellulase)

Endocellulases/Endoglucanases act in a haphazard manner on the amorphous regions of the cellulosic fiber to cause hydrolysis of the internal 1,4-β-glycosidic linkages liberating cello-oligosaccharides. This causes a hasty decrease in polymer length and a steady increase in the reducing sugar concentration. Endocellulases are also denoted as carboxymethyl cellulases (CMCase), after the artificial substrate used for determining the enzyme activity. The action of endocellulases renders cellulose more susceptible to attack by cellobiohydrolases by providing new free chain ends [147]. Numerous studies have reported the production of multiple EGs by fungi. For example, *T. reesei* was reported to produce a minimum of 5 EGs (EGI/Cel7B, EGII/Cel5A, EGIII/Cel12A, EGIV/Cel61A and EGV/Cel45A) while three EGs have been reported from white-rot fungus *P. chrysosporium* (EG28, EG34, and EG44) [148, 149] Additionally, various studies have shown that EGs exist with or without a CBM. For example, four out of five EGs in *T. reesei* have CBM for e.g. EGI, EGII, EGIV and EGV have a CBM while EGIII lacks it [139].

2.5.1.2 Exo 1,4-β-glucanase (EC 3.3.1.91, exocellulase) (Exo 1,4-β-D-glucan cellobiohydrolases, CBH)

For the degradation of native cellulose, cellobiohydrolases (EC 3.2.1.91) appears to be a key enzyme [150]. Cellobiohydrolases attack the insoluble crystalline cellulosic substrates at the reducing or the nonreducing ends to liberate cellobiose as the primary product from the cellulose chain ends [25]. This causes a rapid release of reducing sugars but little change in polymer length. Cellobiohydrolases have been observed to slightly affects the Degree of

Polymerization (DP) of cellulose [50]. CBH activity is generally estimated using Whatman filter paper as a substrate and expressed in terms of filter paper activity (FPAase).
Investigative studies on *T. reesei* have shown that it produces two distinct types of cellobiohydrolases, Cel7A (former CBHI) which attacks the reducing end and Cel6A (former CBHII) which attacks the non-reducing end of the cellulosic chains. This makes *T. reesei* an efficient cellulolytic degrader. Cellobiose, produced as the end-product of CBHs, acts as a competitive inhibitor, which can limit the ability of the enzymes to degrade all of the cellulose molecules in a system [139, 148, 151].
The collective action of endocellulases and exocellulases on cellulose releases cello-oligosaccharides and cellobiose, which act as substrates for attack by β-glucosidases.

2.5.1.3 1,4-β-glucosidases (EC 3.2.1.21)

β-glucosidases (BGL) hydrolyze cellobiose and cello-oligosaccharides to glucose, and as such are prone to end-product (glucose) inhibition. BGL activity is measured using cellobiose as the substrate. Regulation of the whole cellulolytic process is arbitrated by this enzyme. During the enzymatic hydrolysis of cellulose, the reaction catalyzed by BGL is a rate-limiting factor as both endoglucanase and cellobiohydrolases activities are often inhibited by the accumulation of cellobiose [152-154]. β-glucosidases have been isolated from diverse fungal species such as *T. reesei*, and basidiomycetes such as white-rot and brown-rot fungi. Based on their amino acid sequence, BGLs have been placed in glycoside hydrolases families 1 and 3 [155]. BGLs belonging to both families employ the retaining mechanism for hydrolyzing β-1,4-glycosidic bonds [156]. Amongst the various cellulolytic enzymes, BGLs have been shown to exhibit maximum variation in terms of their structure and localization. For example, BGLs from *Pleurotus ostreatus* were observed to possess simple monomeric structures [157] while those isolated from *Sporobolomyces singularis* [158] and *Pisolithus tinctorius* [159] were observed to be dimeric and trimeric respectively. Additionally, BGLs also exhibit varying degrees of glycosylation. In terms of localization, BGLs can be classified as intracellular, cell wall-associated and extracellular [160]. For e.g. *T. reesei*, produces two β-glucosidases (BGLI/Cel3A & BGLII/Cel1A) which are primarily found attached to the cell wall [161].
Some of the obstacles to the commercial development of the enzymatic hydrolysis of cellulose are the high cost of cellulase, the low yield of product, substrate inhibition, thermal inactivation, and product inhibition. Although numerous cellulolytic organisms are known,

most commercial cellulases are produced using *Trichoderma ressei* (glucanases) and *Aspergillus niger* (β-D-glucosidase) [162]. There is a growing demand for the development of thermostable, environmentally friendly, product and substrate tolerant cellulases having enhanced specificity and activity suitable for application in the conversion of cellulose to glucose in the fuel ethanol industry. Research has shown that the rate of enzymatic hydrolysis and sugar yield, can be enhanced by optimizing the hydrolysis process and increasing the cellulase activity [163-165]. In addition to the hydrolytic enzymes, oxidative enzymes are also involved in the efficient degradation of cellulose [135] such as quinone oxidoreductase also known as cellobiose dehydrogenase reduces quinones and phenoxy radicals in the presence of cellobiose, which in turn is oxidized to produce cellobiono-δ-lactone. Cellulose degradation by *Phanerochaete chrysosporium* was enhanced by the external addition of cellobiose dehydrogenase [148]. Cellobiose oxidase is another example which oxidizes cellobiose and longer cello-oligomers to their corresponding acids by using molecular oxygen. Lytic polysaccharide mono-oxygenases (LPOMs), a novel oxidative enzyme class, was also found to be associated with cellulose degradation. The presence of extensive LPOMs in fungal genomes has resulted in the creation of a new class for auxiliary activities in the Carbohydrate-Active enzymes (CAZy) database to accommodate the LPOMs [166].

2.5.2 Hemicellulases – the hemicellulose-degrading enzymes

Hemicellulases which are either glycosyl hydrolases or carbohydrate esterases are classified into (a) depolymerizing enzymes (hydrolases) that attack the hemicellulose backbone and (b) debranching enzymes also known as accessory enzymes (esterases) that attack the polymer branches [50]. Given the structural complexity of hemicelluloses, different glycoside hydrolases, as well as carbohydrate esterases, act in synergism to bring about their degradation [167]. Complete biodegradation of hemicellulose into monosaccharides requires the collective action of endo-β-l,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and numerous accessory enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.131), acetylxylan esterase (EC 3.1.1,72), ferulic acid esterase (EC 3.1.1.73) and p-coumaric acid esterase, required for degrading various substituted xylans [168]. The action of endo-β-1,4-xylanase (EC: 3.2.1.8) on β-1,4 linkages present between D-xylose residues in the main chain of xylan produces oligomers which are further acted upon by β-xylosidase to form xylose (Figure 2.10) [42, 169].

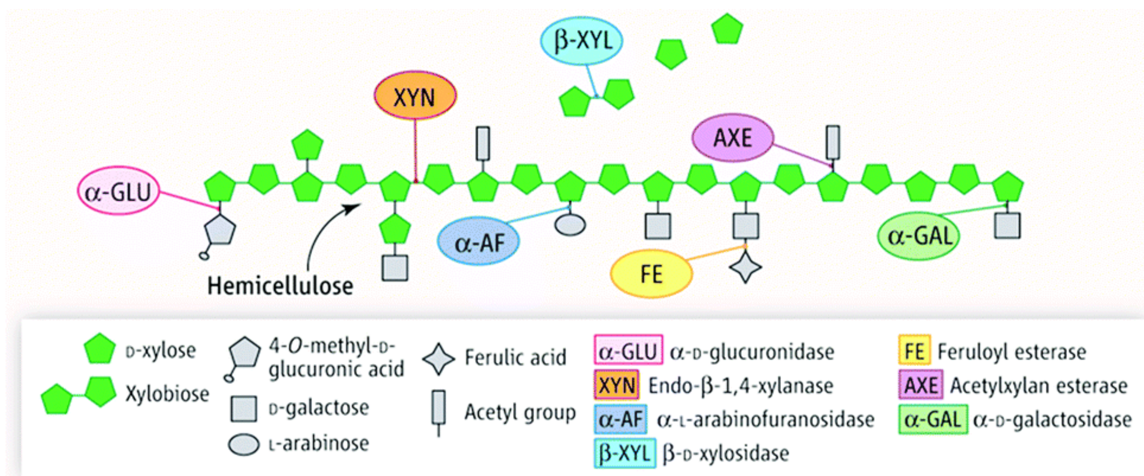


Figure 2.10 Classical model of enzymatic hemicellulose degradation

Other enzymes playing an important role in xylan degradation include α-arabinofuranosidase and α-glucuronidase, acetylxylan esterase, ferulic acid esterase, p-coumaric acid esterase, β-Mannanase and β-mannosidase. Fungi of genera *Trichoderma*, *Aspergillus*, *Fusarium* and *Pichia along with various* basidiomycetes produce large amounts of a full complement of enzymes required for degradation of hemicelluloses and as such are considered significant producers of xylanases. White-rot basidiomycetes such as *Pleurotus ostreatus*, *Pleurotus sajor-caju,* and *Pleurotus florida* have also been investigated for their xylanase enzyme production abilities [170]. Other microorganisms known to produce complete xylan-degrading enzyme systems are *Penicillium capsulatum* and *Talaromyces emersonii* [171].

2.5.3 Ligninases– the lignin-degrading enzymes

Efficient removal of lignin from lignin-carbohydrate complex (LCC) has also been deemed as an important step in the utilization of agro wastes for the production of value-added products. The white-rot fungi’s ligninolytic system collectively termed as “ligninases” is a heterogeneous system comprising of two ligninolytic families; (a) peroxidases [lignin peroxidases (LiPs; E.C.1.11.1.14), manganese peroxidases (MnPs; E.C.1.11.1.13)] and versatile peroxidases (VPs; E.C.1.11.1.16) and (b) phenol oxidase [laccases (E.C.1.10.3.2)] [124]. The numerous enzymes participating in lignin degradation have been collectively grouped into sequence-based families and integrated into the Fungal Oxidative Lignin enzymes (FOLy) database [137].

A lot of information regarding the degradation of lignin has been gathered from studies dealing with their biodegradation by white-rot fungi like *Phanerochaete chrysosporium, Trametes versicolor, Phlebia radiata, Pleurotus ostreatus* The majority of enzymes involved in lignin degradation use hydrogen peroxide as the oxidizing substrate (Figure 2.11).

Wood-rotting fungi which include white-rot basidiomycetes, brown-rot basidiomycetes, and soft-rot ascomycetes/deuteromycetes have been known to produce some or all of these enzymes and their isozymes [127, 172]. For example, while *P. chrysosporium* produces only lignin peroxidase and manganese peroxidase for lignin degradation [173], *Ceriporiopsis subvermispora* produces laccase (EC 1.10.3.2) and MnP isozymes [174]. Innumerable studies have identified white-rot basidiomycetes like *Coriolus versicolor* [175], *P. chrysosporium* and *T. versicolor* [176] as the most efficient degraders of lignin.

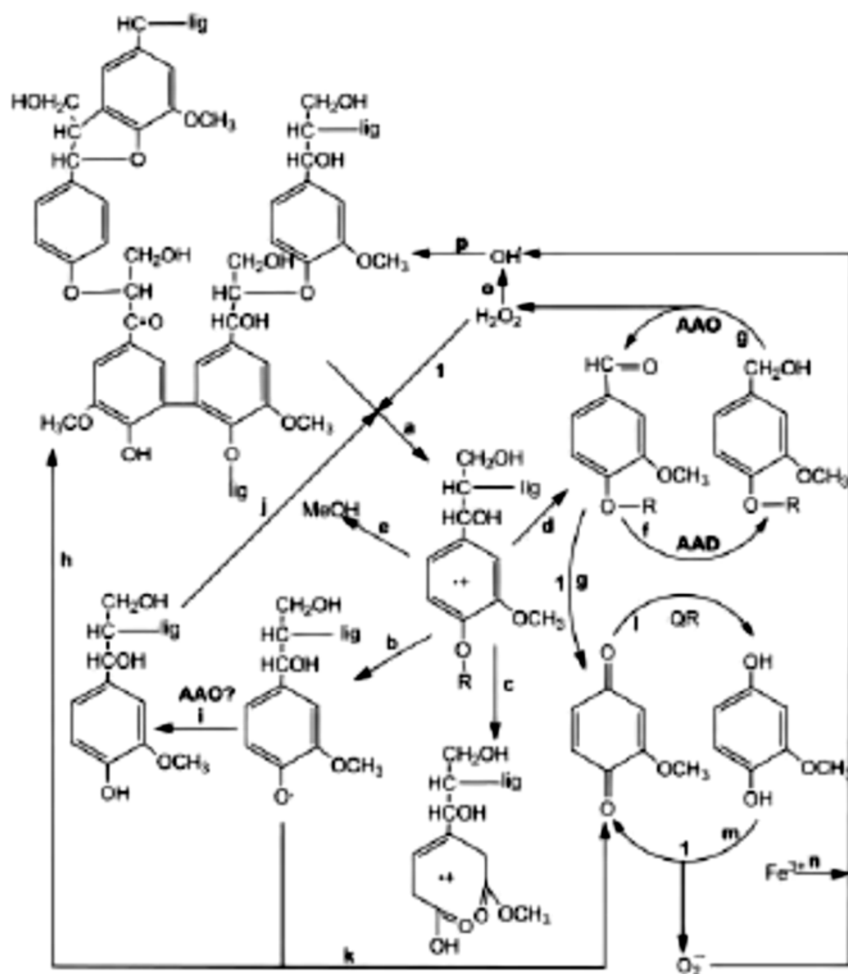


Figure 2.11 The complex interaction of enzymatic and nonenzymatic reactions in lignin degradation by fungi. Initial steps: (a) generation of aromatic radicals by lignin peroxidase, manganese peroxidase, and laccase; (b) C4–ether breakdown; (c) aromatic ring cleavage;

(d) C–C-cleavage; and (e) demethoxylation.(f, g) Hydrogen peroxide formation by AAO from the aromatic aldehydes formed in the reactions before or synthesized by the fungus itself, using a redox cycle that also involves aryl alcohol dehydrogenases (AAD); (h) repolymerization of lignin from its phenoxy radical degradation products; (i) reduction to phenolic products, which prevents (j) reoxidation of phenolic compounds by laccases or peroxidases; (k) C–C cleavage of phenoxy radicals, which yields p-quinones; (l, m) oxygen activation in redox cycling reactions involving quinine reductases, laccases, and peroxidases; (n) thereby, ferric ions are reduced; (o) and hydroxyl radicals formed. (p) The latter can initiate a further attack on lignin [124].

2.5.3.1 Laccases

The complex lignin polymer cannot be degraded solely by the action of peroxidases. Their efficient degradation requires additional enzymes such as laccase (phenol oxidase, benzenediol:oxygen oxidoreductase (E.C.1.10.3.2) [177]. Laccases belong to a family of copper-containing polyphenol oxidases and generally referred to as multicopper oxidases. Laccases are glycoproteins having a molecular weight in the range of 60-80 kDa [127]. The structural analysis of laccase revealed that it consists of three cupredoxin-like domains, and resembles ascorbate oxidase [178]. In 1883, Laccase for the first time was discovered in *Rhus vernicifera*, the Japanese lacquer tree [179]. Since then, numerous laccases and laccase-like proteins have been identified in a variety of plants, fungi, arthropods and bacteria [180]. Studies have shown that phytopathogenic, soil, and fresh water inhabiting ascomycetes along with a variety of basidiomycetes, including some mycorrhizal and brown-rot fungi contain laccase. Almost every species of white-rot and litter-decomposing fungi have been shown to produce laccase [181]. However, the quantity of laccase produced varies significantly among different species [129]. The catalytic mechanism includes electron transfer with nonspecific oxidation of phenolic groups to phenoxy radicals (Figure 2.12) [182, 183] which subsequently polymerize via radical coupling, followed by ring cleavage.

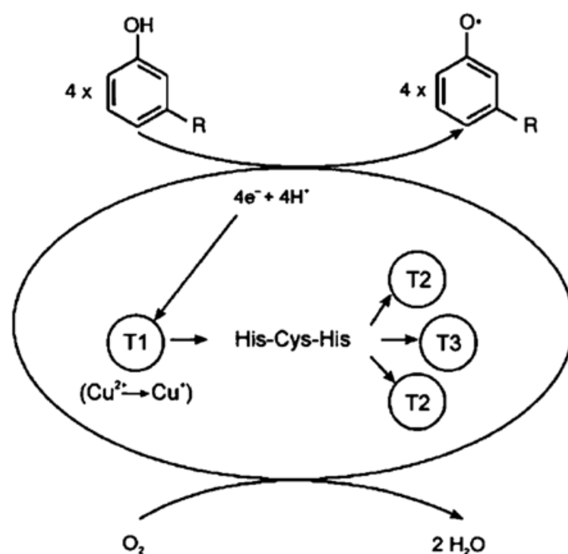


Figure 2.12 Mechanism of laccase catalysis

Below is a summary of the laccase catalysed reaction:

1. Type I Cu reduction by substrate
2. Electron transfer from type I Cu to the type II Cu and type III Cu trinuclear cluster
3. Reduction of oxygen to water at the trinuclear cluster

Laccase-mediated depolymerization has also been explored for their practicality in bioremediation of pollutants [184]. The unceasing requirement for screening new isolates having the ability of enhanced production of a proficient enzyme system capable of withstanding extreme conditions of temperature, pH, etc. has been suggested by various researchers [55, 185, 186]. As such, some laccase producing fungal isolates from diverse natural sources have been reported. These fungi include *Pycnoporus sanguineus* isolated from oil-polluted tropical habitat [187], *Myrothecium verrucaria* from the soil of a pine forest [188], *Marasmius quercophilus* from cork oak litter [189], *Fomitiporia Mediterranean* from wood rot of grape [190], *Funalia trogii* from decayed timber [191], *Phlebia radiator* from forest soil [192].

Laccase production by white-rot fungi is inducible by the addition of Cu^{2+} [193] or aromatic compounds such as veratryl alcohol [194] and 2,5–xylidine [195, 196]. In some fungi such as *C. subvermispora* [197] and *Ganoderma lucidum* [198], laccase production was found to be elevated in the presence of the lignocellulosic material.

2.6 Production of lignocellulolytic enzymes using wheat bran as substrate under submerged and solid-state fermentation

Fungi are regarded as the foremost microbial sources of all the three key lignocellulolytic enzymes viz. cellulase, hemicellulase, and laccase, although a few bacteria have also been reported to yield these enzyme components. Many earlier studies have reported the production of cellulases, hemicellulases, and laccase from fungi in submerged as well as solid state fermentations, individually or in a mixture of few enzymes by employing expensive substrates including pure carbohydrates, nitrogen sources, and a lot of minerals. For large scale production submerged fermentation is an acceptable method which yields about 90% of enzyme and is also cost effective. Although there are many reports in the production of cellulolytic enzymes using a wide range of agro wastes, the table below depicts the recent literature using fungal strains in both submerged and solid state fermentation using wheat bran as substrate.

Table 2.6 Recent reports on the production of lignocellulolytic enzymes using wheat bran as substrate under Solid State Fermentation

Enzymes	Fungal strain	Conditions	References
Cellulase	*T. viride* ATCC 13.631	3.18 FPU/mL at 50°C, pH 4.8	[199]
	A. niger NS-2	Endo-glucanase (18 CMC U/mL and β-glucosidase (3 U/mL at 30°C, pH 4.0	[200]
	Melanoporia sp. CCT 7736	0.89 IU/gds at 60 °C, pH (6.5)	[201]
	Penicillium sp. AKB-24 and *Aspergillus nidulans* AKB-25	134 IU/gds at 30°C, pH. 7.0	[202]
Xylanase	Isolates C and E	697 IU/mL, (131.25 IU/mL at 30°C, pH 6.5	[203]

	C. disseminatus	727.78 and 227.99 IU/mL at 37°C, pH 6.4	[204]
	Penicillium citrinum MTCC 9620	123.54 U/gds at 30°C, pH 5.0	[205]
	Aspergillus fumigatus strain (SCB4	180 U/gds at 45°C, pH.4.5	[206]
Laccase	*T. versicolor* *P. ostreatus*	63.5 and 58.2 U/g at 30°C, pH 4.5	[207]
	C. versicolor	107 U/g at 30°C, pH 3.6	[208]
	Pleurotus ostreatus	32,450 IU/g at 29°C, pH 5.0	[209]
	Pycnoporus sanguineus RP15	138.6 U. g^{-1}. at 74.4°C, pH 4.4	[210]

Table 2.7 Recent reports on the production of lignocellulolytic enzymes using wheat bran as substrate under Submerged Fermentation

Enzymes	**Fungal strain**	**Conditions**	**References**
Cellulase	*A. flavus*	3.3 IU/mL at 30°C, pH 6.0	[211]
	P. sajorcaju	0.89 U/mg at 30°C, pH 5.0	[212]
	Acrophialophora nainiana *Ceratocystis paradoxa*	2,200 IU/L 2,016 IU/L	[213]
	Trichoderma harzianum	Endoglucanase 20.06±0.86 IU/gd at 34°C, pH 5.5	[214]
Xylanase	*C. disseminatus*	30.32 IU/mL at 40 °C, pH 7.0	[215]
	T. basicola MTCC 1467	59.6 IU/mL at 30°C, pH 5.0	[216]
	C. oxysporum GQ-3	55.92U/mL at 30°C, pH 5.0	[217]

	A. niger	326.151 U/mg at 40°C, pH .50	[218]
Laccase	*S. ostrea*	1.26 U/g at 30°C, pH 7.0	[219]
	G. lucidum 447	97340 U/L at 25°C, pH 5.0	[220]
	P. candolleana HLS-2	12000 U/L.	[221]

2.7 Evolution of enzyme cocktails for enhanced hydrolysis of lignocellulosic biomass

If paradigms observed in nature are to be harnessed along with the optimization of these enzymes to industrial conditions, it is imperative to recognize how enzyme cocktails work together synergistically, in addition to understanding the functioning of single enzymes. The clues to unraveling this mystery are without any doubt contained within organisms and microbial communities capable of manipulating their enzyme cocktails to different substrates [222].

Since the enzymatic saccharification process is complicated by the recalcitrant nature of lignocellulosic substrates, employing an enzyme cocktail might prove to be an efficient means for effective saccharification of biomass with reduced amounts of enzyme preparation [223, 224].

Ethanol is obtained from the biomass by fermentation of the constituting carbohydrates. In the case of first generation biofuels, this carbohydrate is primarily in the form of sucrose or starch which can be readily degraded into simpler fermentable sugars. However, in the case of second generation biofuels, the fermentable sugars can only be yielded once the multifaceted lignin-cellulose-hemicelluloses matrix of the biomass has been disintegrated with the subsequent hydrolysis of the exposed carbohydrate polymers. This hydrolysis stage is characteristically catalyzed by enzyme cocktails comprising of cellulases, hemicellulases and other accessory enzymes that specifically target the cell wall components.

Customization, as well as identification of optimum ratios of enzyme mixtures involved in the degradation of less abundant linkages existing within plant cell walls, is an essential prerequisite [21]. Till date majority of the enzyme cocktails that have been formulated, combine enzymes from different organisms so as to compensate for one or the other missing component essential for biomass hydrolysis. For example, enhancement of *T. reesei* cellulases with crude enzyme preparations from other fungi is well documented and may eventually aid in the identification of novel accessory enzymes for biomass hydrolysis

[225]. Less energy and chemical demanding processes that allow for higher fermentable sugar recovery can be developed by employing accessory enzymes that assist in the complete utilization of plant biomass. Recently, Novozymes have developed a nifty enzyme cocktail with augmented catalytic activity and thermostability by combining cellobiohydrolase II and beta-glucosidase into a *T. reesei* cellulase mixture [226].

In the near future, bioproducts formation via biomass-based routes is expected to make a substantial impact on the manufacture of majority chemicals. This would require the development of optimized and cost-effective technologies capable of efficiently degrading biomass polysaccharides to fermentable sugars. Additionally, enzyme cocktails targeting a broad range of lignocellulosic biomass have to be made accessible at truncated costs. Availability of a diverse collection of cellulase and other accessory enzymes would enable the formulation of such cocktails [6].

An enzyme cocktail consisting of xylanase and laccase, obtained from the co-culture of *Penicillium oxalicum* and *Pleurotus ostreatus*, was shown to be effective even in the absence of any mediator [227]. The collective action of these two enzymes was shown to improve the quality of fiber, wherein the fiber morphology following enzyme treatment exhibited increased porosity, swelling, separation and peeling of pulp fibers compared to the smooth surface of untreated pulp [227].

Another instance where substantial delignification using an enzyme concoction was detected was during the collective action of MnP and xylanase [228]. Studies concerning the combined activity of MnP and xylanase evidently showed the inefficiency of single enzymes in mimicking the whole biological system [229, 230].

Colletotrichum graminicola produces significant quantities of β-glucosidase, β-xylosidase and xylanase enzymes under SSF using a cheap media which contained wheat bran as the carbon source and agricultural residues as the supplements. Simultaneous production of all the three enzymes at respectable levels suggested their further utilization in composing potent cocktails without any additional processing for lignocellulosic material hydrolysis.

A cocktail of hydrolytic and oxidizing enzymes from the fungal consortium, reported by Dhiman *et al*. (2015), suggested that the pretreatment and saccharification steps can be effectively combined in a process termed as Simultaneous pretreatment and saccharification (SPS) [231]. This process entirely eradicates the use of hazardous chemicals making it an eco-friendly process. As pretreatment and saccharification are performed in the same vessel, the process becomes more economically sound with reduced energy requirements and ease of residual biomass removal.

2.7.1 Application of lignocellulolytic enzyme cocktail in decolourisation of synthetic dyes

Synthetic dyes, used primarily in industries like cosmetics, paper, pharmaceuticals, and printing [232] Virtually all synthetic dyes, particularly the azo group of dyes and pose a grave danger to the environmental safety because along with their degradation products they are potentially toxic and carcinogenic [233] which has necessitated extensive research for developing biological or enzymatic methods for the degradation of these perilous dyes. As decolourisation of dyes contained within the effluents using physical or chemical methods such as adsorption and precipitation, chemical degradation or photodegradation is financially as well as methodologically demanding, time-consuming and largely ineffective [234], application of enzymes for efficient removal of these coloured textile effluents has proven to be an economic and environmentally friendly technique as it requires low energy inputs and has a minimal impact on the ecosystem.

Decolorization of a wide range of dyes biologically is achieved primarily through the application of fungi especially white-rot fungi (WRF) as they produce a variety of extracellular oxidases such as laccase, Mn-peroxidase (MnP) and lignin peroxidase. The comprehensive substrate specificity of these enzymes allow efficient degradation of many recalcitrant compounds including complex pollutants such as dyes. The two most frequently employed fungal mechanisms for dye decolourisation include physical adsorption (binding of dyes to the fungal hyphae) and enzymatic degradation [235]. Compared to enzymatic degradation [236], decolourisation via physical means has been shown to be a superior and the primary mode of decolorization [235]. Several fungal isolates such as *P. chrysosporium, T. versicolor, C. versicolor, C. polymorpha* [237], *T. polyzona* [238]and *Coprinus comatus* [239] have been extensively explored for their ability to decolorize synthetic dyes. Since the efficacy of the white-rot fungal enzymatic system is reliant on strains and optimization of crucial parameters (temperature, pH, incubation time, etc.), it becomes all the more essential to explore new white-rot fungi for their ability to degrade dyes [240].

Ligninolytic enzyme based decolourisation of synthetic dyes has been achieved by using enzymatic extracts, purified or semi-purified enzymes, and commercial or native enzymes. Additionally, enzymatic decolorization can be performed by using either individual or a mixture of ligninolytic enzymes. Of the various enzymes employed in decolourisation of dyes, laccase was reported to be most efficient enzyme followed by MnP and LiP.

CHAPTER 3

MATERIALS AND METHODS

3.1 Glassware

Glassware from Borosil and Scott Duran, India, were used throughout the study, which was washed twice with detergent with final rinsing in distilled water. All the glassware's were then dried in hot air oven at 70°C and used in the experiments.

3.2 Chemicals and reagents

All the chemicals and reagents used in the study were of high purity obtained from Hi-Media, Mumbai Sigma-Aldrich, USA, Merck, Germany SRL, India.

3.3 Procurement and maintenance of microbial cultures

A collection of nineteen pre-identified fungal strains collected from different geographical regions of North-Western Himalayan ranges in Himachal Pradesh were procured from Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (CSKHPKV), Palampur, India. The yeast culture of *Saccharomyces cerevisiae* MTCC 174 and standard fungal culture of *Trichoderma* reesei MTCC 164, were procured from Institute of Microbial Technology (IMTech), Chandigarh, India. All the fungal cultures were maintained on peptone dextrose (PDA) agar at 4°C.

3.4 Lignocellulosic substrate selection

The biomass sources *viz.* lolium grass and bromus grass were obtained from Institute of Himalayan Bioresource Technology (IHBT), Palampur (H.P) while dried pine needles and wheat bran were obtained from the local outlets & surroundings of Jaypee University of Information Technology, Waknaghat, Solan (H.P.).

3.5 Chemical analysis of the substrate

3.5.1 Determination of cellulose content

The cellulose content of wheat bran was determined by the method of Crampton and Maynard (1938) [241]. One gram of oven dried sample was taken in a 250 mL Erlenmeyer flask. 25 mL of acetic nitrate reagent (acetic acid 650 mL; nitric acid 80

mL; distilled water 150 mL) was added to the flask, and the contents were boiled till the evolution of brown fumes. The residue was then transferred into a dried pre-weighed crucible and filtered using a vacuum pump. After filtration, three washings each of water, alcohol, and acetone were given. The crucible was then placed in an oven (150°C) for l hr. After cooling in a desiccator, it was again weighed. Finally, the crucible was kept in a muffle furnace at 450°C for l hr, cooled and weighed. The loss in weight was observed as the amount of cellulose present in the samples.

3.5.2 Determination of hemicellulose content

Hemicellulose content of wheat bran was determined by estimating the percentage of NDF (Neutral Detergent Fibre) and ADF (Acid Detergent Fibre) by the method of Georing and Vansoest (1970) [242].

Determination of NDF

Reagents: NDS - Neutral detergent solution (SDS 30 g/L, EDTA 18.61 g/l, sodium borate decahydrate 6.8l g/l, disodium hydrogen phosphate 4.56 g/L, 2-ethoxy ethanol 10 mL).

Preparation of NDS: EDTA and sodium borate decahydrate were taken in a beaker containing 500 mL of distilled water and dissolved by heating. Sodium lauryl sulphate and 2-ethoxy ethanol were dissolved separately in boiling distilled water and then mixed with above solution. Disodium hydrogen phosphate was separately dissolved in boiling water and then added to the above-prepared solution. The pH of the solution was adjusted to 7.0, and the final volume was adjusted to 1 L with distilled water.

Method: One gram of the dried sample was taken in a spoutless beaker. Then 100 mL of neutral detergent solution, 2 mL decaline, and 0.5 g sodium sulphite were added in sequence. The contents were boiled for 5 to 10 min. and refluxed slowly for 1 hr. The refluxed sample was filtered by transferring it into the pre-weighed crucible (Al) placed on the filtering apparatus. It was then rinsed with hot water, followed by acetone. The crucible was then dried at 105°C for 12 hrs and weighed (A2). The neutral detergent fiber content was calculated as:

NDF (%) = (A2−Al/ W) x 100

Where W is the weight of the sample taken.

Determination of ADF

Reagent: ADS- Acid detergent solution (Cetyl trimethyl ammonium bromide (CTAB) 20 g in l000 mL of l N sulphuric acid).

Method: One gram of the sample was taken in a 500 mL spoutless beaker. 100 mL acid detergent solution and 2 mL decaline were then added to it. The contents of the beaker were boiled for 10 min. and thereafter refluxed slowly for l hr. The digested sample was filtered into a previously weighed crucible (A3) which was set on a filtering apparatus. The residue was washed first with hot water, then alcohol and finally with acetone. The crucible was then dried at 105°C for 12 hrs, cooled in a desiccator and weighed (A4). The dried weight of the residue was recorded as ADF given by:

ADF (%) = (A4−A3/ W) x l00,

Where W is the weight of the sample taken. Finally, hemicellulose content (%) was calculated as per the formula

Hemicellulose (%) = NDF (%) − ADF (%)

3.5.3 Determination of lignin content

Lignin content of wheat bran was determined by estimating the percentage of ADL (Acid Detergent Lignin) according to the method of Van Soest (1963) [243]

Determination of ADL

The crucible containing ADF was kept on a 500 mL conical flask containing water. The contents of the crucible were covered with 20 mL of 72% sulphuric acid and stirred with a glass rod to form a smooth paste. The crucible was then refilled with acid and kept on ice bucket carefully. After 3 hrs, excess acid was filtered off using a vacuum pump. The residue was then washed with hot water till it was acid-free. The crucible was dried at 100°C, cooled in a desiccator and weighed (A5). The crucible was then placed in a muffle furnace at 500°C for 3 hrs, cooled and weighed (A6). The acid detergent lignin content was computed as:

ADL (%) = (A5−A6/W) x 100

Where W is the weight of the sample taken

The ADL content (%) worked out to be equivalent to lignin content (%).

3.6 Enzyme assays

The enzyme filtrate was assayed for cellulase, xylanase, laccase and filter paper activity according to the protocols described below:

3.6.1 Carboxymethyl cellulase activity assay

Carboxymethyl cellulase activity of the enzyme was worked out as suggested by Mandels *et al.* (1976) [244]. The test tubes containing a mixture of 1% CMC solution (0.5 mL) and cultural supernatant (0.5 mL) were incubated at 50°C for 30 min in 50 mM sodium acetate buffer at pH 5.0. A control with inactivated enzyme was measured simultaneously. 3 mL of DNS was then added to stop the reaction. The reducing sugars were then estimated by DNS method according to the method of Miller (1959) [245]. A blank with no enzyme and a control with inactivated enzyme were also incubated simultaneously under same reaction conditions. The production of glucose was estimated using D-glucose as standards. One unit of activity (U) was defined as the µmoles of glucose released per minute under assay conditions. All the tests were done in triplicates, and the results were represented as mean ± S.D.

3.6.2 Xylanase activity assay

The xylanase assay of enzyme supernatant was performed as suggested by Bailey *et al.* (1992) using birch wood xylan as the substrate [246]. The assay mixture which contained 500 µL of 1% birch wood xylan (Sigma-Aldrich, USA X0502) prepared in 50 mM Na-acetate buffer with pH 5.0 and 500 µL cultural supernatant was incubated at 50°C for 30 min. The reaction was stopped by the addition of 3 mL dinitrosalicylic acid (DNS) reagent and the contents were boiled for 15 min. The developed color was read at 540 nm. The amount of reducing sugar liberated was quantified using the xylose standard. A blank with no enzyme and a control with inactivated enzyme were also incubated under similar reaction conditions. One unit of activity (U) was defined as the µmoles of xylose released per minute under assay conditions. All the tests were done in triplicates, and the results were represented as mean ± S.D.

3.6.3 Laccase activity assay

Laccase activity was determined spectrophotometrically with ABTS as the substrate according to the method of Bourbonnais and Paice (1990) [247]. The assay mixture contained 2 mL ABTS (0.5 mM) and 2 mL aliquots of appropriately diluted culture

supernatant. The oxidation of ABTS was monitored by following the increase in A420 (ε = 3.6 × 104 M-1·cm-1). A blank with no enzyme and a control with inactivated enzyme were also incubated under same reaction conditions. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute. All the tests were done in triplicates & results were represented as mean ± S.D.

3.6.4 Filter paper activity

Filter paper activity of cultural supernatant was assessed by the method of Mandels *et al.* (1976) [244]. The test tubes containing 0.8 mL of 0.05 M citrate buffer (pH 4.8), 0.2 mL cultural supernatant and a rolled strip (1 x 6 cm) of Whatman no.1 filter paper were incubated at 50°C for l hr. A 3 mL of DNS was then added to sojourn the reaction. The tubes were heated in boiling water bath for 10 min, and the absorbance was read at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute. A blank with no enzyme and a control with inactivated enzyme was also incubated under identical reaction conditions. All the tests were done in triplicates & results were represented as mean ± S.D.

3.7 Screening of fungal cultures for lignocellulolytic enzyme activities

All the 19 fungal isolates were screened qualitatively by agar plate assay and quantitatively by a spectrophotometric method for the presence of cellulase, xylanase and laccase enzyme activities using carboxymethyl cellulose (CMC) [244], birchwood xylan [246] and ABTS/guaiacol [247]/[248] respectively as substrates.

3.7.1 Qualitative assay

The congo red clearing zone assay is suitable for qualitative display of cellulase and xylanase activity. The overnight incubated carboxymethyl cellulose and birchwood xylan agar plates containing circular batches of fungi were flooded with 0.1% congo red solution and left for 15 min with intermittent shaking. The plates were then rinsed with water initially and finally with 1 M NaCl solution. Washing with NaCl solution reveals a zone of clearing as it elutes the dye from the regions where the substrates had been hydrolyzed due to the activity of cellulase and xylanase. The extent of clear zones developed around the fungal growth was indicative of the presence and the relative amount of cellulase and xylanase activity in different fungal isolates.

The ability of the fungal strains to secrete extracellular laccase was visualized according to the method of Kiiskinen *et al.* (2004) [248]. The assay plate contained potato dextrose agar amended with 0.01% of guaiacol. The plates were incubated at 30°C for 1–3 days. The appearance of brick red color around the mycelium of a fungal isolate established it as a laccase secreting organism.

3.7.2 Quantitative assay

The cellulase, xylanase, laccase, and filter paper activity in the cultural supernatant was estimated as discussed in section 3.6.1, 3.6.2, 3.6.3 and 3.6.4.

3.8 Morphological and molecular identification of most potential lignocellulolytic enzyme producing fungi

3.8.1 Morphological identification

The cultural characteristics such as colony appearances, mycelial textures on potato dextrose agar plates were observed after 3–7 days of incubation at 30°C. For microscopic observation, mycelial plugs of *C. pannosa* were transferred onto lactophenol cotton blue stain and examined under low and high, magnification for the presence of characteristic mycelia and spores

3.8.2 Molecular identification using Internal transcribed spacer (ITS) region

DNA extraction and PCR amplification

The fungus was inoculated on potato dextrose agar (PDA) for two weeks and then transferred into potato dextrose broth (PDB) at room temperature for one week. The mycelium was filtered and washed with sterilized water. The biomass was frozen and grounded into a fine powder with pre-chilled mortar and pestle. The genomic DNA was extracted using CTAB method [249] with some modifications.

The following solution and reagents were used: Liquid nitrogen; extraction buffer solution (200 mM Tris-HCl, pH 7.5; 25 mM EDTA and 250 mM NaCl and 0.5% SDS); cold phenol:chloroform (1:1); chloroform; cold isopropanol and cold 70% ethanol.

The DNA extraction procedure consisted of the following steps.

- Purified colonies of strain designated as F6 were grown in PDB broth for 3 days in a shaker at 30°C.

- The 3-day-old mycelium was allowed to drain for about 3 min after being placed on a filter paper. The mycelium was then ground separately with mortar and pestle in liquid nitrogen to a fine powder.
- The frozen powder was then transferred to a 2 mL centrifuge tube, and 500 μL of extraction buffer solution was added.
- The reaction mixture was vortexed for 5 sec and kept at room temperature for 30 min.
- The reaction mixture was centrifuged at 13,000g for 1 min.
- The supernatant was then transferred to a new centrifuge tube, and an equal volume of cold phenol and chloroform was added to it.
- The reaction mixture was vortexed briefly and centrifuged again at 13,000g for 2 min.
- The supernatant was again transferred to a new centrifuge tube and re-extracted twice with 300 μL of chloroform and centrifuged again.
- The final supernatant was then transferred to a new centrifuge tube; 300 μL of cold isopropanol was added to it and gently mixed by inverting the tubes twice or thrice.
- The reaction mixture was incubated for 30 min at –80°C or –20°C.
- The nucleic acids were then recovered by centrifugation at 13,000g for 5 min.
- The supernatant was discarded and the pellet obtained was washed with 70% cold ethanol and dried for 15 min at 37°C.
- Finally, the isolated DNA was resuspended in 50 μL of sterile water and stored at –20°C for further use.
- Ten microliters of total DNA solution was loaded onto a 1% agarose gel and electrophoresed to separate DNA.

The ITS region was amplified with fungal specific primer: ITS1 and ITS4 [250]. The PCR amplification was carried out using PCR conditions of 1 cycle at 94°C for 5 min; 30 cycles (95°C for 45 s; 53°C for 1 min; 72°C for 2.5 min); 1 cycle at 72°C for 10 min; hold at 4°C in thermocycler (Bio-Rad T100). The PCR products were then analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1X Tris-acetate-EDTA buffer with 0.5 mg/mL of ethidium bromide at a constant voltage of 80 V for 45 min, then visualized under UV light to confirm the presence of the amplified DNA. The amplified PCR

fragments were purified with DNA purification kit (Real Biotech Corporation), following the manufacturer's instruction and then sequenced.

Sequence alignment and phylogenetic analysis

The ITS regions were employed to search the closest sequences from the GenBank database (http://www.ncbi.nlm.nih.gov) using a BLAST search [251] to clarify the generic and species level of the fungal isolate. DNA sequences were multiple aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) [252]. The phylogenetic analysis was carried out on multiple sequence alignment in order to construct phylogenetic trees by UPGMA method. Bootstrap confidence values were generated using 100 permutations of the data set to derive the nucleotide sequence similarities.

3.9 Optimization of lignocellulolytic enzyme activities of *Cotylidia pannosa* under submerged and solid-state fermentation using one factor at a time approach

The activity of lignocellulolytic enzymes was assessed in submerged as well as solid-state fermentation using wheat bran as the substrate for identifying optimal process parameters such as temperature, pH and incubation time.

3.9.1 Effect of saccharification parameters on enzyme production under submerged fermentation (SmF)

A 2.0 g of wheat bran was added to 100 mL of growth medium (YEP) consisting of 1% yeast extract and 2% peptone followed by sterilization at 121°C for 15 min at 15 psi. A spore inoculum of *C. pannosa* (2.9 x 10^8 fungal spores /mL) was inoculated aseptically in 100 mL of sterilized growth medium followed by incubation for 96 hrs at 30°C. A 5 mL aliquot of hydrolyzed broth was withdrawn from the flask after a regular interval of 12 hrs up to 96 hrs and centrifuged at 4°C at 7000g for 15 min. The supernatant collected was analyzed for cellulase, xylanase enzyme activities by estimating the amount of reducing sugars produced using dinitrosalicylic acid method [4] and laccase activity by measuring the oxidation of ABTS indicated by an increase in absorbance at 420 nm. The lignocellulolytic enzyme production from *C. pannosa* using the most potent substrate was optimized at different fermentation parameters such as temperature (25 – 40°C), incubation time (0 – 108 hrs) and pH (3.0 – 6.0).

3.9.2 Effect of saccharification parameters on enzyme production under solid-state fermentation (SSF)

For solid state fermentation, a spore inoculum of *C. pannosa* (2.9 x 10^8 fungal spores/mL) was inoculated in 2 g of wheat bran moistened with growth media resulting in 100% moisture level. Subsequently, the flasks were incubated at variable growth parameters i.e. temperature (25 – 42°C), time (0 – 108 hrs) and pH (3.0 – 6.0). After incubating the flasks under static conditions, the enzyme was extracted using sodium acetate and phosphate buffer (pH 5.0) by shaking each flask for 1 hr. After proper mixing had been done, the supernatant was filtered using a muslin cloth and subjected to centrifugation at 7,000g for 20 minutes. The supernatant thus obtained was subjected to enzyme activity analysis as described in section 3.6.1 to 3.6.4.

3.10 Optimization of lignocellulolytic enzyme activities of *Cotylidia pannosa* under submerged fermentation using Response Surface Methodology (Multifactorial approach)

Since the maximum enzyme activities using one factor at a time approach were obtained under submerged fermentation condition, further optimization of enzyme activities of *C. pannosa* using RSM approach was performed only under conditions of submerged fermentation.

3.10.1 Modeling and optimization of lignocellulolytic enzyme production through RSM

For optimization study, 2% wheat bran was added to the YEP culture media consisting of 1% yeast extract and 2% peptone followed by sterilization at 121°C for 15 min at 15 psi. A spore inoculum of *C. pannosa* (2.9 x 10^8 fungal spores/mL) was inoculated aseptically into 100 mL of sterilized growth medium. Based on the Central Composite Design (CCD) of RSM (Table 3.1 & Table 3.2), four independent variables, including temperature (A, 25 − 40°C), pH (B, 4 − 6), incubation time (C, 24 − 120 hrs), and agitation rate (50 − 150 rpm) were studied at four different levels, based on preliminary results on lignocellulolytic enzyme production under submerged fermentation using one factor at a time approach. An aliquot of hydrolyzed broth was withdrawn from the flask after a regular interval and centrifuged at 4°C at 7000g for 15 min. The supernatant after

centrifugation was collected and used for determination of cellulase, xylanase and laccase enzyme activities.

Table 3.1 Experimental range of variables for the central composite design in terms of actual and coded factors

Variables	**Symbol coded**	**Range of variables**		
		Low (-1)	Mid (0)	High (+1)
Temperature (°C)	A	25.0	32.5	40.0
pH	B	4.0	5.0	6.0
Incubation Time (hrs)	C	24	72	120
Agitation (rpm)	D	50	100	150

The values of SmF variables for the CCD design of RSM were selected based on literature review and initial screening data through MINITAB 16 software. In the present study, the outputs included the cellulase, xylanase and laccase activities. The set of experiments based on the CCD was executed in triplicate runs. The nonlinear regression analysis was carried out based on the experimental results of cellulase, xylanase and laccase activities using MINITAB 16 which resulted in a second-order polynomial equation. The individual, square and interaction effects of SmF variables on cellulase, xylanase and laccase activities were studied through significance and analysis of variance (ANOVA) tests. The adequacy of the developed model was further checked through R2 and adjusted R2 values. For prediction of a set of SmF variables, the response optimizer function of MINITAB 16 software was utilized to predict maximal cellulase, xylanase and laccase activities [253].

Table 3.2 Composition of the various runs of the central composite design, actual and predicted values of the different compression parameters

Input parameters				
Run	**Temp[a] (°C)**	**pH**	**IT[b] (hrs)**	**Agtn[c] (rpm)**
Order	(A)	(B)	(C)	(D)

1	25	4	120	150
2	32.5	5	72	100
3	40	4	120	50
4	25	4	24	150
5	25	6	24	150
6	32.5	5	120	100
7	25	6	120	150
8	40	4	24	50
9	32.5	4	72	100
10	32.5	6	72	100
11	40	4	120	150
12	25	5	72	100
13	32.5	5	72	100
14	25	6	24	50
15	25	4	24	50
16	32.5	5	72	100
17	40	6	120	50
18	40	6	24	150
19	32.5	5	72	100
20	40	4	24	150
21	32.5	5	72	100
22	32.5	5	72	100
23	32.5	5	72	50
24	40	6	24	50
25	40	6	120	150
26	32.5	5	72	100
27	32.5	5	72	150

[a] Temperature; [b] Incubation Time; [c]Agitation Rate;

The non-linear regression analysis was carried out based on the data collected as per CCD planning for responses, namely cellulase, xylanase and laccase activity using MINITAB 16 software which resulted in a second-order polynomial equation. The

coefficient of the non-linear regression model can be determined using the method of least squares.

The effect of the parameters and their interaction terms on the response has been studied by conducting the significance tests, and ANOVA has been carried out on each response to check the adequacy of the model. The detailed analysis of the effect of parameters and their interactions on the response was also done through the surface plots using MINITAB 16 software. The optimized variables for the higher cellulase, xylanase and laccase activities (U/mL) have been chosen through the response optimizer function of the MINITAB 16 software.

3.11 Characterization of activities of crude lignocellulolytic enzyme cocktail produced by *Cotylidia pannosa* under submerged fermentation using optimized parameters

3.11.1 Production of crude enzyme cocktail under optimized parameters

A 2.0 g of wheat bran was added to 100 mL of growth medium (YEP) consisting of 1% yeast extract and 2% peptone followed by sterilization at 121°C for 15 min at 15 psi. A spore inoculum of *C. pannosa* (2.9 x 10^8 fungal spores /mL) was inoculated aseptically in 100 mL of sterilized growth medium followed by incubation for 77 hrs, at 31°C, pH 5.0 and 140 rpm agitation rate. A 5 mL aliquot of hydrolyzed biomass was withdrawn from the flask after 77 hrs and centrifuged at 4°C at 7000g for 15 min.

3.11.2 Preparation of crude enzyme cocktail precipitate using ammonium sulphate precipitation

The cell-free supernatant obtained from fermentation of YEP containing 2% wheat bran after 77 hrs of incubation at 31°C, 140 rpm and pH 5.0 was precipitated with 80% ammonium sulphate. Ammonium sulphate was slowly added to the sample kept at 4°C on a magnetic stirrer to bring the final concentration to 80%. For complete precipitation, the mixture was kept at 4°C overnight with gentle stirring. The mixture was centrifuged at 15,000g for 15 min at 4°C. The pellet obtained was reconstituted in 1 mL of sodium acetate buffer (pH 5). The protein content of the reconstituted sample was estimated by Lowry’s method of protein estimation [254].

3.11.3 Analysis of enzyme activities in the precipitated crude lignocellulolytic enzyme cocktail

Cellulase, xylanase and laccase activity in the ammonium sulphate precipitated crude enzyme was estimated as discussed in section 3.6.1, 3.6.2 and 3.6.4.

3.11.4 Effect of temperature and pH on enzyme activities

For crude cellulase and xylanase, optimum conditions supporting maximum activity were identified by incubating the enzyme at varying temperature (25 – 70°C) and pH (3.0 – 7.0). Similarly, for crude laccase the optimum conditions supporting maximum activity were identified by varying temperature from 25 − 60°C and pH from 3.0 − 6.0.

3.11.5 Zymogram analysis

For zymogram analysis, the crude enzyme cocktail was subjected to native-PAGE analysis. The crude enzyme preparation was mixed with protein loading buffer and separated on a discontinuous gel system (Bio-Rad Life Sciences, USA) containing 12% resolving gel and 5% stacking gel (Table 3.3) under non-denaturing conditions according to Laemmli [255]. After PAGE, the gel was divided into two pieces: one was stained with 0.05% Coomassie brilliant blue R-250, and the other piece was subjected to activity staining. Activity staining for cellulase and xylanase was performed by incubating the gels in 50 mM sodium acetate buffer (pH 5.0) containing 2% CMC and 2% birchwood xylan respectively for 1 hr at 55°C. After incubation, both the gels were stained with 0.1% Congo red for 30 min. For laccase activity determination the other half of gel was incubated in 20 mM ABTS dissolved in sodium acetate buffer of pH 5.0 at 32°C till green color band corresponding to the presence of laccase developed.

Table 3.3 Recipe for preparing stacking and resolving gels for native-PAGE

12% Resolving Gel		**5% Stacking Gel**	
Component	For 10 mL	**Component**	For 4 mL
Distilled H_2O	3.175 mL	**Distilled H_2O**	2.66 mL
30% Acrylamide	4 mL	**30% Acrylamide**	670 µL
1.5 M Tris (pH = 8.8)	2.503 mL	**1 M Tris (pH = 6.8)**	500 µL

10 % APS	100 μL	**10 % APS**	40 μL
TEMED	4 μL	**TEMED**	4 μL

3.12 Saccharification of wheat bran for bioethanol production

3.12.1 Saccharification potential of fungal biomass

An Erlenmeyer flask containing wheat bran (2%) in YEP media and *C. pannosa* (2.9 x 10^8 fungal spores/mL) was taken and incubated for 72 hrs at optimum conditions 30°C, pH 5.0 and 120 rpm. The hydrolyzed broth was collected from a flask at every 12 hrs interval for determination of enzyme (cellulase, xylanase, and laccase) activity and glucose content to determine the optimum time at which maximum saccharification was obtained. After identifying the optimized time, a fresh flask containing 2% wheat bran and *C. pannosa* was taken and incubated at optimum conditions for the optimum time period identified (56 hrs) to bring about maximum saccharification, after which the contents were centrifuged and the hydrolysate obtained was subjected to fermentation by *Saccharomyces cerevisiae* MTCC 174 inoculated at the level of 0.25 optical density (OD 600nm) at 30°C and 120 rpm for 72 hrs. An aliquot of fermented broth from the flask was withdrawn after 72 hrs incubation and estimated for ethanol content.

3.12.2 Saccharification potential of crude enzyme cocktail

An Erlenmeyer flask containing wheat bran (2%) in minimal media at 30°C, pH 5.0 and 120 rpm was subjected to hydrolysis by crude enzyme mixture at similar levels of activity units as obtained in section 3.12.1. The hydrolyzed broth was collected from a flask at every 12 hrs interval up to 72 hrs for estimation of glucose content to determine the optimum time at which maximum saccharification was achieved. Following this, a fresh flask containing 2% wheat bran and crude enzyme mixture having enzyme units similar to that used in “Saccharification potential of fungal biomass” was subjected to hydrolysis for the optimized time (36 hrs) identified above to bring about maximum saccharification. After the incubation period, the hydrolysate was collected and fermented with *Saccharomyces cerevisiae* MTCC 174 at the level of inoculum size equal to 0.25 optical density (OD 600nm) at 30°C and 120 rpm for 72 hrs. An aliquot

of fermented broth from the flask was withdrawn after 72 hrs incubation and estimated for ethanol content.

3.13 Decolorization of synthetic dyes

3.13.1 Dye decolorization by fungal mycelia

For decolorization experiments, five agar plugs (6 mm diameter) of active mycelium from PDA plate were transferred aseptically into 500 mL Erlenmeyer flasks containing 100 mL of potato dextrose broth with 60 mg/L of Congo red dye (CR), bromophenol blue dye (BB), coomassie Brilliant Blue R-250 (CBB R-250) and orange G (OG) dye and incubated at 30°C for 96 hrs at 120 rpm. A biotic (sterilized medium, without dye addition) and abiotic (sterilized medium containing the dye but not inoculated with the fungus) control experimental were also prepared and maintained in parallel with the decolorization experiments. Culture samples were withdrawn at a defined interval of 24 hrs up to 96 hrs, centrifuged at 10,000g for 20 min at 4°C and the supernatant obtained were used for decolorization assay. The intensity of dyes was measured at their maximum absorbance of 500 nm, 590 nm, 592 nm and 478 nm for CR, BB, CBB R-250 and orange G, respectively.

3.13.2 Dye decolorization by crude laccase

Stock solutions of CR, BB, CBB R-250 and OG dyes were prepared in sterilized distilled water and diluted to the required concentration of 60 mg/L. The decolorization of dyes by crude enzymes was carried out in 5 mL of reaction mixture containing 0.5 mM sodium acetate buffer (pH 5.0), 0.5 U/mL of the crude enzyme with either CR, BB, CBB R-250 or OG dye. The reaction tubes were incubated at 30°C for 240 min. The absorbance of the mixture at maximum wavelength was recorded at the wavelength specified above against each dye. A control test containing the same amount of a heat-denatured laccase was performed in parallel.

Decolorization efficiency (DE)

The decolorization efficiency (DE) was calculated according to the following formula: $DE = (A1-A2)/A1 \times 100\%$, where A1 represented the absorbance of the control, A2 represented the absorbance of the corresponding untreated sample, and DE was the dye color removal rate.

3.14 Statistical analysis

All the experiments were done in triplicates, and the results were analyzed statistically using Graph Pad Prism version 6.00 for Windows, Graph Pad Software, La Jolla California USA, www.graphpad.com.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Fungal cultures

A collection of nineteen pre-identified fungal strains collected from different geographical regions of North-Western Himalayan ranges in Himachal Pradesh were obtained from CSKHPKV, Palampur, India and were analyzed for lignocellulolytic activity. These nineteen fungal strains listed in Table 4.1 were screened for the presence of lignocellulolytic enzymes.

Table 4.1 List of nineteen fungal strains that were procured from CSKHPKV

S. No.	Strain Id	Fungal strains
1	3414	*Dardalea* sp.
2	1773	*Polyporus* (a) sp.
3	1674	*Mycopous* sp.
4	3300	*Astraeus* sp.
5	2687	*Hydnum* sp.
6	3216	*C. pannosa/ Thelephora sp.*
7	3250	*Peziza* sp.
8	2051	*Xylaria* sp.
9	2680	*Flammunlin* sp.
10	2974	*Chlorospelinium* sp.
11	3215	*Hypholoma* sp.
12	3328	*Centinus* sp.
13	2742	*Polyporus* (b) sp.
14	1678	*Coriolus* (a) sp.
15	3262	*Coriolus* (b) sp.
16	2249	*Stereum* sp.
17	3367	*Stereum* sp.
18	3367	*Lycogyla*
19	3325	*Sparasis sp.*

4.2 Chemical analysis of the substrate

The lignocellulosic compositions of selected substrates such as lolium grass, bromus grass, pine needles and wheat bran determined using standard protocols are presented

in Table 4.2. The determination of cellulose, hemicellulose and lignin content has been the focus of many other studies as it helps to ascertain the most suitable biomass for obtaining value added products such as bioethanol [20, 55, 61, 62].

Table 4.2 Composition of different lignocellulosic biomass used for optimization of lignocellulolytic enzyme production in the present study

Lignocellulosic substrates	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Lolium grass	31	28	12
Bromus grass	40	30	10
Pine needles	28	21	29
Wheat bran	33	29	5

The percentage of cellulose, hemicellulose, and lignin detected in wheat bran in our study fell in around the content reported by Gawande and Kamat (1999), Brijwani *et al.* (2010) and Maes *et al.* (2002) [256]. As wheat bran has a good hemicellulosic content rich in arabinans [257], it acts as a good source of soluble sugar like arabinose in addition to xylose and glucose. Also, hemicellulose as a whole itself is a good inducer of cellulolytic enzyme system [258].

In pine needles, the amount of cellulose detected was similar to that reported by Soma and Saito (1983) [259] and Johansson (1995) [260], amount of hemicellulose detected was in congruence with that reported by Johansson (1995) [260] and in close proximity to that reported by Font *et al.* (2009) [261], and the amount of lignin detected was similar to that reported by Dawson *et al.* (1998) [262], Ray *et al.* (2010) [263], and Ghosh and Ghosh (2011) [264].

In the temperate grasses, bromus and lolium, the amount of cellulose, hemicellulose and lignin obtained were in the range of that reported by Betts *et al.* (1991) [265] and Xu *et al*. (2007) [266].

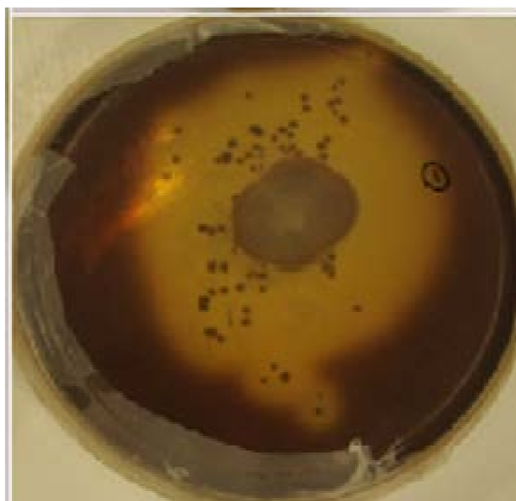
4.3 Screening of fungal cultures for lignocellulolytic enzyme activities

4.3.1 Qualitative assay

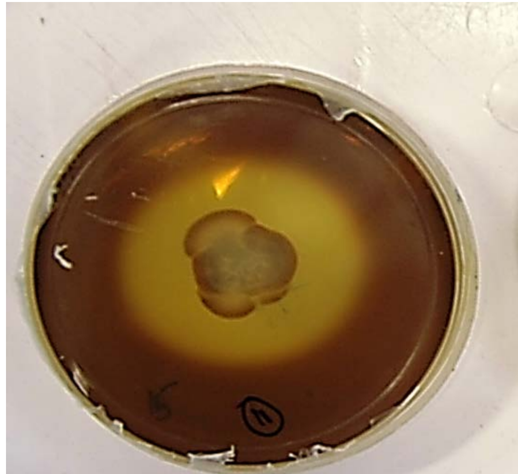
Fungal isolates were screened using plate assay technique, to evaluate the lignocellulolytic enzyme (cellulase, xylanase, and laccase) production by measuring the diameter of (a) hydrolysis zones in case of cellulase and xylanase and (b) brown colored halo in the case of laccase. Among the 19 fungal isolates screened for lignocellulolytic enzyme production, Strain F6 later identified as *C. pannosa* was observed to be the most efficient producer of lignocellulolytic enzymes. Qualitative assay based on agar plate method (Figure 4.1) showed that *C. pannosa* formed an 80 mm zone of clearance on CMC containing agar plate (cellulase activity), a 50 mm zone of clearance on birchwood xylan-containing agar plate (xylanase activity) and a brown coloured halo on guaiacol (0.25mM) containing agar plate (Laccase activity). Compared to our strain, *C. pannosa*, *Trichoderma reesei* MTCC 164 produced a smaller zone of clearance (38 mm) on CMC agar plates indicating the presence of lower cellulase activity.

Plate screening method has been reported as a suitable technique for screening of fungi capable of producing hydrolytic enzymes [267-269]. Plate assays are powerful tools employed in screening fungi for lignocellulose degrading enzyme production. These assays give a positive or negative indication of enzyme production. They are particularly useful in screening large numbers of fungal isolates for several classes of enzyme, where definitive quantitative data are not required. The reagents required are all commonly available and relatively inexpensive [270, 271].

a)



(b)



(c)

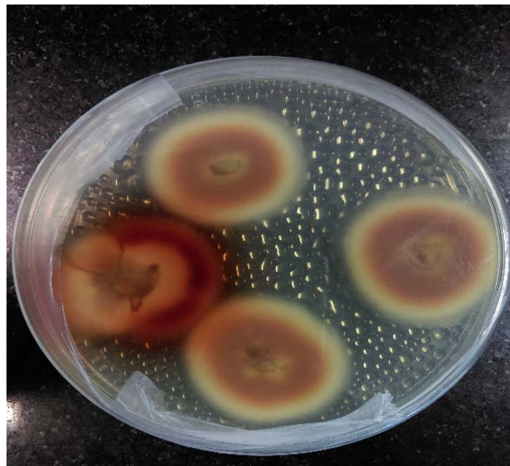


Figure 4.1 Plate assay showing the presence of (**a)** cellulase (80 mm zone), **(b)** xylanase (50 mm zone) and **(c)** laccase enzymes activities in *C. pannosa*

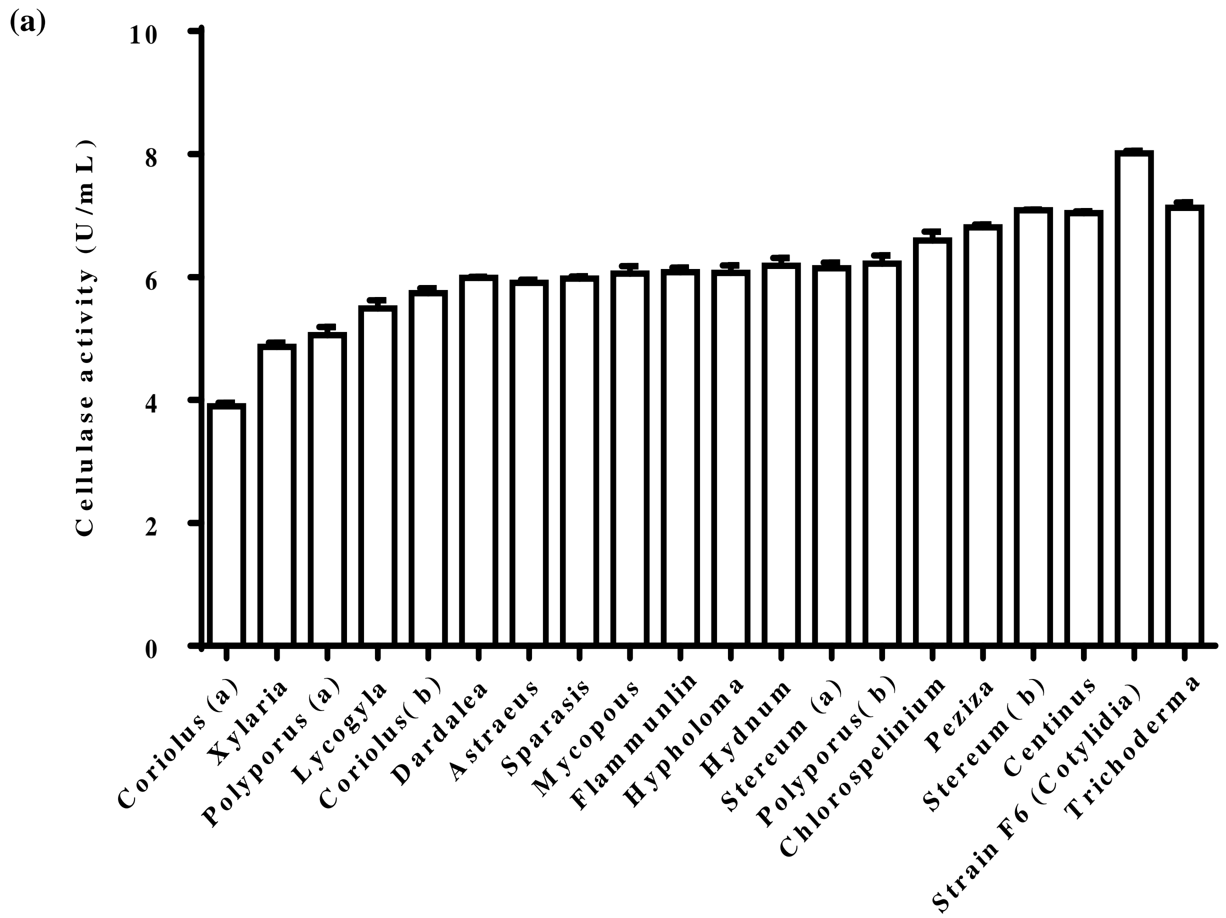
Plate assay based qualitative technique has frequently been used for screening of lignocellulolytic fungi. For example, a novel strain of *Trichoderma harzianum* and *Trichoderma viride* was screened for cellulase activity by carboxymethyl cellulose plate assay coupled with gram iodine staining [272, 273]. Kasana *et al.* (2008), reported gram iodine based plate assay as an easy and rapid method for detection of microbial cellulase [274]. This approach has also been used for screening fungal strains such as *Aspergillus niger, Trichoderma harzianum*, *Fusarium oxysporum*, *Aspergillus terreus*, *Aspergillus fumigatus* and *Aspergillus tamarii* for xylanase production [275].

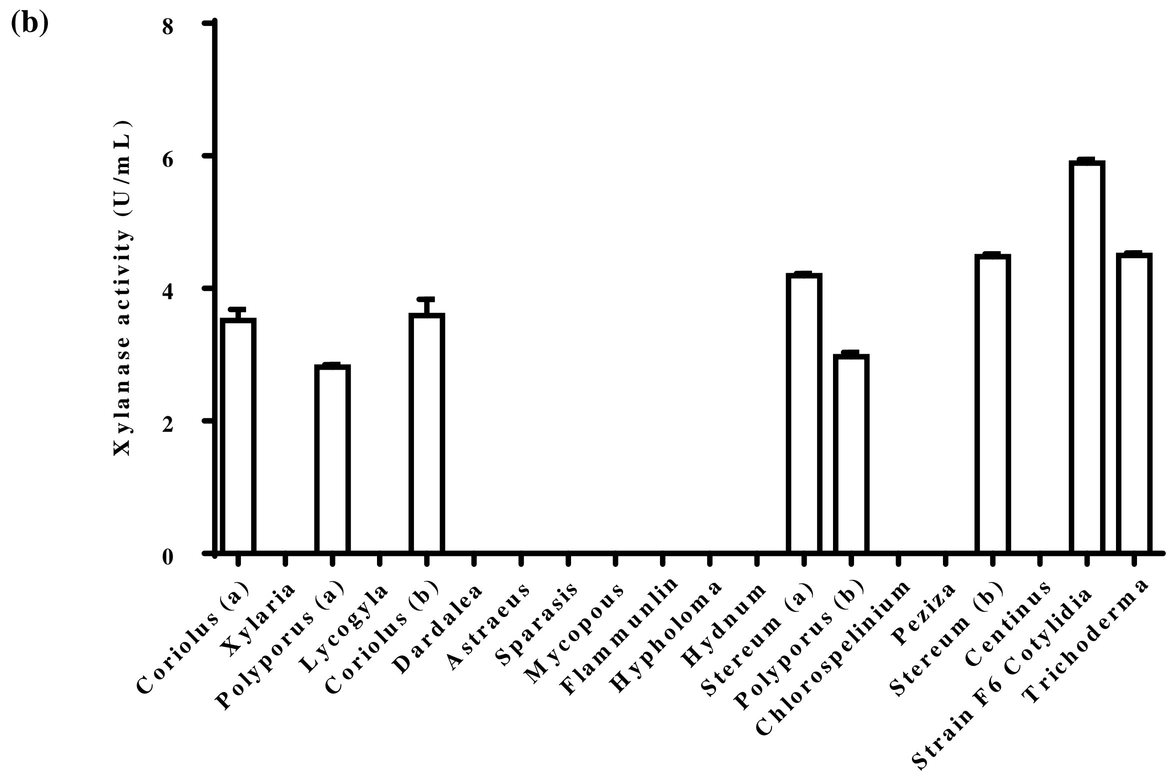
Guaiacol based plate assay for screening of laccase production has been reported by Devasia and Nair (2016). Guaiacol gave a positive reaction for laccase production as

indicated by the formation of a reddish-brown halo [276]. Guaiacol is an acutely sensitive substrate that permits rapid screening of fungal strains producing extracellular guaiacol oxidizing enzymes by means of a color reaction [277, 278].

4.3.2 Quantitative assay

Qualitative assay generally gives a more or less qualitative result of the lignocellulolytic enzyme producers with the clear (cellulase and xylanase) and colored (laccase) zones only able to distinguish producers from non-producers among the isolates. There was, therefore, a need for a more quantitative result to be able to distinguish the producers of lignocellulolytic enzymes among the isolates used. The results of the quantitative screening of selected strains are presented in Figure 4.2. The quantitative assay based on spectrophotometric method also indicated that the fungal isolate F6 possessed maximum cellulase (8.01 U/mL), xylanase (5.89 U/mL) and laccase (5.01 U/mL) activity. For rest of the 18 fungal strains, cellulase activity was observed within a range of 3.0 U/mL to 7.0 U/mL which was lower than the activity detected in strain F6 (later identified as *C. pannosa*). Furthermore, compared to our strain *C. pannosa*, *Trichoderma reesei* MTCC 164 which is one of the most commercially exploited strain, also exhibited a lower cellulase activity of 7.1 U/mL and xylanase activity of 4.5 U/mL respectively with no detection of laccase activity.


(a)
Cellulase activity (U/mL)
10
8
6
4
2
0
Coriolus (a)
Xylaria
Polyporus (a)
Lycogyla
Coriolus(b)
Dardalea
Astraeus
Sparasis
Mycopous
Flammunlin
Hypholoma
Hydnum
Stereum (a)
Polyporus(b)
Chlorospelinium
Peziza
Stereum(b)
Centinus
Strain F6 (Cotylidia)
Trichoderma


(b)
Xylanase activity (U/mL)
8
6
4
2
0
Coriolus (a)
Xylaria
Polyporus (a)
Lycogyla
Coriolus (b)
Dardalea
Astraeus
Sparasis
Mycopous
Flammunlin
Hypholoma
Hydnum
Stereum (a)
Polyporus (b)
Chlorospelinium
Peziza
Stereum (b)
Centinus
Strain F6 Cotylidia
Trichoderma

(c)

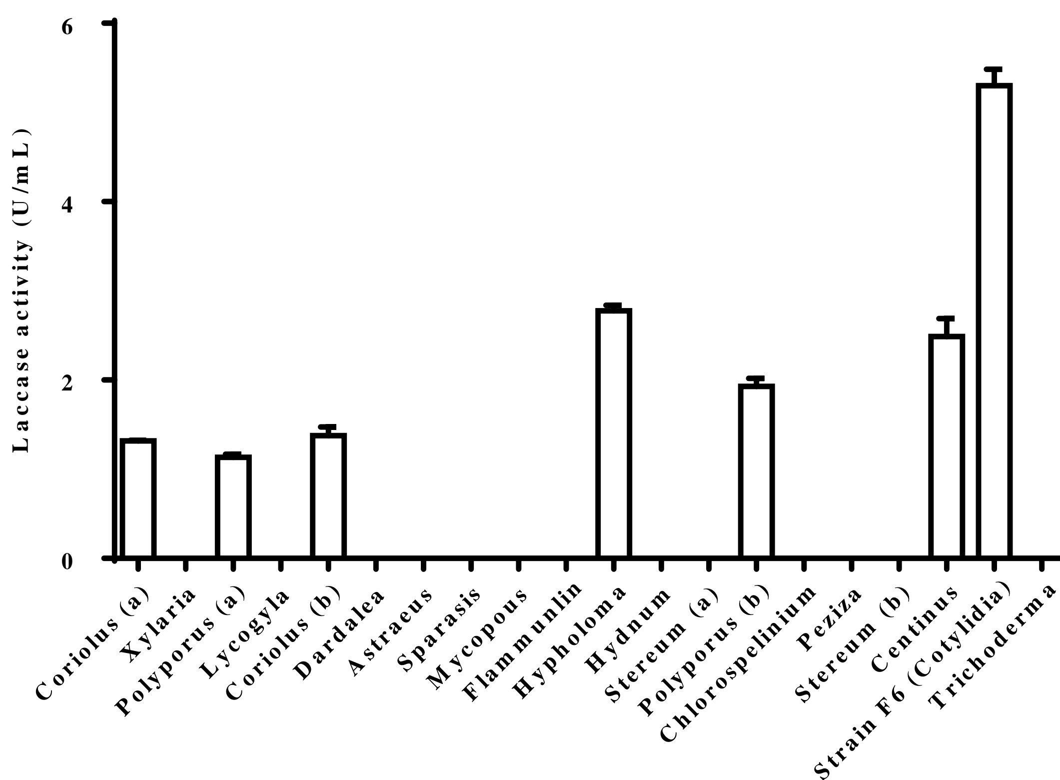


Figure 4.2 Quantitative assay intended for screening of 19 fungal isolates for **(a)** cellulase activity, **(b)** xylanase activity and **(c)** laccase activity

4.4 Morphological and molecular identification of most potential lignocellulolytic enzyme producing fungi

4.4.1 Morphological identification

The selected fungal strain F6 was observed to produced whitish mycelial on PDA. The spores began to appear approximately after 5 days of incubation at 30°C. The microscopic examination of the fungal isolate in a lactophenol cotton blue mount revealed the presence of ellipsoidal shaped spores and aseptate hyphae. When the isolate was directly inoculated on a smear of PDA medium on a microscopic slide, clamp connections were not observed (Figure 4.3). As the morphology was uninformative and ambiguous, molecular identification using internal transcribed spacer (ITS) region was performed.

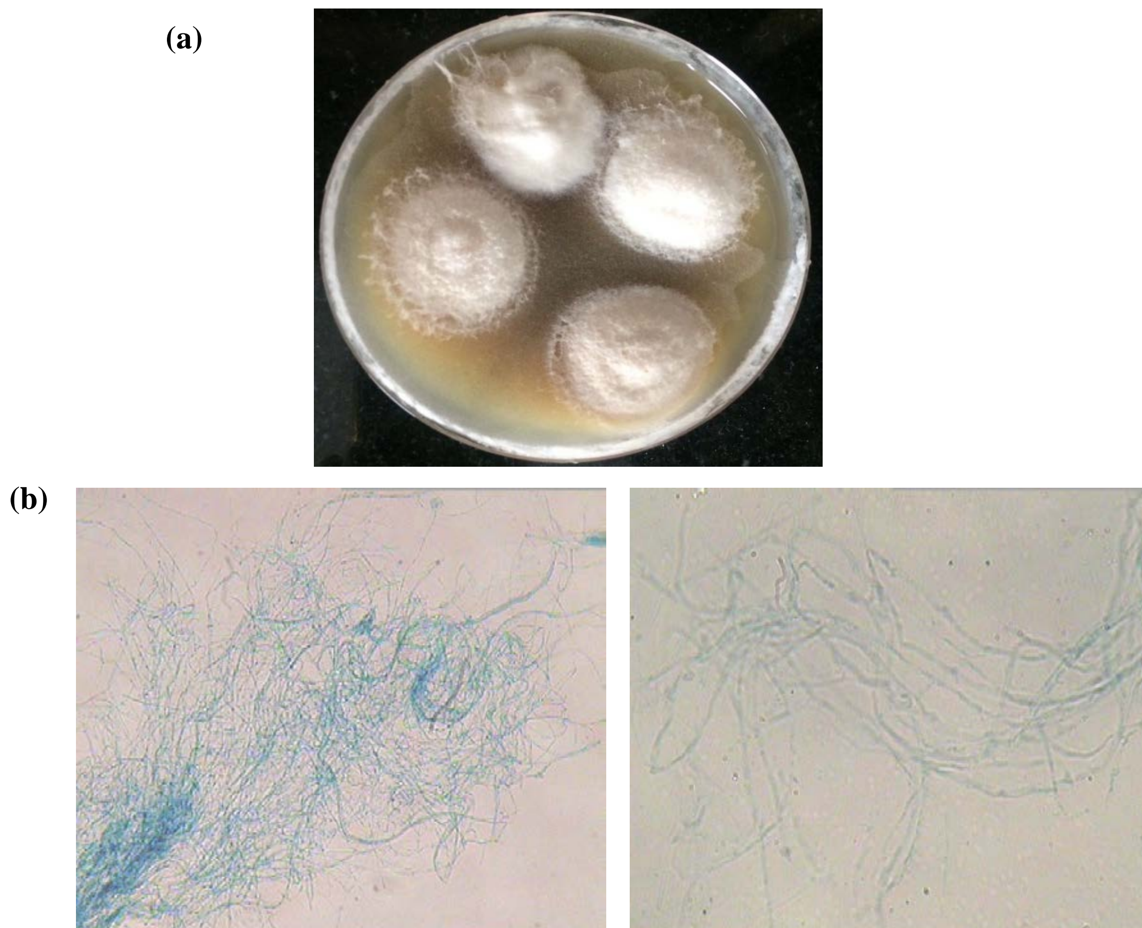


Figure 4.3 Morphological characteristics of *C. pannosa* **(a)** Mycelium morphology of *C. pannosa* on PDA plate and **(b)** Microscopic observation of hyphae of *C. pannosa* (10 x, 40 x)

4.4.2 Molecular identification using Internal transcribed spacer (ITS) region

Among the 19 fungal isolates, strain designated as F6 exhibited maximum lignocellulolytic enzyme activity. Hence, it was subjected to molecular identification. DNA for the selected fungal isolate was isolated. The ITS region of rDNA was amplified using PCR by employing universal primers (ITS1 and ITS4). It has been reported that in comparison to sequences from coding regions, using ITS regions gives a clearer taxonomic resolution [279]. Fungal DNA contains large copy numbers of ITS region as part of the tandemly repeated nuclear rDNA. This ITS region together with PCR forms a sensitive assay. The amplified product was 656 bp in length. Similar sequences were collected using BLAST program in NCBI database, and a phylogenetic tree was constructed from a dataset consisting of 50 sequences aligned with fungal

strain F6. BLAST search of the complete sequence of ITS region revealed that strain F6 identified as *Cotylidia pannosa* was in close homology of 96% with *Podoscypha petalodes* isolate DK09. The sequence of the isolate was deposited in the GenBank database and assigned the accession number KT008117. The bootstrapped unrooted tree was structured by the neighbor joining method from the distance data generated by alignment of the nucleotide sequence. Our fungal strain identified as *C. pannosa* formed a clade with *Podoscypha petalodes* isolate DK09 with an alignment score of 96.93%, based on Clustal analysis (Figure 4.4).

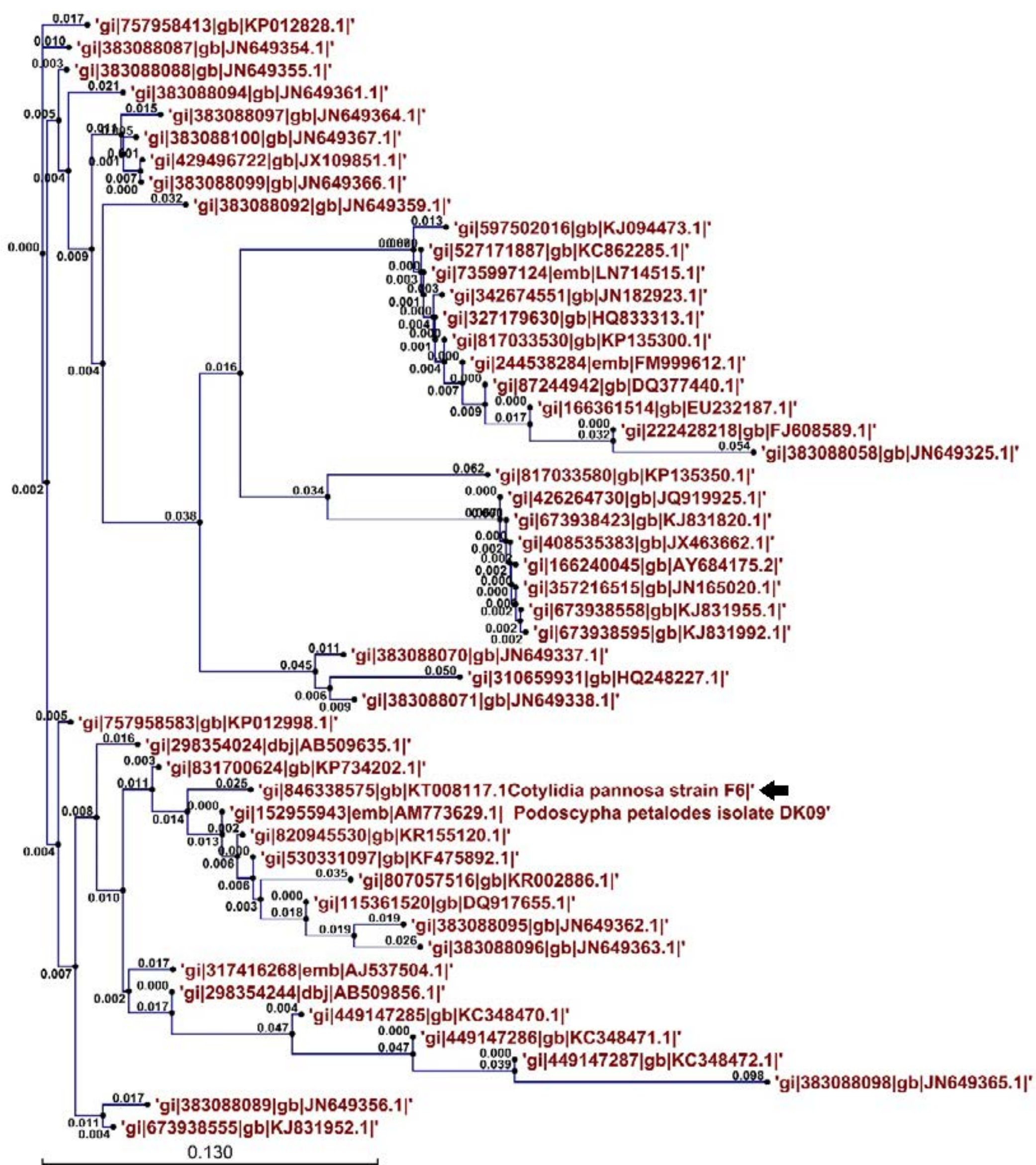


Figure 4.4 Maximum likelihood phylogenetic tree derived from Internal Transcribed sequence data of select F6. Bootstrap support values above 50% are indicated above the branches

4.5 Total cellulase activity (Filter paper activity)

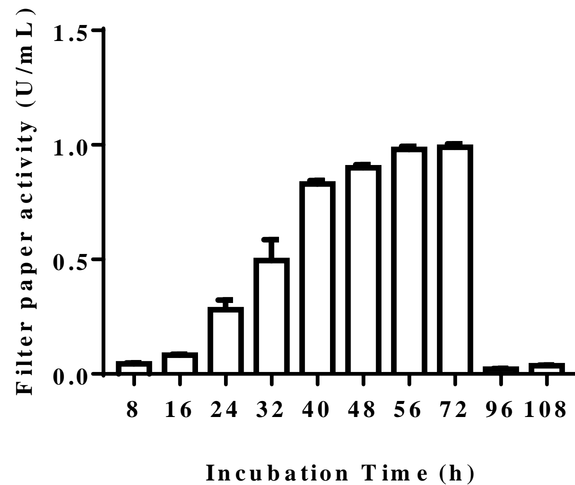


Figure 4.5 Total cellulase activity (Filter paper activity) of *C. pannosa* in submerged fermentation using wheat bran as substrate

To compare the efficacy of cellulase activity between microorganisms or their secreted enzymes, techniques for measuring total cellulase activity are required. Globally, screening of total cellulase activity is done chiefly using the International Union of Pure and Applied Chemists (IUPAC) standard filter paper assay (FPA). This assay employs the dinitrosalicylic acid (DNS) method to estimate the reducing sugars released from 50 mg of Whatman #1 filter paper by a complex cellulase mixture [244]. The filter paper assay is the key technique for analyzing total cellulase activity. Our stain *C. pannosa* exhibited a total cellulase activity of 1.0 U/mL after 96 hrs as shown in Figure 4.5. This activity was found to be better than most other fungi as can be seen from the following examples. Oguntimein *et al.* (1992) reported a filter paper activity of 0.142 U/mL with Chinese bagasse and 0.073 U/mL with Mexican bagasse as substrates using *Neurospora sitophila* [280]. Wu *et. al.* (2016) reported a total cellulase activity of 0.96 U/mg in *A. wangii* at week 2 [281]. The white rot fungus *Irpex lacteus* CD2 was reported to possess a filter paper activity of ~2.5 IU/g [282]. In a study by Elisashvili *et al.* (2009) white rot fungi *C. maxima, C. polyzona, F. trogii, P. coccineus,* and *P. ostreatus* were found to have a filter paper activity of 1.8±0.2 U/mL, 1.0±0.1 U/mL, 1.7±0.2 U/mL, 1.0±0.1 U/mL and 2.6±0.2 U/mL using tree leaves as substrate [283]. *Sporotrichum pulverulentum and Trichoderma reesei* QM9414 have been shown to possess a filter paper activity of 0.22 U/mL and 0.55 U/mL respectively [284].

4.6 Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged and solid-state fermentation using one factor at a time approach

4.6.1 Selection of lignocellulosic substrates

The cellulase activity in *C. pannosa was* estimated by supplementing 2% of different lignocellulosic substrates in YEP medium, the results of which are shown in Figure 4.6 The data indicated wheat bran as the potential substrate for production of cellulase by *C. pannosa* in comparison to other substrates (filter paper taken as reference). The cellulase produced by *C. pannosa* using wheat bran as substrate was found to have a maximum activity of 8.48 U/mL. On the basis of these results, wheat bran was selected for further optimization study.

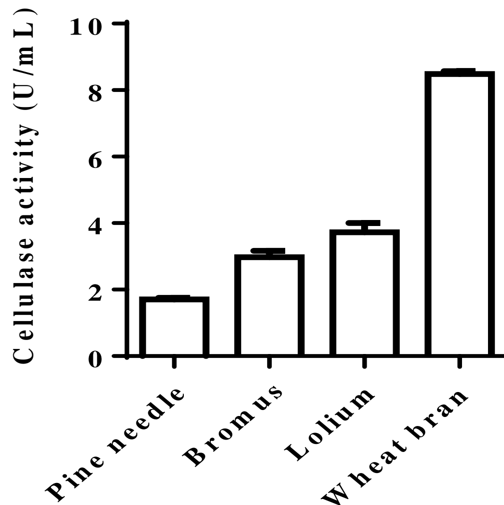


Figure 4.6 Effect of the different substrates on cellulase activity of *C. pannosa*

Among the various lignocellulosic substrates, wheat bran resulted in the maximum cellulases activity. The carbon source such as wheat bran plays an important role in enzyme production which is evident from several studies carried out using various cellulolytic microorganisms. The characteristic features of wheat bran that make it a promising substrate for inducing production of cellulases are high protein content (13–19%), high hemicellulose content (higher than 30%), and low lignin content (3–6%) [285]. The high protein content makes wheat bran a substrate with well-balanced C/N

ratio, which is crucial to obtain specific bioproducts from submerged state fermentation [286].

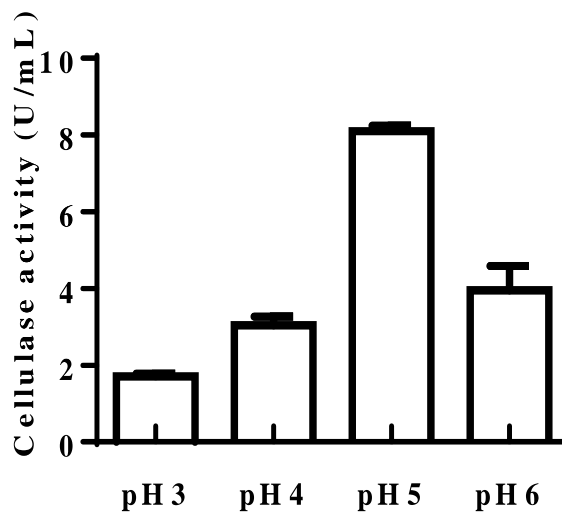
4.6.2 Effect of saccharification parameters on enzyme production under submerged fermentation (SmF)

Based on the results obtained from the qualitative and quantitative assays, the effects of saccharification parameters on lignocellulolytic enzyme production in submerged fermentation using *C. pannosa* were studied. Among the different fermentation parameters for production of enzymes, the pH of the substrate, incubation temperature and time play a vital role in the metabolic activity of the microbial cell. Therefore, the aforementioned parameters were taken into consideration for optimizing lignocellulolytic enzyme production by *C. pannosa*.

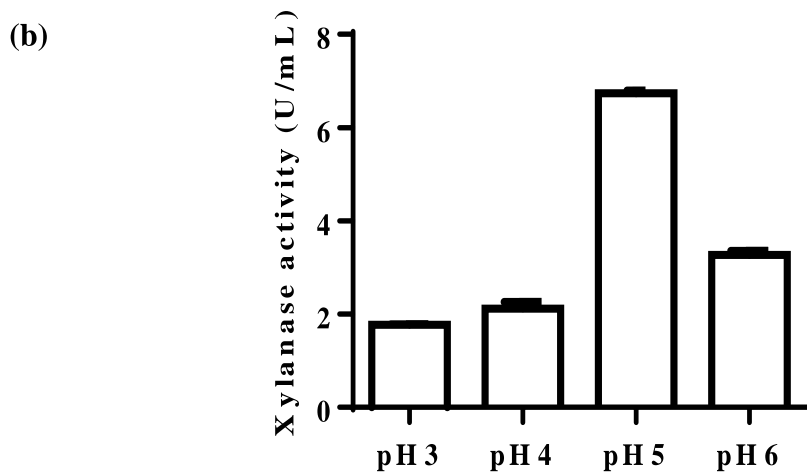
4.6.2.1 Effect of pH

In order to establish a suitable application in saccharification of lignocellulosic wastes, the production of lignocellulolytic enzymes by *C. pannosa* was optimized under submerged fermentation conditions using wheat bran as the carbon source. The maximum lignocellulolytic activity for all the three enzymes i.e. cellulase, xylanase, and laccase was detected at a pH of 5.0 (Figure 4.7).

(a)



(b)



(c)

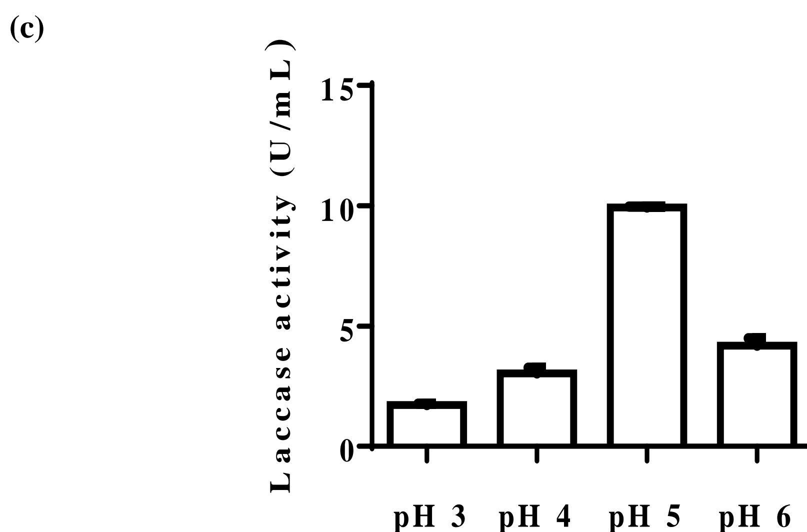


Figure 4.7 Effect of pH on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *C. pannosa* in submerged fermentation

It has been observed that pH higher or lower than the optimum pH causes a decrease in the production of enzymes primarily because of the fact that enzyme production by mold cultures is known to be very sensitive to pH [287]. pH is a major factor affecting the production of enzymes as well as the growth of microorganisms. The enzymes being proteins contains ionizable groups, consequently the pH of the culture medium affects their structure and function. Thus, development of an optimal pH control strategy is helpful in obtaining higher enzyme production by efficient fungal strains.

The maximum cellulase enzyme activity for *C. pannosa* using wheat bran (2%) was detected at pH 5.0 which is in accordance with the work done on *Aspergillus niger* wherein the authors reported maximum cellulase enzyme activity of 5.57 U/mL at pH 5.0 using wheat bran as the substrate [288]. Our results were comparable to those of the positive control strain *T. reesei* MTCC 164 showing a cellulase activity of 7.1 U/mL with wheat bran at 30°C [289].

Studies involving *Penicillium* sp. [290, 291] and various other fungal species [292, 293] have shown that the most appropriate pH value for xylanase activity lies within the acidic region. In a study by Izidoro *et al.* (2014), *Aspergillus niger* was reported to exhibit maximum xylanase activity (4.72±0.04 U m/L) at a pH of 5.0 [294] which is in congruence with the observations reported in the present study wherein maximum xylanase activity was also obtained at pH 5.0. In yet another study, *Colletotrichum graminicola* was also reported to produce maximum xylanase (34.6±4.9 U /mL) activity at pH 5.0 [295].

The maximum laccase activity in YEP with 2% wheat bran was also detected at a pH of 5.0. It has been reported that when fungi are grown in a medium pH optimal for their growth, laccase is generally produced in excess as was observed in *A. bisporus* [296]. Fungal laccases are reported to be generally active at low pH values (pH 3.0 to pH 5.0) [297]. This observation is also in accordance with the identification of an optimum pH of 5.0 for laccase production by *T. versicolor* [298].

4.6.2.2 Effect of temperature

Another important factor governing the production of enzymes is the temperature of the medium. The maximum lignocellulolytic activity for all the three enzymes i.e. cellulase, xylanase, and laccase was detected at a temperature of 30°C (Figure 4.8).

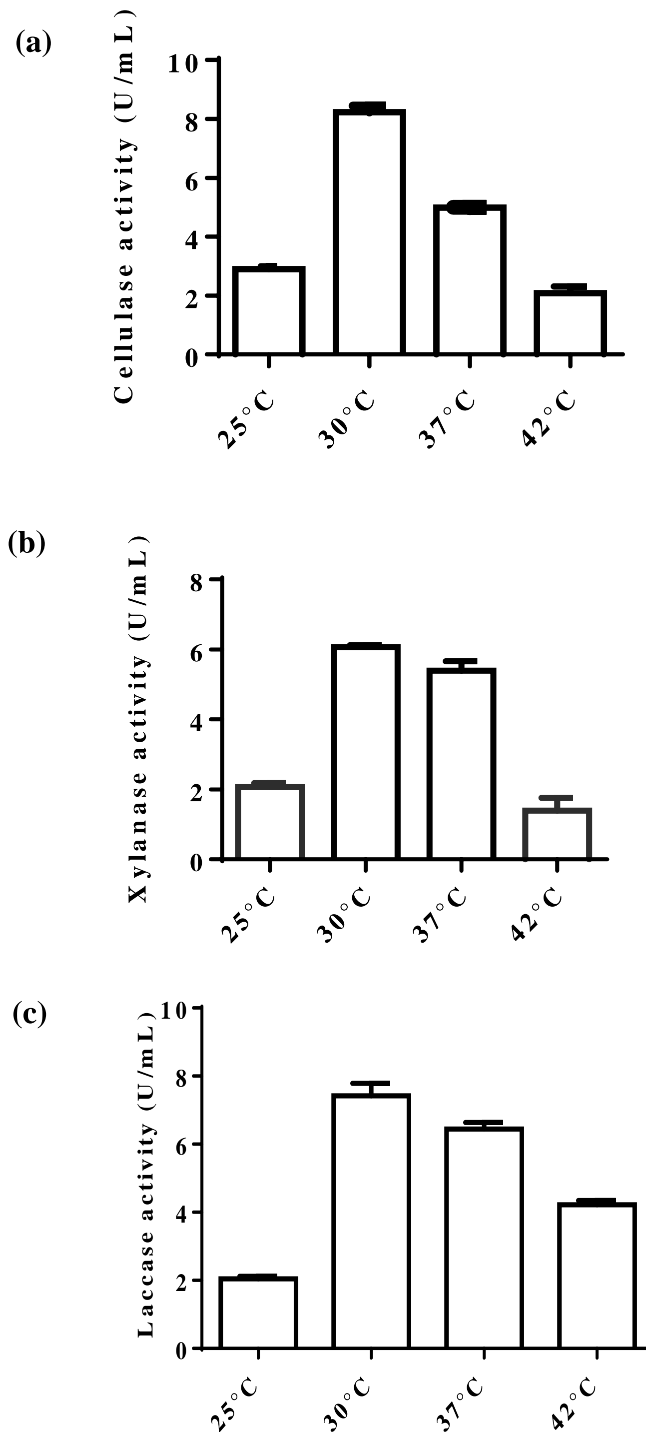


Figure 4.8 Effect of temperature on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *C. pannosa* in submerged fermentation

For all the three lignocellulolytic enzymes there was an increase in enzyme activity as the temperature was raised to 30°C followed by a decrease in activity as the temperature was increased beyond the optimum level. The decline in enzyme activity on increasing

the temperature beyond optimal levels is probably due to alterations in the membrane composition, protein catabolism and induction of sporulation which hampers the mycelial growth in fungus [30, 299].

This observation is in accordance with a previous study in which an increased cellulase production (540 IU/L) was observed with wheat bran using *Ceratocystis paradoxa*, as the temperature was raised to 30°C and thereafter the production of enzyme was observed to decline [213]. In an another study, maximal cellulase activity of 1.30 IU/mL was reported by submerged fermentation of wheat bran at 30°C using *Trichoderma reesei* QM9419 mutant [300]. Even though the optimum temperature (30°C) in the above cases was similar to that in our study with *C. pannosa*, the enzyme activity was lesser than that obtained in our study. The optimum temperature for cellulase production was akin to those of other mesophilic fungi such as *Aspergillus japonicas* C03 [301], *Aspergillus glaucus* XC8 [302], *A. niger* MS82 [303], *Trichoderma reesei* Rut C30 [304], *Trichoderma viride* strain EU2-77J [305], *Penicillium echinulatum* [306], and *Fusarium oxysporum* [307].

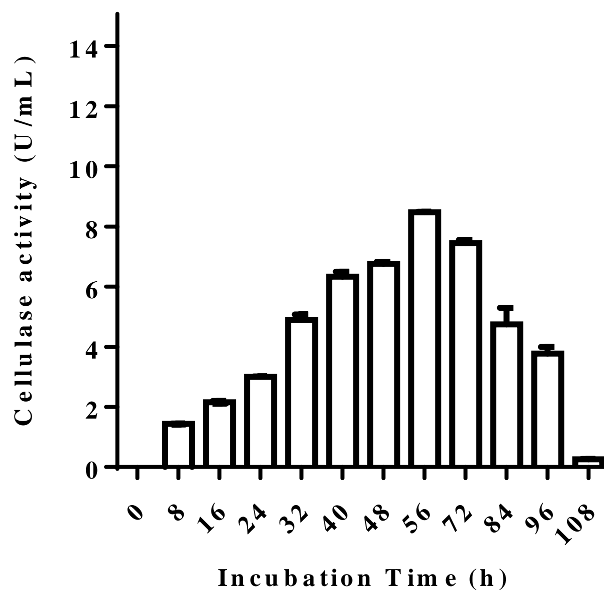
For xylanase production, a lower activity has been reported when the cultivation temperatures were lower or above the ambient temperature. The results obtained indicated that the enzyme production corresponded closely to temperature optima around room temperature. A similar observation was made by Chirstakopoullos *et al.* (1996) and Biswas *et al.* (1988 and 1990) who exhibited that the highest xylanase activities were obtained at temperatures that were optimum for the growth of the *Fusarium solani F7* fungi at room temperature [308-310]. Our study also revealed similar observations. *Phanerocheate chrysosporium*, *Pleurotus ostreatus,* and *Ganoderma lucidum* have also been reported to have a similar optimum temperature (25-35°C) for growth as well as the production of lignocellulose degrading enzymes [311].

The maximum laccase activity in YEP with 2% wheat bran was detected at a temperature of 30°C. As observed for cellulase and xylanase, further increase in temperature resulted in a decrease in laccase activity which is in congruence with a report on *Agaricus bisporus* where the maximum laccase is produced at 30°C [296]. In general, the fungi are cultivated at temperatures between 25°C and 30°C for optimal laccase production [312]. When cultivated at temperatures higher than 30°C the activity of ligninolytic enzymes was found to diminish [313].

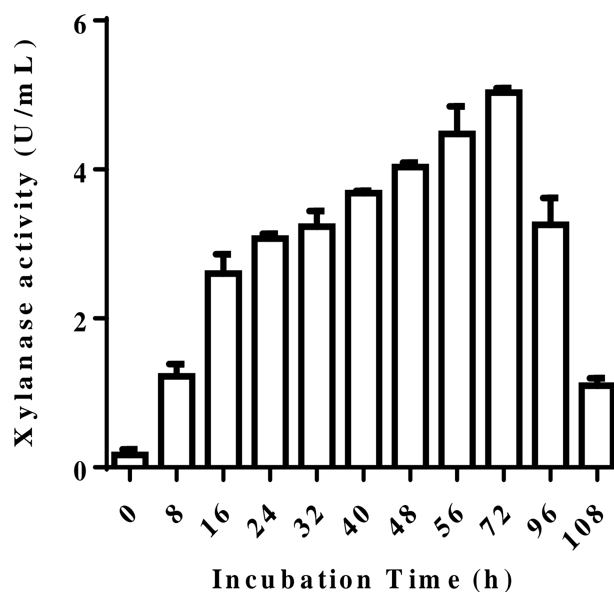
4.6.2.3 Effect of incubation time

As shown in Figure 4.9, *C. pannosa* showed maximum cellulase activity of 8.44 U/mL after an incubation time of 56 hrs, xylanase activity of 5.0 U/mL and laccase activity of 7.23 U/mL after 72 hrs.

(a)



(b)



(c)

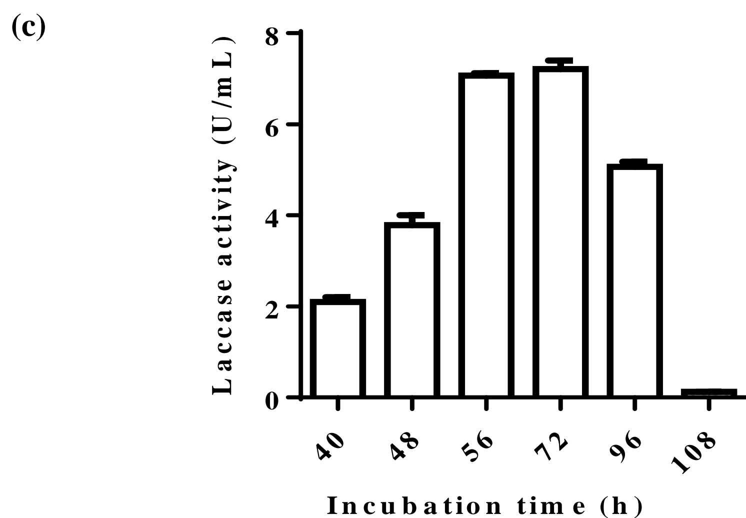


Figure 4.9 Effect of incubation time on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *C. pannosa* in submerged fermentation

A short optimum incubation time for achieving maximum enzyme activity is preferable because the high amount of enzyme activity can be achieved in reduced time. The high enzymatic yield in a short production period would be advantageous in terms of its industrial applications over other fungal lignocellulolytic enzymes requiring longer production periods.

In numerous studies, the maximum cellulase production was achieved after a prolonged incubation time in comparison to *C. pannosa* (56 hrs). For example, *Aspergillus awamori* 2B.361 U2/1 has been shown to give maximum cellulase enzyme activity (0.000248 U/mL) when the incubation was carried out for 96 hrs using wheat bran [314]. In another study, *Trichoderma ressei* NCIM 992 was reported to give maximum cellulase enzyme activity of 2.63 U/mL with wheat bran after the sixth day of incubation [315].

In a study by Saleem *et al.* (2014), maximum xylanase activity of 272.74, 278.52 and 292.86 IU/mL was obtained at pH 5.0 after 216 hrs by *P. sordida* MRL3, *L. pigrinus* MRL6, and *P. caliatus* MRL7 respectively [316]. *C. pannosa*, on the other hand, gives maximum activity at pH 5.0 in relatively lesser time (72 hrs). In an another study, *C. versicolor* was reported to exhibit a continuous increase in xylanase activity throughout the fermentation process with maximum activity (2565 U/L) being achieved on the 14th

day [317]. Okafor *et al.* (2007) reported that the maximum xylanase activity (0.95 U/mL) obtained by *Aspergillus niger* ANL 301 was at 96 hrs [318].

Earlier reports on *Trametes hirusta* indicated maximum laccase enzyme activity (7.614 U/mL) after an incubation time of 20 days using wheat bran [319]. In another study, *G. lucidum* was reported to give maximum laccase enzyme activity of 2.7 U/mL after the 14th day of incubation with wheat bran [220]. According to the observations on other white rot fungal laccases, our strain holds a potential of higher activity using wheat bran as the substrate in relatively reduced time (72 hrs).

The attainment of maximum enzyme activity in the present study under moderate conditions of temperature and pH (30°C and 5.0) in comparatively reduced incubation time (Table 4.3) makes *C. pannosa* a potential candidate for application in various industries such as bioethanol, textile, cellulose, and paper industries.

Table 4.3 Optimized variables with the corresponding lignocellulolytic enzyme activities in submerged fermentation

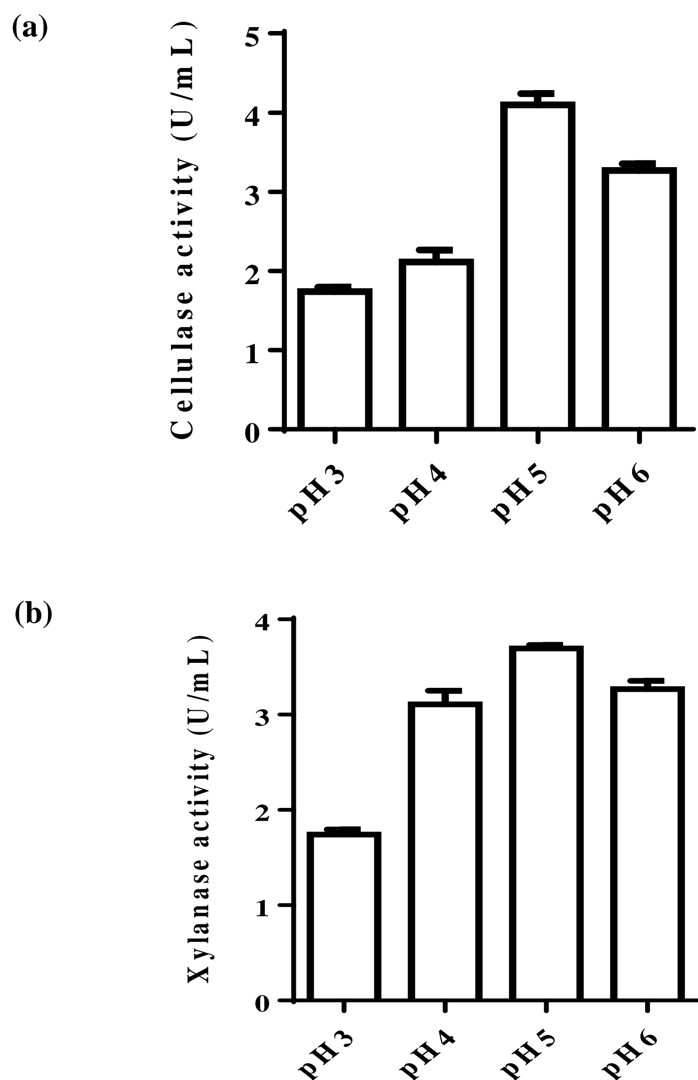
Lignocellulolytic enzyme activity	**pH 5.0**	**Temperature 30 (°C)**	**Incubation time (hrs; 56 hrs for cellulase and 72 hrs for xylanase and laccase)**
Cellulase activity (U/mL)	8.1	8.22	8.44
Xylanase activity (U/mL)	6.74	6.0	5.0
Laccase activity (U/mL)	10.0	7.42	7.23

4.6.3 Effect of saccharification parameters on enzyme production under solid state fermentation

SSF has been reported for higher yields of enzymes from fungal isolates. Therefore, the lignocellulolytic enzyme production from *C. pannosa* was also explored using SSF. SSF was conducted with different parameters such as pH, temperature, incubation time at a moisture level of 100% to investigate if there was any increase in lignocellulolytic enzyme production.

4.6.3.1 Effect of pH

The production of lignocellulolytic enzymes by *C. pannosa* was also optimized under solid state fermentation using wheat bran as the carbon source at different pH. The maximum lignocellulolytic activity was detected at a pH of 5.0 (Figure 4.10).



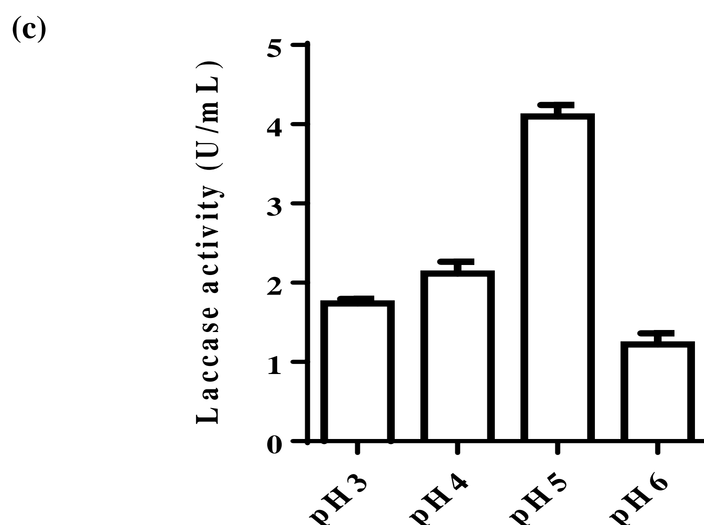


Figure 4.10 Effect of pH on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *C. pannosa* in solid state fermentation

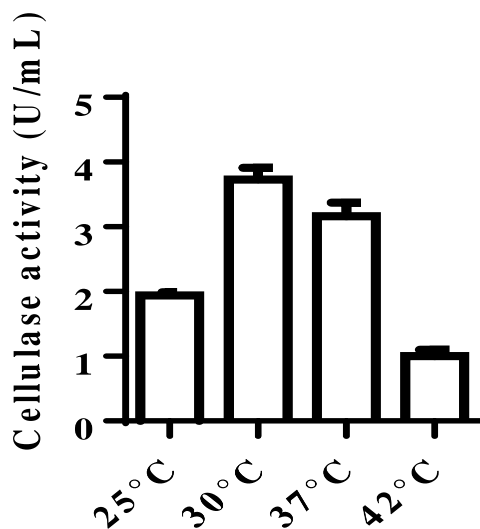
In a study where lignocellulolytic enzyme production was performed under conditions of solid state fermentation, *Trichoderma viride* showed a cellulase activity of 3.18 U/mL at pH 4.8 [199]. Amid the numerous factors influencing enzyme production, the pH of the medium is connected to the enzymatic adsorption, and hence significantly affects the saccharification progression. Another study reported the maximum cellulase activities of 9.50 IU/mL in the case of *A. flavus* AT-2 and 12.73 IU/mL in the case of *A. niger* AT-3 at pH 4.8 and 5.3, respectively [320]. A further increase in pH reportedly diminished the cellulase activity of fungal strains. The primary reason for this observed decrease in enzyme activity at higher pH has been accredited to the proteolytic inactivation of the cellulase. Effect of pH on the production of lignocellulolytic enzymes by fungi has been reported in numerous studies where it was reported that while slightly acidic pH values favored enzyme production, the activity decreased gradually as the pH was increased further [321, 322]. This observation substantiates our results wherein a similar pattern of lignocellulolytic enzyme production was observed with respect to varying pH. The H+ concentration in the fermentation medium thereby had a profound effect on the enzyme production.

4.6.3.2 Effect of temperature

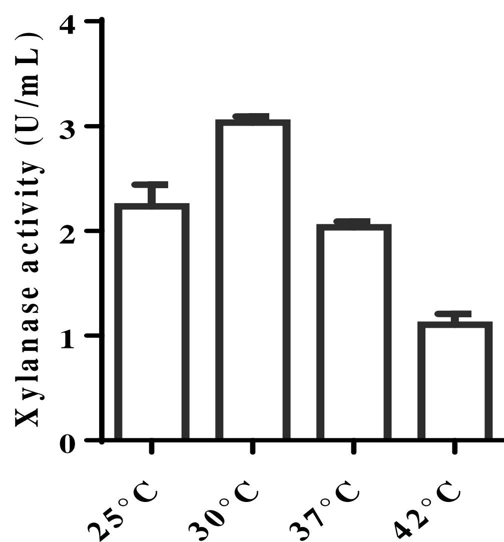
Effect of temperature on lignocellulolytic enzyme activity was also studied in solid state fermentation. Maximum lignocellulolytic activity was obtained at a temperature of

30°C (Figure 4.11) which is in congruence with various reports according to which the majority of the white-rot fungi explored till date are reportedly mesophilic in nature having an optimum temperature in the range 15°C to 35°C [323].

(a)



(b)



(c)

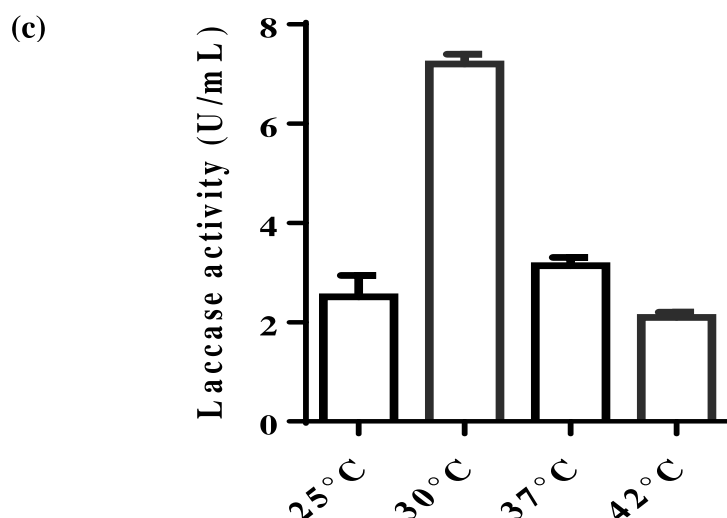


Figure 4.11 Effect of temperature on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *C. pannosa* in solid state fermentation

Numerous studies have identified varying optimal temperatures for production of enzymes by white-rot fungi under SSF conditions. For example, the optimal temperatures for producing the maximum amount of lignocellulolytic enzymes using *P. chrysosporium* have been reported as 39°C [324] and 30°C [325]. In yet another study the optimum temperature at which *P. ostreatus* produces the maximum amount of laccase was reported to be 28°C [326]. The observed variance in the optimal temperature of different fungi under SSF for the biological pretreatment of lignocellulosic biomass is correlated with fungal physiology, fungal strain, and substrate type. The metabolism of white-rot fungi's during delignification engenders heat which may raise the fermenting material's temperature to the extent that it inhibits lignocellulolytic enzyme production by limiting the growth of microorganisms [327].

4.6.3.3 Effect of incubation time

Lignocellulolytic enzyme activities were also estimated in solid state fermentation at varying incubation times. As shown in Figure 4.12, *C. pannosa* showed maximum cellulase activity of 4.33 U/mL after an incubation time of 96 hrs, xylanase activity of 5.23 U/mL after an incubation time 56 hrs and laccase activity 5.22 U/mL after 72 hrs.

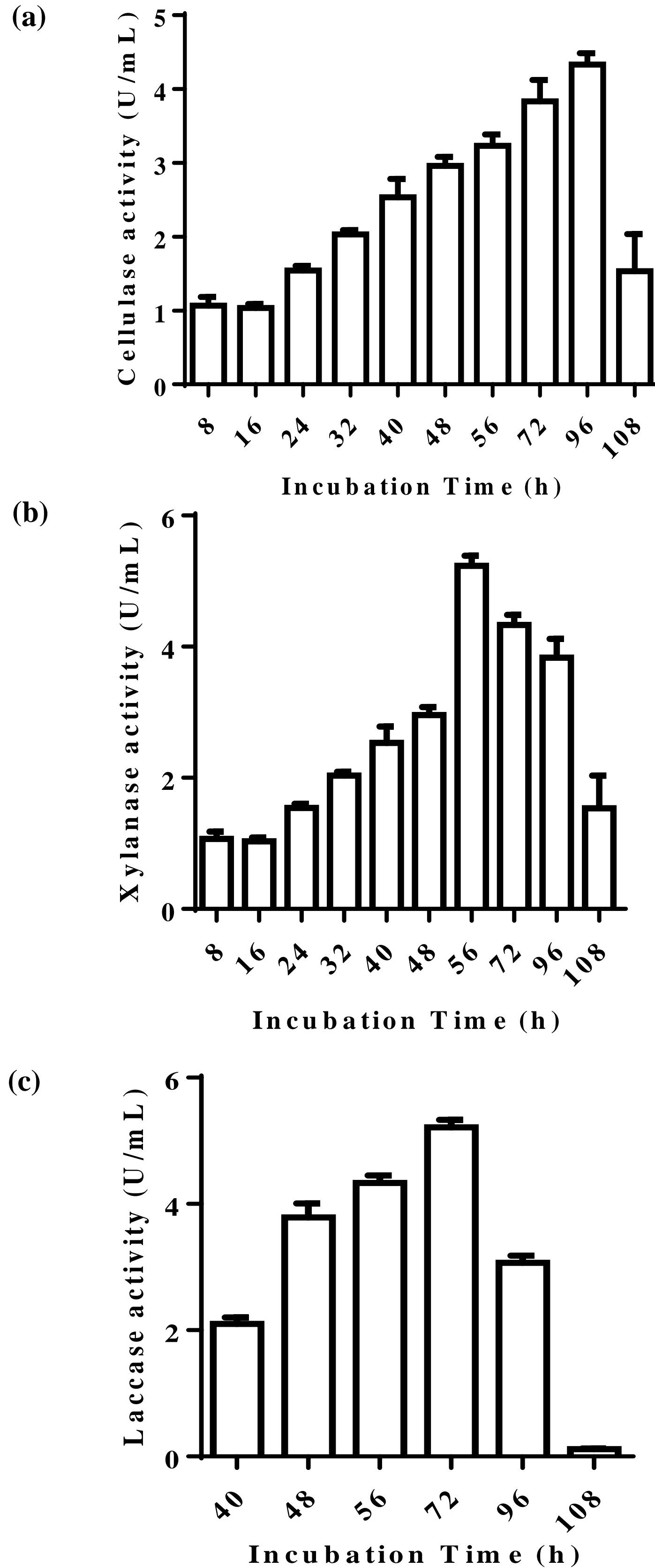


Figure 4.12 Effect of incubation time on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *C. pannosa* in solid state fermentation

The optimum time in which maximum lignocellulolytic enzymes were produced was determined by assaying samples collected at different time intervals. In our case, the maximum lignocellulolytic activities were achieved in relatively reduced time of incubation compared to various other reports. For example, maximum cellulase activity of 2.63 U/mL by *Trichoderma reesei* NCIM 992 under SSF was obtained after an incubation time of 6 days [328]. Similarly, Ghoshal *et al.* (2016) reported an incubation time of 5 days as the optimum time required for attaining maximum xylanase production in SSF by *P. citrinum* [205]. *T. versicolor*, an excellent producer of ligninolytic enzymes, was also found to produce maximum laccase and MnP activity of 1.54 U/mL and 1.63 U/mL, respectively, after 14 days of fermentation [329].
The optimum parameters identified for solid-state fermentation with the corresponding activities obtained at those parameters have been summarized in Table 4.4

Table 4.4 Optimized variables with the corresponding lignocellulolytic enzyme activities in solid-state fermentation

Lignocellulolytic enzyme activity	**pH 5.0**	**Temperature 30 (°C)**	**Incubation time (hrs; 96 hrs for cellulase and 56 hrs for xylanase and 72 hrs laccase)**
Cellulase activity (U/mL)	4.1	3.07	4.33
Xylanase activity (U/mL)	3.6	3.0	5.23
Laccase activity (U/mL)	4.1	7.2	5.22

Investigating the production of lignocellulolytic enzymes in submerged and solid-state fermentation revealed that *C. pannosa* performs more efficiently under conditions of submerged fermentation as compared to solid-state fermentation (Table 4.5).

Table 4.5 Comparison of Lignocellulolytic enzyme activities using SmF and SSF

Lignocellulolytic enzyme activity	**Maximum activity (U/mL) obtained in SmF**	**Maximum activity (U/mL) obtained in SSF**
Cellulase activity (U/mL)	8.44	4.33

Xylanase activity (U/mL)	6.74	5.23
Laccase activity (U/mL)	10.0	7.0

In submerged fermentation microbes are cultivated on solid substrates dissolved or submerged in liquid. The limited availability of water in SSF makes it pretty different from SmF. In comparison to solid state fermentation, the majority of investigations on enzyme production methods involve the production of fungal or bacterial biomass in submerged conditions. In SmF transfer of heat and mass is more efficient. Scaling-up of the process is also a lot easier [330]. It is simpler to control pH, temperature and nutrient levels under conditions of submerged fermentation when the fungi are growing in the presence of cellulosic feedstock [331]. The requirement of water by microorganisms for growth and metabolic activities along with the potential of water activity of the medium in controlling biotechnological processes is well established [332]. Thus it is anticipated that the elevated moisture content of the medium would result in an increased productivity of the system. Recent investigations have shown disparities in productivity of enzymes by organisms due to differing biochemical and physiological responses of these organisms in SSF v/s SmF.

4.7 Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged fermentation using Response Surface Methodology (Multifactorial approach)

Temperature, pH, incubation time and agitation rate are the critical factors in SmF and their importance in enzyme production has been well established. The results obtained from One-factor-at-a-time approach was used to identify the concentration levels of these parameters using RSM. The process variables of SmF, i.e., temperature (Temp), pH, incubation time (IT) and agitation rate (Agtn) were selected as input variables, and experiments were executed based on CCD for developing a second order polynomial response surface model for lignocellulolytic enzyme production by *C. pannosa* (Table 4.6).

Table 4.6 Central Composite design matrix with actual values of variables along with the observed and predicted values of cellulase, xylanase and laccase activities

Input parameters					**Response** (U/mL)					
Run	Temp[a] (°C)	pH	IT[b] (hrs)	Agtn[c] (rpm)	Cellulase		Xylanase		Laccase	
Orde	(A)	(B)	(C)	(D)	(Exp.)	(Predict.)	(Exp.)	(Predict.	(Exp.)	(Predict.)
1	25	4	120	150	16.24	15.81	14.25	13.05	2.78	2.87
2	32.5	5	72	100	16.5	16.51	14.7	15.6	12.1	11.77
3	40	4	120	50	4.86	4.95	6.59	6.23	0.4	0.54
4	25	4	24	150	12.22	11.85	6.21	6.95	0.13	0.28
5	25	6	24	150	11.33	11.02	2.16	1.44	1.38	0.90
6	32.5	5	120	100	13.45	13.35	12.89	13.55	6.9	6.74
7	25	6	120	150	11.12	11.80	3.40	4.25	2.86	3.06
8	40	4	24	50	0.18	-0.70	2.14	2.05	0	-0.21
9	32.5	4	72	100	14.9	15.77	14.56	15.07	11.9	12.1
10	32.5	6	72	100	15.7	16.47	11.48	12.26	10.9	12.08
11	40	4	120	150	8.1	8.51	5.4	6.3	1.62	2.11
12	25	5	72	100	11.8	12.9	8.6	9.16	4.19	4.90
13	32.5	5	72	100	16.5	16.51	14.7	15.57	12	11.77
14	25	6	24	50	9.45	8.85	1.71	1.58	0	-0.50
15	25	4	24	50	4.7	4.97	5.1	4.94	0	0.05
16	32.5	5	72	100	17.5	16.5	16.7	15.57	12	11.77
17	40	6	120	50	7	7.18	6.1	6.12	0.05	-0.11
18	40	6	24	150	6.2	6.06	2.91	3.27	0.55	0.94
19	32.5	5	72	100	17.5	16.51	16.7	15.57	13	11.77
20	40	4	24	150	5.3	5.38	3	2.25	0.54	0
21	32.5	5	72	100	17.5	16.51	16.7	15.57	13	11.77
22	32.5	5	72	100	17.5	16.51	16.7	15.57	13	11.77
23	32.5	5	72	50	17.26	18.33	17.56	18.1	11.3	12.58
24	40	6	24	50	4.49	4.70	5.1	5.21	0	-0.43
25	40	6	120	150	6.51	6.02	4.97	4.04	3.02	2.63
26	32.5	5	72	100	17.5	16.51	16.7	15.57	11.6	11.77
27	32.5	5	72	150	20.63	21.19	17.3	18.05	14	14.07

[a] Temperature; [b] Incubation Time; [c]Agitation

These experiments were performed in triplicates and cellulase, xylanase and laccase activity of *C. pannosa* were expressed as a non-linear function of the input process parameters as follows:

Cellulase (U/mL) = -121.7 + 6.948 Temp (°C) + 6.15 pH + 0.4592 IT (hrs) - 0.0775 Agtn (rpm) - 0.1152 Temp (°C)*Temp (°C) - 0.389 pH*pH - 0.002070 IT (hrs)*IT (hrs) + 0.001302 Agtn (rpm)*Agtn (rpm) + 0.0505 Temp (°C)*pH - 0.000577 Temp (°C)*IT (hrs) - 0.000537 Temp (°C)*Agtn (rpm) - 0.01651 pH*IT (hrs) - 0.02357 pH*Agtn (rpm) 0.000262 IT (hrs) *Agtn (rpm)

Xylanase (U/mL) = -141.3 + 7.341 Temp (°C) + 12.89 pH + 0.4048 IT (hrs) - 0.1069 Agtn (rpm) - 0.1279 Temp (°C)*Temp (°C) - 1.907 pH*pH - 0.001635 IT (hrs)*IT (hrs) + 0.001002 Agtn (rpm)*Agtn (rpm) + 0.2178 Temp (°C)*pH - 0.001424 Temp (°C)*IT (hrs) - 0.001204 Temp (°C)*Agtn (rpm) - 0.01705 pH*IT (hrs) - 0.01074 pH*Agtn (rpm) 0.000014 IT (hrs)*Agtn (rpm)

Laccase (U/mL) = -116.8 + 8.081 Temp (°C) - 3.98 pH + 0.3824 IT (hrs) - 0.1490 Agtn (rpm) 0.1251 Temp (°C)*Temp (°C) + 0.319 pH*pH - 0.002497 IT (hrs)*IT (hrs) + 0.000625 Agtn (rpm)*Agtn (rpm) + 0.0109 Temp (°C)*pH - 0.000331 Temp (°C)*IT (hrs) - 0.000015 Temp (°C)*Agtn (rpm) - 0.00222 pH*IT (hrs) + 0.00583 pH*Agtn (rpm) + 0.000142 IT (hrs)*Agtn (rpm)

The predictive ability of developed non-linear regression models was further confirmed through significance test and ANOVA tests. Significance test results for cellulase, xylanase and laccase activities are tabulated in Table 4.7. In the case of cellulase, individual, square and interaction effects of SmF variables seemed to have a significant effect ($P<0.05$) except the individual, square and interaction effects of pH. In the case of xylanase, except the individual and interaction effects of agitation, the individual, square and interaction effects of other SmF variables (Incubation time, temp and pH) determined the significant impact ($P<0.05$). Compared to cellulase and hemicellulase, the important factors that determined the laccase activity included only the individual and square effects of incubation time and agitation.

Table 4.7 Results of significance test on the non-linear model-coefficients, standard errors, T statistics, and *P* values for cellulase (U/mL), xylanase (U/mL) and laccase (U/mL)

Term	Coef	SE Coef	T - Value	P - Value
Cellu.[a], xylan.[b], Lacc.[c]	Cellu.[a], xylan.[b], Lacc.[c]	Cellu.[a], xylan.[b], Lacc.[c]	Cellu.[a], xylan.[b], Lacc.[c]	Cellu.[a], xylan.[b], Lacc.[c]
Constant	16.51, 15.57, 11.77	0.29, 0.31, 0.27	57.55, 50.38, 43.30	0.00, 0.00, 0.00
Temp	-2.86, -0.78, -0.17	0.23, 0.25, 0.22	-12.57, -3.17, -0.80	0.00, 0.01, 0.44
pH	0.39, -1.41, -0.01	0.23, 0.25, 0.22	1.53, -5.73, -0.04	0.15, 0.00, 0.97
IT	1.61, 1.75, 0.73	0.23, 0.25, 0.22	7.06, 7.12, 3.37	0.00, 0.00, 0.00
Agtn	1.43, -0.02, 0.74	0.23, 0.25, 0.22	6.28, -0.08, 3.44	0.00, 0.94, 0.00
Temp*Temp	-6.48, -7.19, -7.04	0.60, 0.65, 0.57	-10.79, -11.12, -12.38	0.00, 0.00, 0.00
pH*pH	-0.39, -1.91, 0.32	0.60, 0.65, 0.57	-0.65, -2.95, 0.56	0.53, 0.01, 0.58
IT*IT	-4.77, -3.77, -5.75	0.60, 0.65, 0.57	-7.94, -5.82, -10.12	0.00, 0.00, 0.00
Agtn*Agtn	3.25, 2.50, 1.56	0.60, 0.65, 0.57	5.42, 3.87, 2.75	0.00, 0.00, 0.01
Temp *Ph	0.38, 1.63, 0.08	0.24, 0.26, 0.23	1.57, 6.27, 0.36	0.14, 0.00, 0.72
Temp*IT	-0.21, -0.51, -0.12	0.24, 0.26, 0.23	-0.86, -1.97, -0.52	0.40, 0.07, 0.61
Temp.*Agtn	-0.20, -0.45, -0.01	0.24, 0.26, 0.23	-0.83, -1.73, -0.02	0.42, 0.10, 0.98
pH*IT	-0.79, -0.82, -0.11	0.24, 0.26, 0.23	-3.28, -3.14, -0.47	0.01, 0.01, 0.65
pH*Agtn	-1.18, -0.54, 0.29	0.24, 0.26, 0.23	-4.87, -2.06, 1.27	0.00, 0.06, 0.22
IT*Agtn	-0.63, -0.03, 0.34	0.24, 0.26, 0.23	-2.61, -0.13, 1.49	0.02, 0.90, 0.16
S = 0.97, R-Sq = 98.20%, R-Sq(adj) = 96.63% (For cellulase)				
S = 1.04, R-Sq = 98.14%, R-Sq(adj) = 96.50% (For xylanase)				
S = 0.91, R-Sq = 98.45%, R-Sq(adj) = 97.10% (For laccase)				

[a] cellulase; [b] xylanase; [c] laccase

From the results of ANOVA test (Table 4.8), significant contributors towards cellulase activity were observed to be the linear, squared, and interaction terms of temp, pH, and agitation. In the case of xylanase activity, the linear, square and interaction effects of temp, incubation time, pH were also observed to play a significant role. The most significant contributions for laccase activity included the linear, square and interaction effects of incubation time and agitation.

Table 4.8 ANOVA for quadratic model for cellulase (U/mL), xylanase (U/mL) and laccase (U/mL)

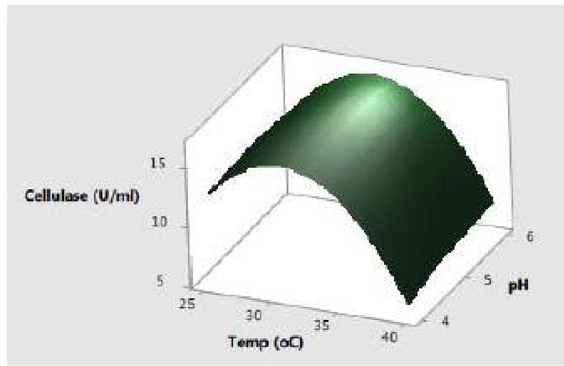
Source	DF	Adj SS	Adj MS	F - Value	P - Value
C. [a], X. [b], L. [c]	C. [a], X. [b], L. [c]	C. [a], X. [b], L. [c]	C. [a], X. [b], L. [c]	C. [a], X. [b], L. [c]	C. [a], X. [b], L. [c]
Model	14, 14, 14	816.61, 914.46, 854.46	58.33, 65.32, 61.03	62.36, 60.15, 72.74	0.00, 0.00, 0.00
Linear	4, 4, 4	233.38, 101.55, 20.00	58.34, 25.39, 5.00	62.38, 23.38, 5.96	0.00, 0.00, 0.00
Temp.	1, 1, 1	147.70, 10.88, 0.53	147.67, 10.89, 0.53	157.90, 10.02, 0.63	0.00, 0.01, 0.44
pH	1, 1, 1	2.178, 35.60, 0.00	2.18, 35.60, 0.00	2.33, 32.79, 0.00	0.15, 0.00, 0.97
IT	1, 1, 1	46.66, 55.06, 9.53	46.66, 55.06, 9.53	49.89, 50.71, 11.36	0.00, 0.00, 0.00
Agtn.	1, 1, 1	36.84, 0.01, 9.94	36.84, 0.01, 9.94	39.39, 0.01, 11.84	0.00, 0.94, 0.00
Square	4, 4, 4	540.98, 747.40, 830.73	135.24,186.85, 207.68	144.59, 172.08, 247.50	0.00, 0.00, 0.00
Temp.*Temp.	1, 1, 1	108.95, 134.24, 128.57	108.95,134.24, 128.57	116.48, 123.63, 153.22	0.00, 0.00, 0.00
pH*pH	1, 1, 1	0.39, 9.44, 0.26	0.39, 9.44, 0.26	0.42, 8.69, 0.32	0.53, 0.01, 0.58
IT*IT)	1, 1, 1	59.03, 36.83, 85.87	59.03, 36.83, 85.87	63.11, 33.92, 102.33	0.00, 0.00, 0.00
Agtn.*Agtn.	1. 1, 1	27.48, 16.28, 6.33	27.48, 16.28, 6.33	29.38, 14.99, 7.54	0.00, 0.00, 0.01
2-Way Inter	6, 6, 6	2.26, 65.51, 3.73	7.04, 10.92, 0.62	7.53, 10.06, 0.74	0.00, 0.00, 0.62
Temp.*pH	1, 1, 1	2.29, 42.69, 0.11	2.30, 42.69, 0.11	2.45, 39.31, 0.13	0.14, 0.00, 0.73
Temp.*IT	1, 1, 1	0.69, 4.21, 0.23	0.69, 4.21, 0.23	0.74, 3.87, 0.27	0.40, 0.07, 0.61
Temp.*Agtn	1, 1, 1	0.65, 3.26, 0.00	0.65, 3.26, 0.00	0.69, 3.01, 0.00	0.42, 0.10, 0.98
pH*IT	1, 1, 1	10.05, 10.72, 0.18	10.05, 10.72, 0.18	10.74, 9.87, 0.22	0.01, 0.01, 0.65
pH*Agtn.	1, 1, 1	22.23, 4.62, 1.36	22.23, 4.62, 1.36	23.76, 4.25, 1.62	0.00, 0.06, 0.22
IT*Agtn.	1, 1, 1	6.35, 0.02, 1.86	6.35, 0.02, 1.87	6.79, 0.02, 2.21	0.02, 0.90, 0.16
Error	16, 16, 16	4.97, 17.37, 13.43	0.93, 1.09, 0.84		
Lack-of-Fit	10, 10, 10	13.54, 11.66, 11.20	1.35, 1.17, 1.12	5.69, 1.22, 3.02	0.02, 0.42, 0.09

Pure Error	6, 6, 6	1.43, 5.71, 2.22	0.24, 0.95, 0.37		
Total	30, 30, 30	831.58, 931.83, 867.88			

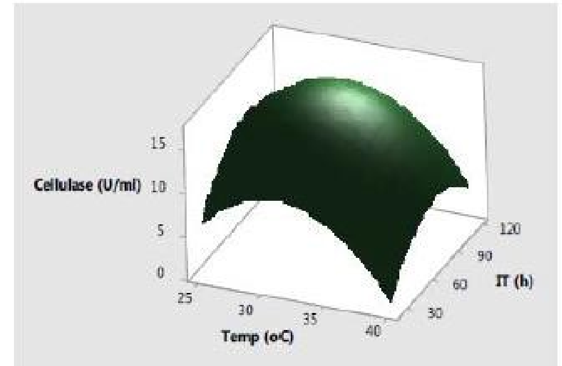
[a] cellulase; [b] xylanase; [c] laccase

The fitness and adequacy of the developed non-regression model was further confirmed through the R^2 and adjusted R^2 values (Cellulase: R^2-98.20%, Adj. R^2 - 96.63%; Xylanase: R^2-98.14%, Adj. R^2 - 96.503%; Laccase: R^2-98.45%, Adj. R^2 - 97.10%). Further, the interaction effects of variables selected for the production of enzymes were studied by plotting three-dimensional surface curves to determine the optimum level of each variable for maximum enzyme activity.

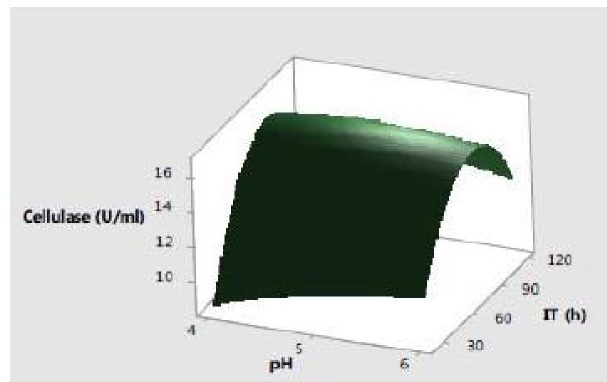
The interaction effects of SmF variables on cellulase activity are depicted in response surface plots from Figure 4.13 (a − c). Figure 4.13 (a) indicated that the higher cellulase activities were observed at intermediate values of temperature and pH. Later on, irrespective of increase or decrease in pH and temperature conditions, no significant effect on cellulase production was observed. The pH of the substrate plays a critical role in the production of lignocellulolytic enzymes by affecting either the microbial growth or denaturating the enzymes. The change in pH is also reported to affect the transport of various components across the cell membrane and denaturation of the enzyme activities. Figure 4.13 (b) showed that the increase in temperature with incubation time resulted in higher cellulase production with a maximum cellulase production at 32°C in 72 hrs. Usually, the optimum temperatures and incubation time for lignocellulolytic enzymes production vary with the use of different strains. The cumulative effect of pH and incubation time on cellulase production (Figure 4.13 (c)) revealed that the higher cellulase production was mainly recorded in the intermediate values of pH and incubation time with decreased trend after pH of 5.5 and prolonged incubation time of 90 hrs. This type of behavior is mainly attributed to the inefficient transport across membranes at higher pH conditions and denaturation of cellulase activity at longer incubation times.



(a)



(b)



(c)

Figure 4.13 Response surface plots showing the effect of **(a)** temperature and pH **(b)** temperature and incubation time and **(c)** pH and incubation time on cellulase production

Figure 4.14 (a) showed that the maximum xylanase production was obtained around 32°C and pH 5.0 after median values of incubation time. Further increase in temperature and incubation time resulted in significant decrease in xylanase activities (Figure 4.14 (b)). From cumulative graph Figure 4.14 (c), maximum xylanase production was observed at pH 5.0 after 92 hrs of incubation.

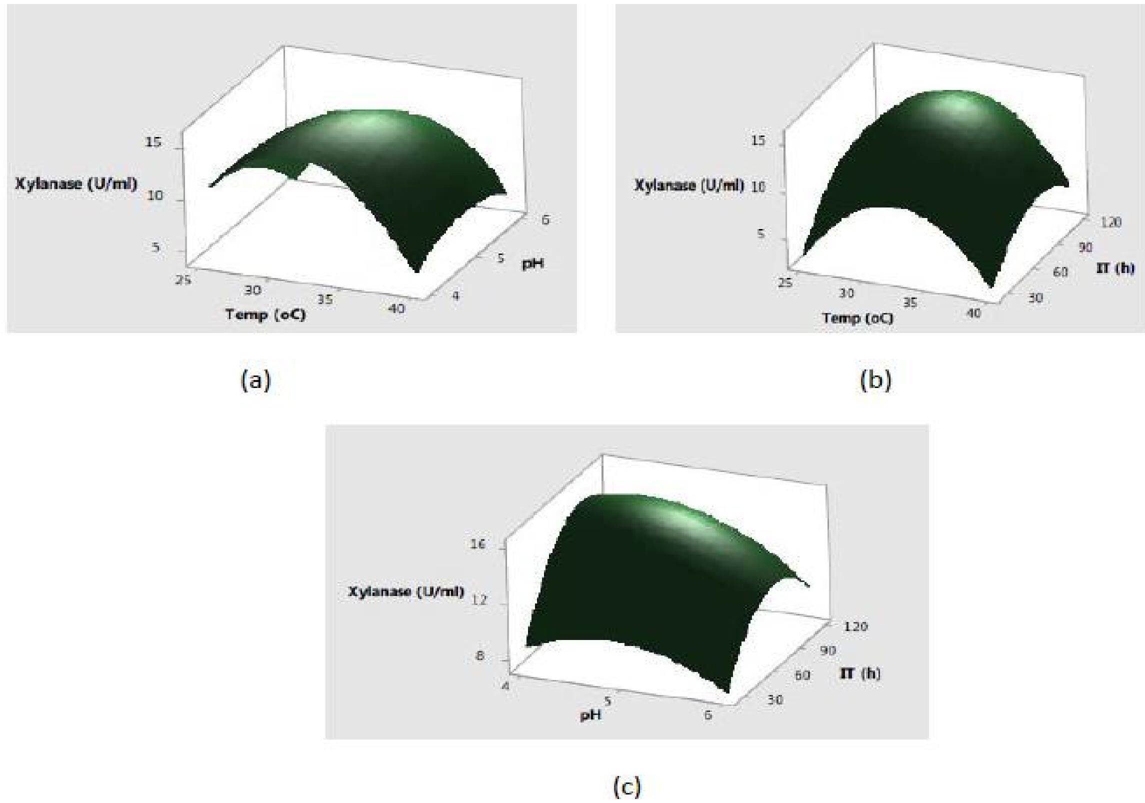


Figure 4.14 Interaction effects of **(a)** temperature and pH (**b**) temperature and incubation time and **(c)** pH and incubation time on xylanase production

The cumulative effect of submerged fermentation variables on laccase production are presented in Figure 4.15 (a − c). The results in Figure 4.15 (a) indicated that the higher laccase activity was obtained at intermediate values of temperature with no significant effect of a change in pH. The combined effect of temperature and incubation time on laccase production (Figure 4.15 (b)) demonstrated that the maximum laccase production could be achieved at average values of temperature and incubation time. Moreover, the significant effect of incubation time and non-significant behavior of change of pH on laccase production was also observed in Figure 4.15 (c).

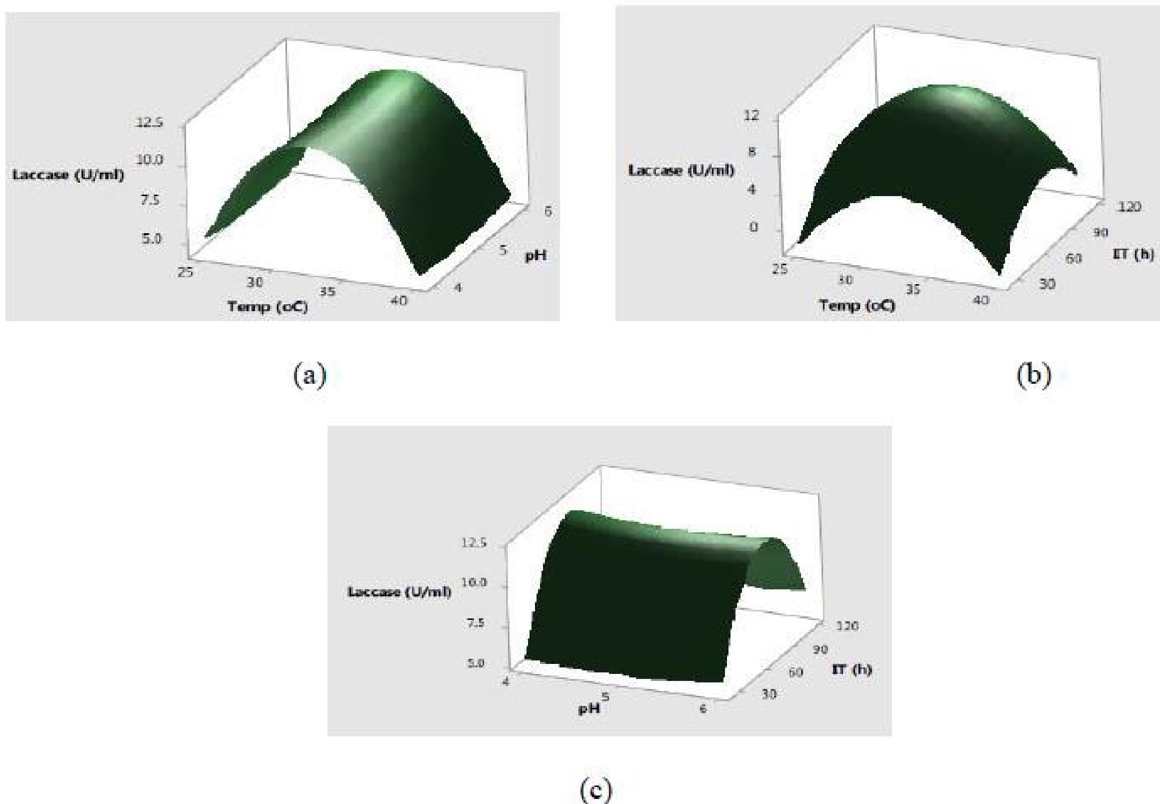


Figure 4.15 Effect of **(a)** temperature and pH, **(b)** temperature and incubation time and **(c)** pH and incubation time on laccase production

Response optimizer function of MINITAB 16 was used to predict the submerged fermentation variables for optimal cellulase, xylanase and laccase activities. It was observed that for all the three lignocellulolytic enzymes the optimum variables identified via RSM (pH of 5.0, temperature of 31°C, agitation rate of 140 rpm and an incubation time of 77 hrs) were identical. The experimental results i.e. 20.6 U/mL of cellulase activity, 17.3 U/mL of xylanase activity and 14 U/mL of laccase activity obtained through triplicate runs by utilizing the predicted variables, were in close agreement with the predicted outputs i.e. cellulase: 20.1 U/mL; xylanase: 17.2 U/mL; laccase: 13.1 U/mL. The close agreement between the predicted and experimental responses indicated the adequacy and accuracy of the model for further scale-up studies. Moreover, the lignocellulolytic enzyme activities attained through this study determined significant improvement over the one variable at a time (OVAT) experiments i.e. cellulase: 10 U/mL; xylanase: 7 U/mL; laccase: 5 U/mL. The higher activities obtained through RSM studies in the case of individual lignocellulolytic enzyme production through SmF have been documented very well in the literature. Overall, the present study introduces a white rot fungal strain, capable of

producing three essential lignocellulolytic enzymes namely cellulase, xylanase and laccase for lignocellulosic bioethanol sector.

The maximum lignocellulolytic activities of fungi have been reported at pH 5.0 in the case of *Agaricus bisporus, Trametes versicolor, Trichoderma harzianum* and *Penicillium janczewskii* [333, 334]. The optimum temperature for lignocellulolytic enzyme production was similar to those of other mesophilic fungi such as *Aspergillus japonicas* C03, *Aspergillus glaucas* XC8, *A. niger* MS82, *Trichoderma reesei* Rut C30, *Trichoderma viride* strain EU2- 77, *Penicillium echinulatum* and *Fusarium oxysporum*. The maximum xylanase production has already been reported in a temperature range of 25-30°C by *Trichoderma viride* [335] and *P. glabrum* [336]. Usually, the maximal reported temperature for these filamentous fungi is 35°C, with an indication of the absence of growth at 37°C [337, 338]. The activity of lignocellulolytic enzymes also increased on increasing the agitation up to 100 rpm. As for the submerged fermentation, mechanical agitation is known to be a crucial factor because of its effectiveness in mixing the contents of the medium, uniform air distribution, and prevention of cell clumping. The lower activity levels at higher agitation rates could be attributed to the possible damage to the filamentous structure thereby lowering the enzyme production. The shear stress sensitivity of mycelium has also been reported in the case of xylanase production by *Thermactinomyces thalophile* subgroup C [339]. Furthermore, the shearing of mycelium at high agitation rates also release intracellular proteins in broth, which increases foam generation during the fermentation process and thus reduce xylanase yield by affecting the oxygen transfer ratios [340].

4.8 Characterization of activities of crude lignocellulolytic enzyme cocktail produced by *Cotylidia pannosa* under submerged fermentation using optimized parameters

4.8.1 Preparation of crude enzyme mixture for characterization studies using ammonium sulphate precipitation

Based on the results obtained from the optimized parameters of RSM study, lignocellulolytic enzyme production was performed under those conditions. The crude enzyme mixture thus obtained was precipitated using ammonium sulphate precipitation method and subjected to enzyme activity determination.

Following this, the precipitated crude enzyme mixture was reconstituted in sodium acetate buffer 50 mM pH .5.0 and subjected to various characterization studies as mentioned below:

4.8.2 Effect of temperature and pH and on the activities of crude enzyme mixture

The effects of different temperature and pH condition on the activities of crude cellulase, xylanase, and laccase enzyme were studied, the results of which are shown in Figure 4.16, 4.17 and 4.18. The maximum cellulase, xylanase and laccase activities were obtained at 50°C with pH of 5.0.

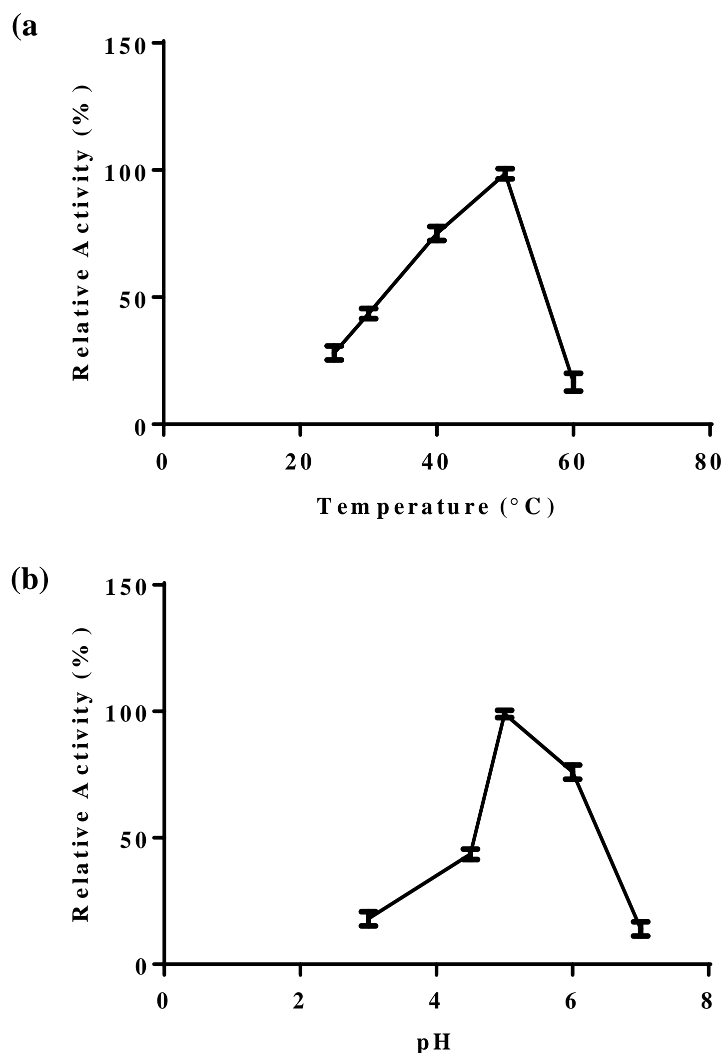
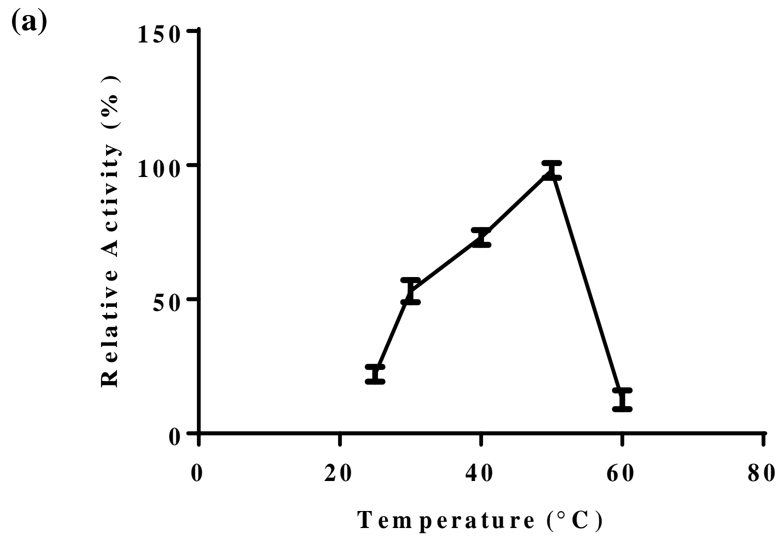


Figure 4.16 Effect of **(a)** temperature and **(b)** pH on the activity of crude cellulase of *C. pannosa*

(a)



(b)

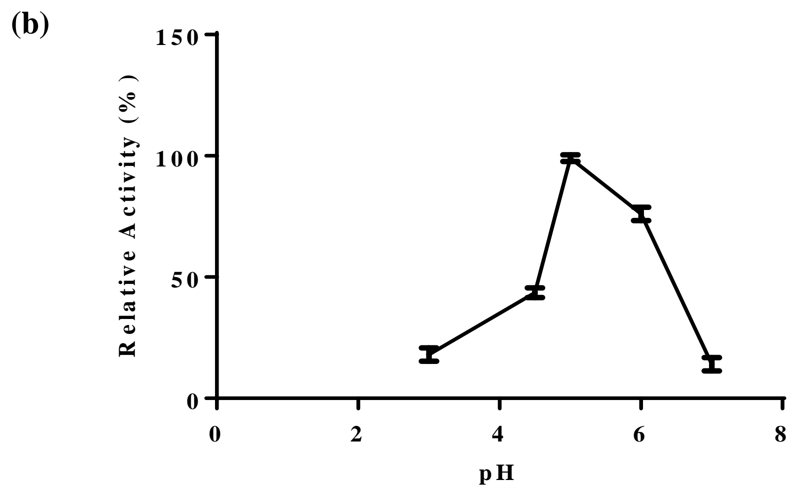
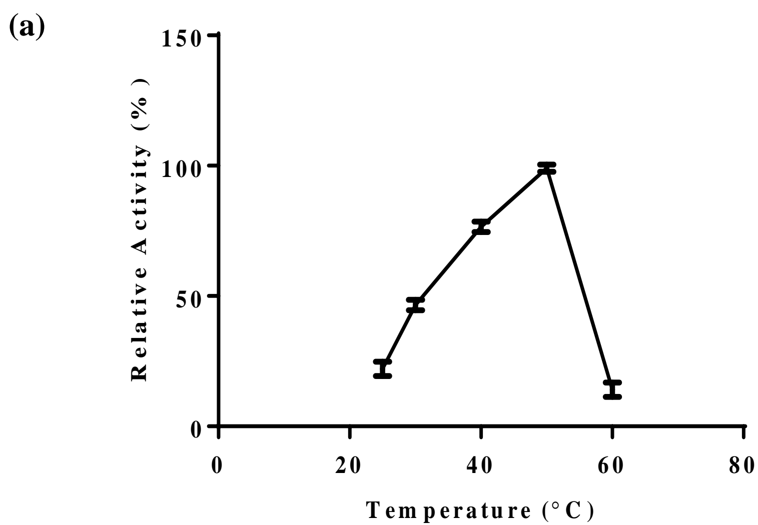


Figure 4.17 Effect of **(a)** temperature and **(b)** pH on the activity of crude xylanase of *C. pannosa*

(a)



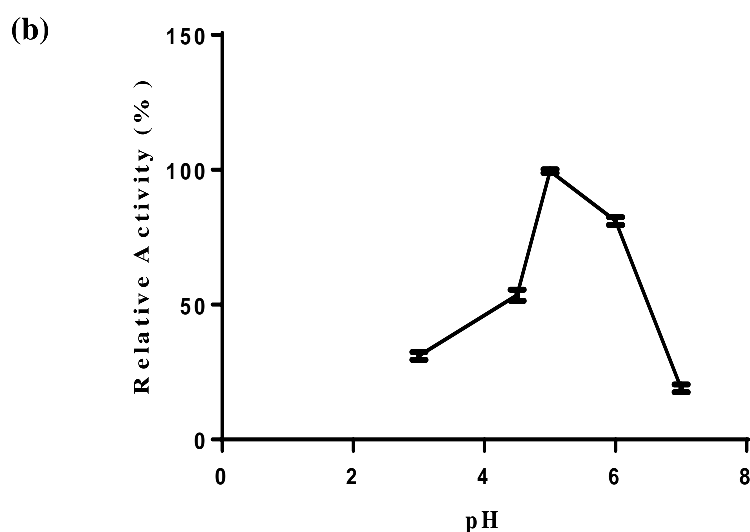


Figure 4.18 Effect of **(a)** temperature and **(b)** pH on the activity of crude laccase of *C. pannosa*

The complete degradation of lignocellulose wastes to glucose or its oligomers requires optimum functioning of lignocellulolytic enzyme complex; therefore, the characterization of the crude lignocellulolytic enzyme was crucial. The application of enzymes in different biotechnological interventions needs the characterization of the enzyme at various biocatalytic parameters such as process temperature and pH. Crude cellulase, xylanase, and laccase enzymes showed maximum activity at 50°C and pH 5.0. Temperature kinetics demonstrated that the activity of all the three enzymes are increased as the temperature was raised to 50°C. Thereafter the activity was found to decrease with further increase in temperature. Similarly, pH kinetics revealed an increase in activity from pH 3.0 to 5.0 followed by a decline in activity with a further increase in pH. As the crude enzyme extract remained stable at moderately high temperature its role in various applications such as pulp and paper, food/feed enzymatic hydrolysis of lignocellulosic materials can be explored as thermostability is an attractive and a necessary characteristic of an enzyme [341].

4.8.3 Zymogram analysis

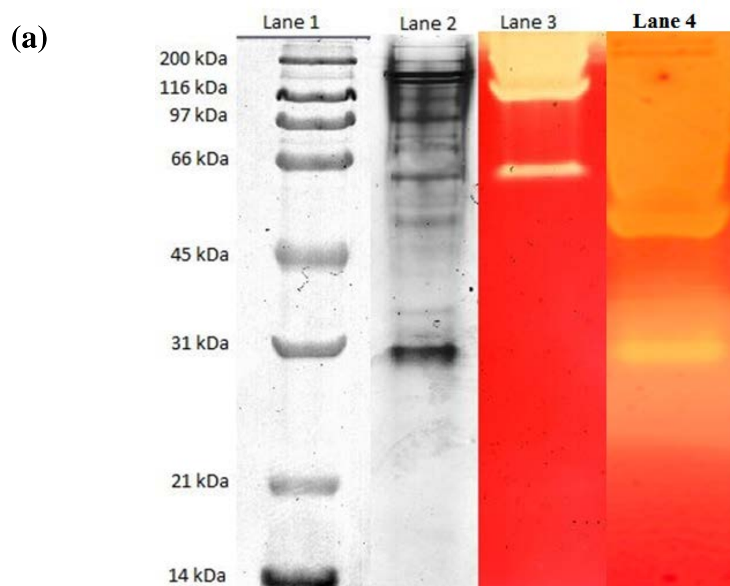
Review of literature revealed that the molecular weight of cellulase, xylanase, and laccase from *C. pannosa* had not been described before. The zymogram analysis to detect the expression of cellulase, xylanase and laccase in the crude enzyme extract produced by *C. pannosa* revealed the presence of multiple cellulase isozymes (Figure

4.19 (a); Lane 3) and xylanase isozymes (Figure 4.19 (a); Lane 4) with the most active cellulase and xylanase being detected at ~60 kDa and ~32 kDa respectively, which appeared as a sharp clear zone against a dark red background (Figure 4.19 (a)). In the case of laccase zymogram, a single ~43kDa laccase was detected which appeared as a green band against a transparent background (Figure 4.19 (b)).

The zymogram analysis using carboxymethyl cellulose as a substrate showed the most active band around ~60 kDa which is in the range of 25–60 kDa of endocellulases reported in *Chrysosporium sp*. [342].

Xylanase zymogram analysis showed that the band pattern obtained was similar to that achieved in other white rot fungi such as *Pleurotus ostreatus* and the ascomycete *Penicillium oxalicum* [343]. Investigation of the xylanolytic system of white-rot fungus *Phanerochaete chrysosporium* by several research workers [344, 345] revealed the presence of at least three xylanases, having a molecular weight of 30, 50 and 52 kDa [345]. Another white-rot fungus, *Ceriporiopsis subvermispora*, has been reported to produce xylanases having a molecular weight of 33 and 80 kDa [346]

In the case of laccase zymogram (Figure 4.19 (b)), comparison of the position of the active band on the gel with the other half of gel on which a broad range of marker was run revealed an ~43 kDa band corresponding to laccase which is in the range of laccase reported in *P. chrysosporium* [347].



(b)

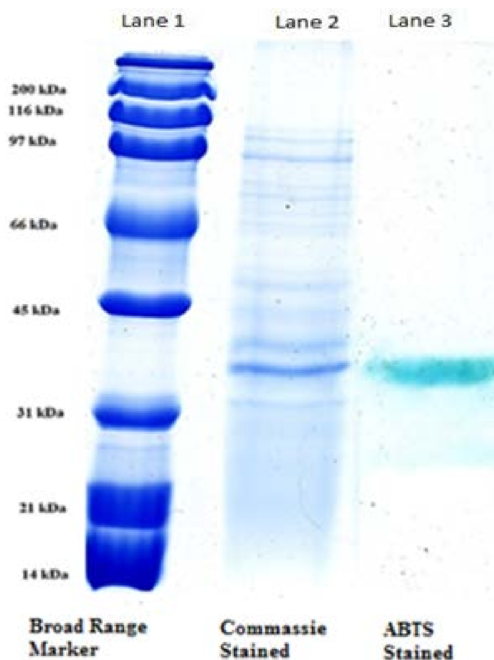


Figure 4.19 Zymogram analysis showing the expression of **(a)** cellulase and xylanase in the crude enzyme extract produced by *C. pannosa*. Lane 1 corresponds to protein standard with molecular weights (kDa) labeled, Lane 2 is coomassie stained sample, Lane 3 is the corresponding cellulase and Lane 4 xylanase zymogram stained with Congo red. **(b)** crude laccase from *C. pannosa.* Lane 1 corresponds to protein standard with molecular weights (kDa) labeled, Lane 2 is coomassie stained sample and Lane 3 is the corresponding laccase zymogram stained with ABTS

4.8.4 Saccharification of wheat bran for bioethanol production

Assessing saccharification efficiency in the presence of fungal mycelia revealed that the flask incubated for 56 hrs exhibited the maximum amount of glucose (8.75 g/L) while in the case of precipitated crude enzyme mixture, maximum glucose was obtained at 36 hrs (10.5 g/L) as can be seen from Figure 4.20. These flasks, when subjected to fermentation by *Saccharomyces cerevisiae* MTCC 174, gave an ethanol yield of 4.12 g/L and 4.8 g/L in case of hydrolysate obtained from flasks containing fungus and crude enzyme cocktail respectively (Figure 4.20)

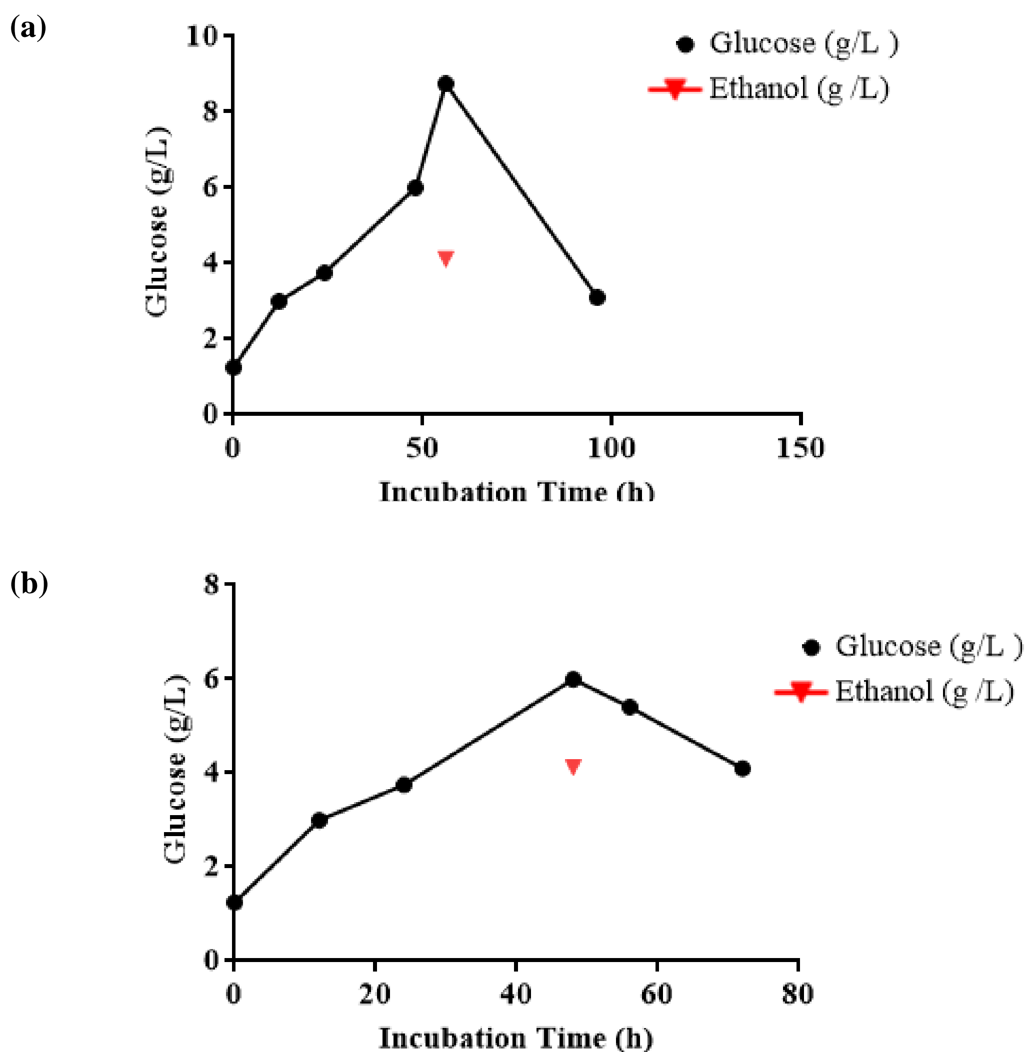


Figure 4.20 Glucose production (●) profile during saccharification of wheat bran (2%) by **(a)** fungal mycelia and **(b)** crude enzyme of *C. pannosa* along with the amount of ethanol produced (▼)

Saccharification of wheat bran for bioethanol production revealed that precipitation of crude enzyme cocktail obtained from *C. pannosa* resulted in efficient saccharification and ethanol production from wheat bran as compared to that in the presence of fungal mycelia (Table 4.9). It was observed that precipitated crude enzyme cocktail produced higher amounts of glucose and ethanol in lesser time as compared to saccharification and fermentation in the presence of fungal mycelia. The ethanol yield obtained in our study using wheat bran as carbon source was found to be far much better than in the earlier report of submerged fermentation of pretreated cotton stalk with *Phanerochaete chrysosporium* with an ethanol yield of 0.027 g ethanol g^{-1} [348]. In yet another study

L. betulinus was reported to produce 0.22 g and 0.16 g of ethanol per gram of rice straw and corn stalks as substrates, respectively [349]. Our results are comparable to work done on *Trametes hirsuta* where an ethanol yield of 4.3 g L^{-1} is reported after 96 hrs cultivation using wheat bran under submerged fermentation via consolidated bioprocessing [350]. This suggests that *C. pannosa* is a suitable candidate for use in the production of ethanol from lignocellulosic biomass.

Table 4.9 Comparison of Saccharification efficiency and ethanol production from wheat bran by fungal mycelium and crude enzyme cocktail

	Maximum glucose released	**Time taken for maximum saccharification**	**Ethanol yield**
Fungal mycelium	8.75 g/L	56 hrs	4.12 g/L
Crude enzyme cocktail	10.5 g/L	36 hrs	4.8 g/L

4.8.5 Decolorization of synthetic dyes by C. pannosa

The aptitude of white-rot fungi to decolorize synthetic dyes has been extensively studied in *P. chrysosporium, T. versicolor, C. versicolor, C. polymorpha* [237], *T. polyzona* [238] and *Coprinus comatus* [239]. To date, according to our literature survey, decolorization ability in *C. pannosa* has not yet been studied. The decolorization results indicate that the mechanism for decolorization by *C. pannosa* consist of a combination of biosorption by fungal mycelia and biodegradation by extracellular laccase. In the case of decolorization by fungal biomass, *C. pannosa* was able to decolourize CR to a maximum extent of 94% with no decolourization of orange G after 96 hrs of incubation (Figure 4.21).

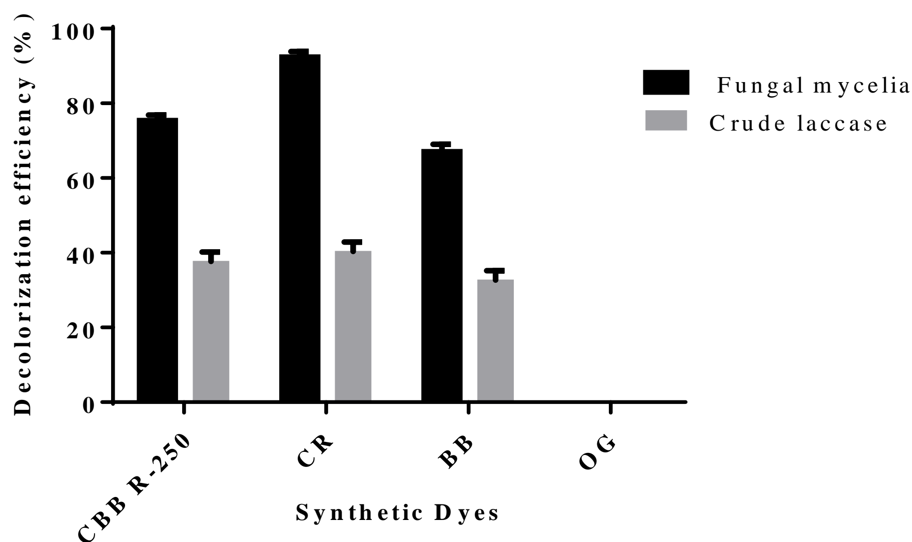


Figure 4.21 Decolorization efficiency of fungal mycelium and crude laccase of *C. pannosa*

The dye decolorization efficiency of crude laccase was 40% for CR with no decolorization of orange G. The CR being an azo dye is decolourized more rapidly than both CBB R-250 and BB which are triphenylmethane dyes. The triphenylmethane dyes are known to be resistant to enzymatic decolorization as compared to azo dyes and hence require more time for decolorization [351]. The resilient nature of orange G has also been reported by Selvam *et al.* (2003) where they indicated around 33% of decolorization after 9 days using similar basidiomycetes named *Thelephora* species [235]. The slow mineralization of orange G might be due to the nature of ring substituents as compared to Congo red. The most common mechanisms for dye decolorization are binding of dyes to the fungal hyphae, physical adsorption and enzymatic degradation [235]. The higher decolorization by fungal biomass indicates that the primary mode of decolorization by *C. pannosa* is through adsorption of the dyes on its cell surface. This type of decolorization has been reported to be the main mechanism of decolorization [235] and has been reported to be superior to decolorization by enzymatic preparations [236].

LIST OF PUBLICATIONS

Publication in peer reviewed journals

- **D. Sharma**, G. Goel, A. Sud, and R. S. Chauhan, "A novel laccase from newly isolated *Cotylidia pannosa* and its application in decolorization of synthetic dyes," *Biocatalysis and Agricultural Biotechnology,* vol. 4, pp. 661-666, 2015. (IF= awaited)
- **D. Sharma**, V. K. Garlapati, and G. Goel, "Bioprocessing of wheat bran for the production of lignocellulolytic enzyme cocktail by *Cotylidia pannosa* under submerged conditions," *Bioengineered,* vol. 7, pp. 88-97, 2016. (IF= 1.676)

Publication in conference

- **D. Sharma**, G. Goel, S. Bansal, R. Mahajan, B. M. Sharma, and R. S. Chauhan, "Exploring white rot fungi isolated from North-Western Himalayas for lignocellulolytic enzyme activities". Presented in International Conference on Emerging Trends in Biotechnology (ICETB-2014), JNU University, Delhi.
- **D. Sharma**, G. Goel, R. S. Chauhan, *Cotylidia pannosa*: a white rot fungal strain with lignocellulolytic potential. Presented in 43rd Annual meeting of the Mycological society of India (November 16-18- 2016) Birla institute of Scientific Research, Jaipur.