Development of an Environment-friendly Process for the Production of Biofuels and Value-added Products from Lignocellulosic Biomass Residues

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CERTIFICATE

This is to certify that the thesis entitled "**Development of an environment-friendly process for the production of biofuels and value added products from lignocellulosic biomass residues**" and submitted by **PAMIDIPATI SIRISHA NAGARAJU PAMIDIPATI**, ID. No. **2013PHXF600H** for award of Ph.D. of the institute embodies original work done by her under my supervision.

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Dedicated to my son…

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ABSTRACT

Development of an Environment-friendly Process for the Production of Biofuels and Value-added Products from Lignocellulosic Biomass Residues

Depletion of fossil fuels due to the decline in conventional energy reserves has created increased interest in biofuels research. Lignocellulosic biomass residues, which include agricultural residues and forest wastes are a promising feedstock for the production of bioethanol, considering their abundant availability and low cost. Lignocellulosic biomass primarily contains cellulose, hemicellulose, lignin, with cellulose linear chains forming the backbone, surrounded by shorter chains of hemicellulose, while lignin acts as a matrix and provides structural integrity to the biomass. Cellulose and hemicellulose fractions undergo hydrolysis to form corresponding monomeric sugars, which further undergoes fermentation to form bio-ethanol. A pre-treatment of the biomass feedstock is required for the removal of lignin and for breaking the cellulose-lignin bonds for increased cellulose availability to hydrolysis. This has emerged as the main processing challenge and is considered to be the single most expensive processing step. Conventional pretreatment methods involve using chemical, physical, or thermal methods, which use strong chemicals and have high energy demands. In recent years, biological pretreatment has received renewed attention since it requires reduced energy demands and offers milder operating conditions. However, lower reaction rates, leading to longer processing times has been the main drawback preventing scale-up and commercialization of such processes.

The current research work deals with developing an integrated biological route for the conversion of agricultural residues to bio-ethanol and other value-added products using a single microorganism. Analytical techniques such as Gravimetric based- Klason's method and reversed-phase high-performance liquid chromatography were used for analyzing lignin content in solid biomass residues and in liquid supernatant fractions respectively. Additionally, mass spectroscopy was used to analyze and characterize solubilized in-process lignin degradation intermediates. The structural differences in lignin and cellulose polymers, such as syringyl-to-guaiacyl ratio, and cellulose crystallinity in biomass residues was studied using Fourier transform infrared spectroscopy. Apart from this, UV-visible spectroscopy was used to for measuring enzymes activities responsible for degrading lignin and cellulose, and also to

determine degraded sugar content. Gas chromatography was used for measuring final ethanol yields in fermentations.

As part of this work, traditionally used white rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* as well as a locally isolated indigenous fungus, *Neurospora discreta* were tested for their ability to degrade agricultural residues such as sugarcane bagasse. Based on the growth characteristics and effectiveness in lignin degradation, *P. chrysosporium* and *N. discreta* were taken forward for a more in-depth evaluation before the final selection of microorganism. The optimum culture parameters for *N. discreta* were identified from a statistical design of experiments study. A detailed comparison of lignin degradation capabilities of *N. discreta* and *P. chrysosporium* was performed using two biomass residues, sugarcane bagasse and cocopeat. This study resulted in two inferences: (1) Sugarcane bagasse was easier to degrade compared to cocopeat and (2) *N. discreta* degraded nearly twice as much lignin as *P. chrysosporium* in sugarcane bagasse. This indicated that biomass structure also plays an important role in microbial delignification. Furthermore, *N. discreta* was found to be a promising alternative to white rot fungi for faster microbial pre-treatment of agricultural residues.

The activities of the key lignin-degrading enzymes such as laccase and cellulose hydrolyzing enzymes (cellulase) were also studied during this work and in-process enzyme activities of *N. discreta* were reported for the first time. Biomass loading was identified as a key process lever, as it impacted the activities of both types of enzymes due to the presence of lignin degradation intermediates during the process. At increased biomass loading, laccase activity was found to be significantly lower, indicating the possibility of product inhibition by lignin degradation products. This effect was confirmed by studying the effect of fungal solubilized lignin on pure laccase enzyme. A more detailed study of the kinetics of laccase inhibition was conducted using pure laccase enzyme which revealed competitive inhibition of the enzyme by highly polar lignin intermediates.

On the other hand, the soluble lignin degradation intermediates were found to have a stimulatory effect on cellulase, enabling a higher extent of cellulose hydrolysis to its monomer sugars, even in a relatively recalcitrant residue such as cocopeat. Despite the lower cellulose content and higher crystallinity indices in cocopeat, the amount of cellulose hydrolyzed was nearly 27% - 40% higher in cocopeat compared with sugarcane bagasse.

Finally, the capability of *N. discreta* to produce ethanol in a single integrated process from the two biomass substrates was demonstrated as a proof of concept. The final ethanol yields using the two biomass residues were found to be between 0.99 – 1.2 g/L for 1% biomass loadings. To improve the process economics, the production of value-added products such as carotenoids from the fungus, and high-value low molecular weight aromatic compounds from degraded lignin were also evaluated.

Keywords: *Neurospora discreta*, lignocellulosic biomass, value-added products, single step bioprocessing, laccase inhibition, enzyme kinetics, lignin degradation intermediates, cellulase stimulation, cocopeat, sugarcane bagasse

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NOMENCLATURE

Abbreviations

[S]: Substrate conentration

[v]: Rate of reaction

1% CP: 1% Cocopeat

1% SB: 1% Sugarcane bagasse

3.3% CP: 3.3% Cocopeat

3.3% SB: 3.3% Sugarcane bagasse

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline- 6-sulfonate

CBP: Consolidated bioprocessing

CP: Cocopeat

DNSA: Di nitro-salicylic acid

DOE: Design of Experiments

ESI: Electron Spray Ionization

FSL: Fungal solubilized lignin

FTIR: Fourier Transform Infrared

HBI: Hydrogen Bond Intensity

K_I: dissociation constant of the enzyme-inhibitor complex

$K_{m,app}$: Michaelis constant, apparent

K_m: Michaelis constant

LC-MS: Liquid Chrmotography-Mass Spectrometry

LOI: Lateral Order Index

NCIM: National Collection of Industrial Microorganisms

ND: *Neurospora discreta*

PC: *Phanerochaete chrysosporium*

PDA: Potato Dextrose Agar

PL: Pure laccase

RT: Retention time

SB: Sugarcane bagasse

SHF: Separate Hydrolysis and Fermentation

SSF: Simultaneous Saccharification and Fermentation

UV: Ultraviolet

$V_{max,app}$: Maximum rate of reaction, apparent

V_{max}: Maximum rate of reaction

ε : extinction coefficient

Symbols

°C: degree Celsius

µL: micro-liter

g: gram

hrs: hours

mg L^{-1}: milligrams per liter

mg: milligram

mL min^{-1}: milli-liter per minutes

ml: milliliter

nm: nanometer

rpm: revolutions per minute

U l^{-1} : Enzyme Units per liter

v/v: volume by volume

µM-min^{-1}: micro-moles per liter per minute

µM-sec^{-1}: micro-moles per liter per second

CHAPTER 1

INTRODUCTION

1.1 Overview

Growing demand for energy and depletion of fossil-based fuels has created an urgent need for the development of sustainable technologies for the production of biofuels such as bio-ethanol and bio-butanol from renewable resources. Conventional biofuels known as 'first generation biofuels' were obtained from food crops such as grains, sugar cane, and vegetable oils. The sugars, oils and starch were converted to biodiesel or bio-ethanol using trans-esterification or fermentation technologies respectively. However, this technology was unsustainable, as farmlands were diverted to grow crops for biofuel production rather than for food supply causing stress on food commodities [1]. This led to the development of second-generation biofuels that utilize agricultural residues and non-food crops such as sugarcane bagasse, rice and wheat straws, husk, wood, grasses, woody biomass etc. as raw material. However, these sources of raw material are far more complex and recalcitrant, resulting in much lower biofuel yields compared to first generation processes. Third generation biofuels were therefore developed, using algal biomass which is a rich source of lipids and carbohydrates which can be converted to biodiesel or bio-ethanol [2]. These processes face their own set of challenges including the cultivation of algal cells at a large scale [3], which either requires large amounts of land and water or specialized photo-bioreactors. In contrast to the first and third generation biofuels, second-generation biofuels have additional long-term environmental sustainability benefits. The global production of agricultural residues is 1395 million tons [4, 5] and their disposal poses a huge environmental burden. The use of such residues for production of biofuels, therefore, provides a more holistic solution [6].

1.2 Second generation biofuels

The raw material for second-generation biofuels is broadly known as 'lignocellulosic biomass' and includes waste byproducts obtained from agriculture (sugarcane bagasse, rice and wheat straws, corn and sorghum stalks etc.), forestry (paper mill discards) and discards from first generation biofuel processes [4]. The major constituents of lignocellulosic biomass are three polymers: cellulose, hemicellulose and lignin. Cellulose is a highly ordered and a linear polymer made of cellobiose units, which are in turn made of glucose monomers. This is the most useful part of the biomass, as its hydrolysis results in the formation of glucose, which can be subsequently fermented to ethanol. Hemicellulose is a carbohydrate polymer made of

different sugars such as glucomannans and xylans and can get easily hydrolyzed to form pentose sugars. The pentose sugars can also be fermented to ethanol, although this is more challenging compared to glucose fermentation. Lignin is an aromatic polymer forming a complex three-dimensional network of phenylpropanoid units [7, 8]. Lignin fraction gives structural rigidity to plants and is the most recalcitrant component making it difficult to degrade.

For obtaining bio-ethanol, lignocellulosic biomass initially is subjected to a pretreatment step for the removal of lignin, making the cellulose fraction more accessible to enzymes or microorganisms in further processing steps [5, 9]. The pretreatment step is one of the most challenging steps as the digestibility of cellulose depends on effective removal of lignin which in turn is difficult to breakdown owing to its structural characteristics. In the next step, the cellulose and hemicellulose fractions are hydrolyzed to respective monomers, i.e., hexoses and pentoses. Once hydrolysis is completed, the resulting cellulose hydrolysate is fermented and converted into ethanol. To further increase the bio-ethanol yield, certain processes employ fermentation of hemicellulose hydrolysate, i.e., pentoses, by suitable enzymes or micro-organisms [10] (Figure 1.1). In the overall process, the removal of lignin (delignification) can be considered to be the most crucial step, as it determines the potential of hydrolyzing cellulose and obtaining fermentable sugars, thereby directly impacting the final ethanol yields [6].

Pretreatment methods currently employed for delignification of biomass can be classified into physical, chemical, physico-chemical and biological pretreatments. Physical pretreatment involves mechanical size reduction techniques such as milling, grinding and chipping employed to breakdown the lignin structure and thus reduce the crystallinity and degree of polymerization of cellulose [6]. These processes, however, require high energy inputs and therefore are not economically feasible. Chemical methods include alkali pretreatment, acid hydrolysis, organosolv treatment, ozonolysis and ionic liquid pretreatment, which either hydrolyze hemicellulose fraction or oxidize lignin thus making cellulose more accessible. However, certain disadvantages of these methods are the usage of chemicals that are strong or expensive, requiring additional recycle, recovery and neutralization steps. Physico-chemical methods include steam explosion and hot water treatment which have a significantly lower environmental impact as compared to other methods. However, each of the above methods discussed generate certain toxic compounds such as furan derivatives and weak acids produced from sugar degradation that could severely impact the subsequent hydrolysis and fermentation

steps. Biological pretreatment method involves using either purified lignin-degrading enzymes such as laccases [11] or whole cell fungal microorganisms such as white rot fungi that secrete the lignin-degrading enzymes which in turn causes lignin degradation [6, 12]. Microbial pretreatment methods require milder operating conditions, lower energy supplies, fewer processing steps and most importantly, do not form any inhibitory compounds that can adversely impact the subsequent steps. While these processes are significantly slower and less efficient compared to other traditional pre-treatment methods, they can be optimized to improve the overall extent of delignification. A major portion of the present thesis, therefore, deals with developing a robust microbial delignification step for the conversion of lignocellulosic residues.

After removal of lignin, the subsequent steps of hydrolysis and fermentation employ either pure enzymes or whole micro-organisms to hydrolyze bio-polymers and ferment resulting sugars. Based on the method employed, the processing strategies can be classified as (a) Separate hydrolysis & fermentation (SHF), (b) Simultaneous saccharification and fermentation (SSF) (c) Consolidated bioprocessing (CBP) [13]. In SHF, the hydrolysis of cellulose and the fermentation are carried out in different steps sequentially (Figure 1.1) while in SSF, the two steps are performed in a single unit. Some advantages of SSF over SHF are decreased capital costs, and an increased hydrolysis rate as degraded sugars are immediately consumed to ethanol. However, as the two steps, hydrolysis and fermentation are integrated into a single step, a major challenge is the maintenance of optimal culture conditions, which could be significantly different for hydrolysis and fermentation. In SSF, externally produced cellulase enzymes are added to hydrolyze cellulose to sugars that add cost to the process. In CBP, a further reduction in capital and operating costs are seen as enzymes are replaced by naturally occurring cellulolytic micro-organisms. Therefore, enzyme production, substrate hydrolysis, and fermentation steps are all consolidated into a single step [6]. Robust cellulolytic fungal strains, or microbial consortia that are capable of hydrolyzing cellulose and ferment both hexose and pentose sugars are being targeted as ideal CBP candidates. The primary challenge in an integrated CBP process, however, is to develop “engineered” strains that can secrete high titer values of cellulases and hemicellulase enzymes and therefore higher ethanol yields [14]. Moreover, this process still requires a pre-treatment of delignification step, similar to other processes discussed above. A further advancement in CBP would be the development of a single step process, which combines the pretreatment of biomass along with the subsequent hydrolysis and fermentation steps as shown in Figure 1.1. Such a process would require a single microorganism capable of delignification, hydrolysis and fermentation. While this could prove

to be challenging, such a process could overcome several of the disadvantages mentioned above and also result in a significant reduction of capital costs.

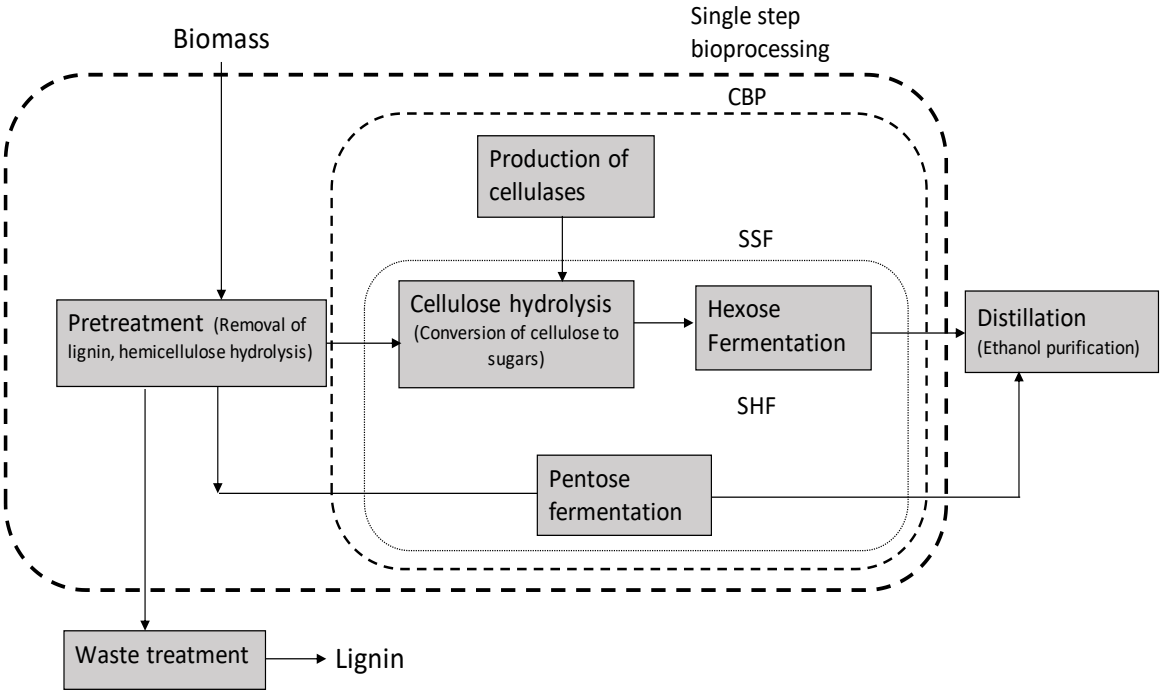


Figure 1.1: Block diagram for bioethanol production from lignocellulosic biomass using various processes (SHF: Separate Hydrolysis and Fermentation, SSF : Simultaneous Saccharification and Fermentation and CBP: Consolidated bioprocessing)

1.3 Gaps in existing Research

- While microbial delignification of biomass has been considered to be a promising alternative to chemical, physical and physico-chemical methods, it has suffered from low rates of delignification and low process yields, thereby increasing the processing times significantly. A major gap in this area is that there has been no significant development in understanding the causes of these low reaction rates.

- As microbial processes are heavily dependent on the activities of the enzymes involved, it is critical to understand the effects of various process parameters and in-process intermediates that the enzymes would be exposed to, during the process. Literature reports studies dealing with the effects of various isolated organic and inorganic molecules on activities of lignin-degrading and cellulose-hydrolyzing enzymes. However, the effects of in-process intermediates have not been evaluated so far, which

is another significant gap in the understanding of how the process impacts enzyme activities, and therefore reaction rates.

- Another major gap in the current state-of-the-art is that the pre-treatment of biomass for delignification has been studied in isolation with respect to the hydrolysis and fermentation steps. While consolidated bioprocessing has been explored, involving the use of a single microorganism for both hydrolysis and fermentation, this has not yet been extended to the pre-treatment of biomass. Such a process would have several advantages of reduced capital costs, decreased risk of inhibitory products being generated during pre-treatment, and a more robust process as it involves only one microorganism.

1.4 Objectives of the Present Research

The primary hypothesis of this research was that a single microorganism could be used for effective conversion of lignocellulosic biomass into bioethanol. This includes biomass delignification, hydrolysis and fermentation of the resulting sugars to ethanol and other value-added products. The key objectives of this research are as follows:

- **Objective 1: To develop a robust microbial pre-treatment step for effective delignification**. The major focus of this research was the development of the delignification step as this is the most challenging part of the overall process. This objective involved the screening and selection of the microbial catalyst and developing robust analytical tools for evaluating the microorganisms in terms of their lignin degradation capability. A detailed comparison of conventional white rot fungi with a novel isolated strain in terms of growth characteristics on solid biomass substrates was conducted as a preliminary screening step followed by optimization of growth conditions using the design of experiments for the novel strain. Later, an in-depth comparison of lignin degradation capabilities was performed using substrates with varying lignin content at different biomass loadings. Klason's method, a gravimetric analysis was performed for measuring lignin before and after fungal treatment and liquid chromatography was used for analyzing solubilized lignin present in the fermentation medium. Another important aspect of this objective was to identify the cause for low delignification rates. This was done by studying the effect of in-process intermediates on the activities of ligninolytic enzymes, which pointed to a severe enzyme inhibition by certain compounds.

- **Objective 2: To integrate the pretreatment process with hydrolysis and fermentation of cellulose and hemicelluloses fractions for production of bio-ethanol:** Once the microorganism was selected, its ability to hydrolyze cellulose and hemicellulose, and to ferment the sugars to ethanol was evaluated. The effect of biomass loading, the nature of biomass, and the presence of in-process intermediates on cellulose hydrolysis and activity of cellulase enzyme were evaluated. Analytical tools such as liquid chromatography, liquid chromatography-mass spectroscopy were used to quantify and characterize in-process lignin degradation intermediates, UV-visible spectroscopy was used to determine degraded sugar content as well as for measuring enzymes activities responsible for degrading lignin and cellulose. Gas chromatography was used for measuring ethanol content. Gravimetric based methods were used to measure cellulose content.

- **Objective 3: To investigate the possibility of value addition from by-product streams**: The overall process economics of the biomass to biofuel process can be improved by evaluating potential value-added products from by-product streams generated during biomass to biofuels process. Two types of value-added products were studied: Lignin, a major byproduct stream (Figure 1.1) is an amorphous polymer with a huge potential for the production of aromatic chemicals such as vanillin [15]. Using liquid chromatography-mass spectroscopy, the low molecular weight compounds from fungal solubilized lignin having a high potential for value addition were identified. Also, carotenoids which are secreted by certain fungal strains having varied applications in pharmaceutical, food and cosmetic industries were studied as a potential value-added product[16].

1.5 Thesis Organization

A detailed literature review on structural chemistry of lignocellulosic biomass, pretreatment methods of lignocellulosic biomass, biological pretreatment and lignin degrading enzymes is provided in chapter 2. Chapter 3 covers the selection of microorganisms for de-lignification of biomass based on growth characteristics and optimization of process parameters such as temperature, pH and media components using the design of experiments for optimal fungal growth. The phylogenetic identification of a novel fungal microorganism using 18s ribosomal RNA sequencing technique is also covered in this chapter. In chapter 4, a detailed comparison of novel strain *Neurospora discreta* and conventional white rot fungi *Phanerochaete chrysosporium* in their lignin degradation capabilities is presented. Experiments were set up

using different biomass residues with varying lignin content and its structural attributes. Lignin content in biomass before and after fungal treatment was measured gravimetrically and solubilized lignin content in liquid samples was measured using liquid chromatography. The final selection of the fungal strain was done based on percentage lignin degradation and growth rate of the fungus. A detailed study on the effect of biomass loadings and enzyme activities of the selected fungus, *Neurospora discreta* is presented in chapter 5 along with the effect of lignin degradation intermediates on lignin degrading enzymes. A thorough kinetic inhibition study of pure laccase enzyme by in-process lignin degradation intermediates is covered in chapter 6 to understand the nature of inhibition. Chapter 7 covers the effect of biomass loading on hydrolysis of cellulose and cellulase activity. The effect of biomass loading on overall biomass degradation is also presented here. Finally, a proof-of-concept single step bio-processing of the two agricultural residues using fungus *Neurospora discreta* is presented in chapter 8 and the potential value-added products from the process are discussed. Figure 1.2 shows the graphical abstract of the work, which includes the details of fermentation set-up, sample preparation and analytical details.

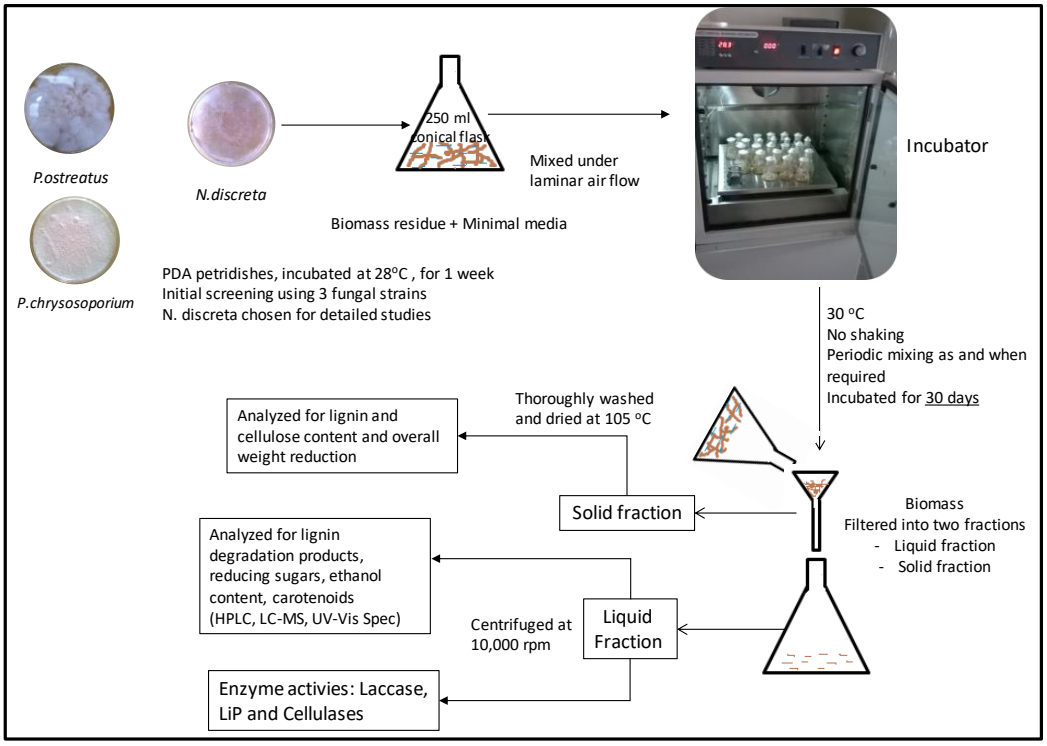


Figure 1.2: Fermentation set-up, sample preparation and analytical details

CHAPTER 2

LITERATURE SURVEY

2.1 Overview

The current chapter begins with a detailed description of the composition and major structural components of lignocellulosic biomass, - cellulose, hemicellulose and lignin. A detailed literature survey on the conventional pretreatment methods for the removal of lignin components along with their advantages and disadvantages is then presented, with emphasis on the biological pretreatment method. The chapter then discusses the processes after pretreatment - namely hydrolysis of cellulose and hemicellulose, enzymes involved in the breakdown of these polymers to sugars and subsequent fermentation to yield bioethanol in the final step in the overall conversion of lignocellulosic residues.

2.2 Structural Chemistry of Lignocellulosic Biomass

The three polymers of lignocellulosic biomass: cellulose, hemicellulose and lignin are strongly intermeshed and chemically bonded by covalent cross-linkages and other non-covalent bonds. While cellulose and hemicellulose are macromolecules made from hexose and pentose units, lignin is an aromatic polymer found majorly in middle lamellae and secondary cell walls [7]. Typically, a plant cell wall consists of an outer most primary layer, three secondary layers (S1, S2 and S3) positioned inwards and the middle lamella, present between two adjacent cells [7, 17]. The composition and percentages of these polymers vary from plant to plant, between the cell wall layers and also depends on the age, growth and on the location within the plant. The bulk compositions of some common agricultural residues are given in Table 2.1 and a brief explanation of the three major polymers are provided in the following sections.

Table 2.1: Chemical composition of common agricultural residues

Substrates	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	References
Sugarcane bagasse	50	25	18.4-25	2.4	[5, 18]
Coco peat	21-36	15	48-53.5	9-9.6	[19, 20]
Rice straw	32-47	19-27	5-24	12.4	[5]
Wheat straw	35-45	20-30	8-15	10.1	[5]

2.2.1 Cellulose

Cellulose is the most abundant polymer found on Earth and is widespread in plants and microorganisms such as algae and bacteria [21]. Cellulose is a high molecular weight, linear polymer composed of D-glucose subunits linked by β-1,4 glycosidic bonds. The repeating unit is the disaccharide cellobiose, which forms long chains of elemental fibrils consisting of 100-15,000 glucose molecules [22, 23]. The presence of a large number of hydroxyl groups on the long chains causes inter and intramolecular hydrogen bonding, resulting in the formation of a highly-ordered aggregate of cellulose fibrils [24]. These cellulose fibrils are in turn, embedded in a matrix of lignin and hemicellulose, making it extremely resistant to enzymatic degradation. While a majority of cellulose is highly ordered and crystalline in nature, a small percentage of non-organized (amorphous) cellulose is also found, which is more susceptible to enzymatic degradation [25]. The degree of polymerization and cellulose crystallinity which varies significantly across the biomass residues are the key parameters that determine the efficiency of cellulose hydrolysis [7, 24].

2.2.2 Hemicellulose

While cellulose is a linear polymer made up of a single component - glucose, hemicellulose is a branched polysaccharide made from different monomer sugars. The basic monomeric sugars of hemicellulose include xylose, mannose, galactose, glucose and arabinose which form short and branched chains, with each chain typically consisting of 500-3000 monomer units [23]. The sugar monomers are majorly linked together by β -1,4 and to some extent by β -1,3-glycosidic bonds. Xylan is the most abundant hemicellulose formed by β-D-xylopyranosyl that are linked by β- 1,4 bonds [25]. Hemicellulose xylan is linked to non-core lignin through ester or ether bonds and forms hydrogen bonds with cellulose chains, thus linking crystalline cellulose and amorphous lignin fractions [26]. Unlike cellulose, hemicellulose is majorly built from pentose sugar units and has an intermediate affinity to water. Because of a lower degree of polymerization and fewer crystalline structures, hemicellulose gets easily hydrolyzed in acidic medium [21].

2.2.3 Lignin

Lignin is the second largest renewable resource available after cellulose, and one of the important components of biomass providing structural support and resistance against external microbial attack [27]. It is majorly found in middle lamella and secondary cell

wall layers [21]. Lignin principally consists of three phenyl-propane units, namely p-hydroxyphenyl (derived from coumaryl alcohol), guaiacyl (G type units derived from coniferyl alcohol), and syringyl (S type units derived from sinapyl alcohol) units, which are extensively cross-linked via ether and carbon-carbon (C-C) linkages (Figure 2.1). As the coupling can occur at various sites due to "resonance delocalization of radicals", an array of structural units are formed from the three precursor units and the final result of polymerization is a heterogeneous complex three-dimensional structure [28, 29]. β-linked structures are formed by the endwise coupling of monomers to the growing polymer, while coupling between two lignin oligomers results in 5-5 and 5-O-4 structures. The phenol-ether bond dominates the lignin structure with β-O-4 coupling creating most abundant structures ranging between 50%-80%. The other phenol-ether linkages include α-O-4, 5-O-4 and other such units. The dominant linkages in C-C type are β-5, β- β linkages followed by β-1, 5-5 units (Figure 2.2) [21, 28]. While the bond energies for phenyl-ether linkages are lower and can, therefore, be cleaved by chemicals, the bond energies for C-C linkages are higher and are therefore resistant to chemical degradation [27]. Moreover, the bulky nature of the heterogeneous lignin polymer strongly reduces enzyme accessibility, thus challenging bio-degradation of lignin. An additional limitation is its non-phenolic aromatic nature, which makes it difficult to oxidize lignin units. In nature, high redox potential peroxidase enzymes secreted by certain fungi are capable of degrading recalcitrant lignin sub-structures [28].

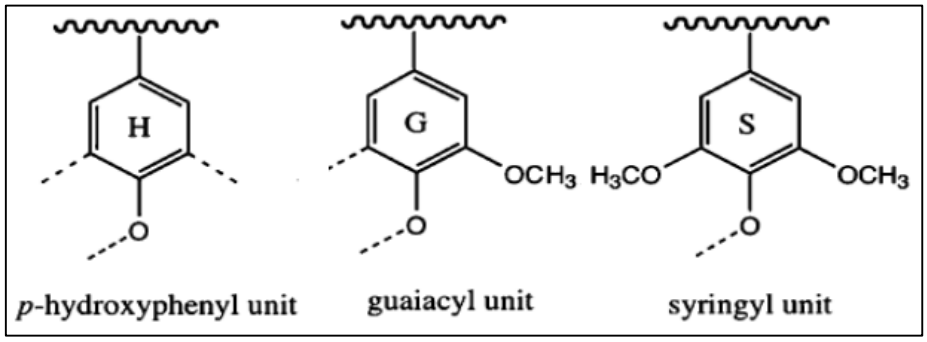


Figure 3.1: Monomeric units of lignin. Adapted from [28]

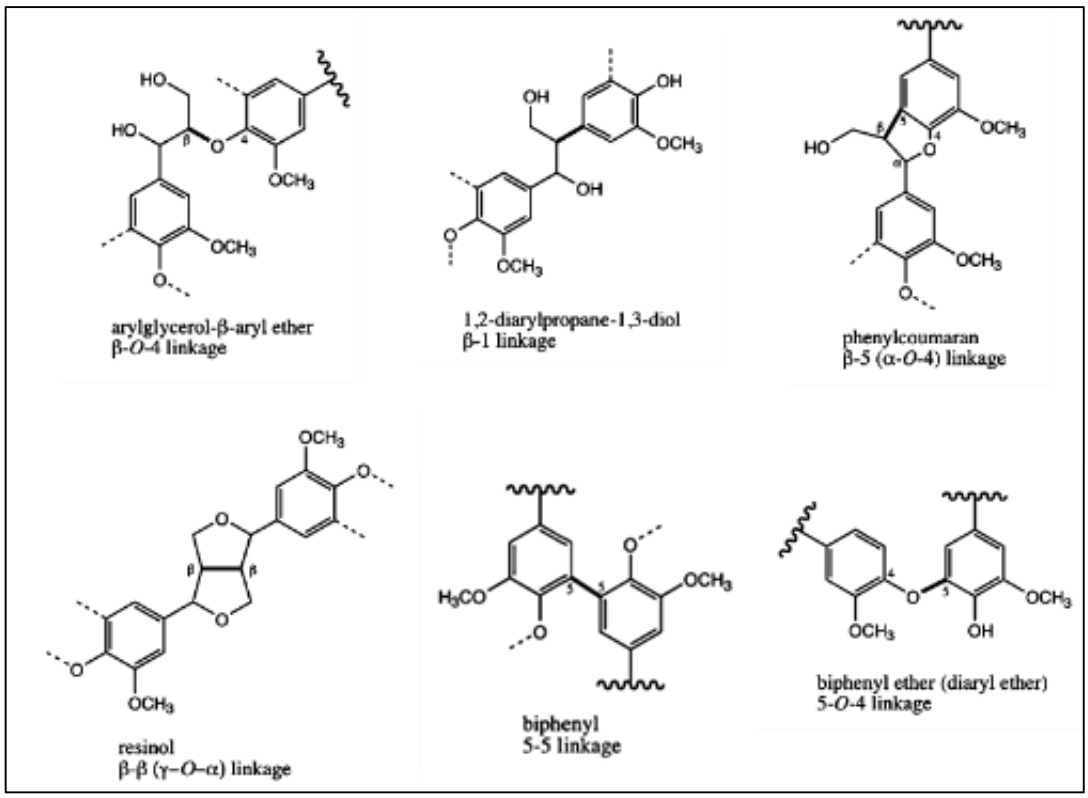


Figure 2.2: Major structural linkages found in lignin. Adapted from [28]

2.3 Pretreatment methods

Pre-treatment of biomass is the first step in the biomass-to-biofuels process. The close association of the main components of the biomass hinders hydrolysis of cellulose and hemicellulose to fermentable sugars. The primary objective of the pre-treatment step is to increase the cellulose accessibility to hydrolysing agents for subsequent conversion to sugars. Therefore, the main goals of the pre-treatment step are to remove lignin fraction, reduce cellulose crystallinity and increase the porosity of the biomass. Pre-treatment methods can be divided into four categories, namely physical, chemical, physico-chemical and biological methods [6, 12]. A short summary of each of the methods is given below.

Physical methods: Physical methods such as chipping, milling, grinding and comminution of biomass causes a reduction in particle size and a decrease in overall crystallinity of biomass. The increase in the specific surface area of the biomass causes an increase in the accessibility of the three polymers to subsequent hydrolysing agents. Also, a reduction in the degree of polymerization of cellulose chains improves its hydrolysis rate. However, the power requirements of such physical methods are relatively high and therefore may not be suitable at large scale [6, 12, 30].

Chemical methods: Chemical pretreatment processes majorly use two routes to degrade lignocellulosic biomass, a) acid hydrolysis which causes complete hydrolysis of hemicellulose and partial hydrolysis of cellulose fractions to corresponding sugars and b) alkaline hydrolysis that solubilizes lignin fraction leaving behind cellulose enriched biomass [6]. Dilute acid hydrolysis of lignocelluloses has been successfully used for agricultural and wood residues. Common acids such as HCl and H_2SO_4 are used at dilute concentrations and the process takes place at higher temperatures of around 160^{o}C [12, 30]. The process completely hydrolyses hemicellulose fraction and significantly improves cellulose hydrolysis. However, the major drawback is the formation of compounds such as furfural, hydroxyl-methyl furfural, carboxylic acids and furans which are known to inhibit the microorganisms used in the fermentation step [31]. The application of NaOH, ammonia and other such alkaline solutions to cause alkaline hydrolysis of lignin has also been investigated. The ester bonds cross-linking lignin and hemicellulose are targeted in this process. Alkaline hydrolysis causes lower sugar degradation the and therefore increases the availability of sugars to subsequent fermentation to ethanol, as compared to acid hydrolysis, but with applicability to only low lignin content biomass residues [6, 12]. However, alkali pretreatment can be conducted at near ambient temperatures unlike acid hydrolysis [12]. In organosolv process, a mixture of organic solvents such as methanol, ethanol, acetone or aqueous solvents is used at high temperatures ranging between 160^{o}C and 220^{o}C to cause delignification of biomass. However, recovery of solvents and their inhibitory nature to subsequent fermentation agents are the major drawbacks.

Physico-chemical and thermo-chemical methods: One of the most commonly used physico-chemical methods is steam explosion. In this method, the biomass is initially contacted with steam at high pressure for a certain period and later subjected to sudden de-pressurization. An explosive decompression opens up the biomass structure and increases its porosity. It is a physico-chemical process as the high-pressure water also hydrolyses hemicellulose fraction which exposes cellulose surface. Although the method is cost-effective, the partial degradation of hemicellulose releases toxic compounds such as weak acids, furan derivatives etc., that could inhibit subsequent fermentation steps. An alternative route is hot water treatment that does not employ rapid decompression. To prevent inhibitor formation, hemicellulose is partially hydrolyzed to form oligomer fractions and minimize monomeric sugar formation. Although the method utilizes low-cost water, the overall efficiency of the method is quite low [6, 12]. Ammonia fiber explosion is another pretreatment method that uses liquid anhydrous ammonia between 60-100^{o}C. A rapid expansion of ammonia gas at lower pressures causes physical

disruption of biomass fibers [6, 30]. The other category of pretreatment is through thermochemical methods that use high temperature and pressure conditions to gasify biomass to syngas which is then passed through a catalytic reactor or anaerobic fermenters to form bio-ethanol or other liquid fuels. Pelletization, torrefaction, pyrolysis of biomass to syngas to further yield Fischer-Tropsch fuels, is another route that has been extensively studied [12, 32]. However, the use of catalysts and high temperature and pressure conditions have been major drawbacks for developing a cost-effective route.

2.4 Biological pre-treatment methods

In recent years, biological pre-treatment has received renewed attention since it requires milder operating conditions and is more environment-friendly. Another advantage is that biological pre-treatment does not result in the generation of fermentation inhibitors. Biological treatment methods involve using either whole cell microorganisms or purified enzymes for the treatment of biomass. Enzymatic pre-treatment using ligninolytic (lignin-degrading) enzymes such as laccase have been studied on low lignin content agro-wastes such as apple peels and potato peels and were found to be effective as it causes selective delignification while keeping the other carbohydrate polymers intact and therefore increasing sugar yields [11]. Enzymatic pre-treatment also prevents the formation of by-products and fermentation inhibitors such as furfural and hydroxymethylfurfural that are usually formed from sugar degradation [33]. However, the overall cost of purified enzymes could prevent the scale-up and commercialization of such processes and biological pre-treatment using microorganisms could, therefore, be a more viable route. A comparison of the pretreatment steps is provided in Table 2.2 that highlights the advantages of biological pretreatment method.

Table 1.2: Comparison of Pretreatment methods

Pretreatment method [6, 30]	**Physical**	**Chemical**	**Biological**
Mode of action	Mechanical size reduction	Acid hydrolysis or alkali treatment	Uses expensive enzymes or whole cell microorganisms
Yields and Selectivity	Low	Higher yields but may have lower selectivity	Highly selective, but lower yields
Energy/Cost	High energy requirement	Medium	Low energy inputs, but expensive if enzymes are used
Impact on environment	Nil	High, Generates toxic intermediates	Nil
Ease of scalability/Process integration	Not easy	Easy. However requires additional neutralization steps	Not enough supporting literature

Degradation of lignin can be caused by certain bacteria such as actinomycetes and proteobacteria, which are considered major decomposers of lignin in the soil. Literature reports the use of several filamentous bacterial species belonging to the *Streptomyces* genus that have the ability to produce several lignin-degrading enzymes including high redox potential peroxidase enzymes [15]. However, bacterial delignification is considered to be slow and limited, while fungal microorganisms are shown to be more efficient in the breakdown of lignin [15]. Literature reports the presence of more than 1600-1700 species of fungi that can degrade wood [26]. In nature, wood rotting fungi are primary lignin degraders, that play an important role in the biosphere, decaying dead plant organic matter thus preventing its accumulation [26].

Most of the biological pre-treatment methods employ wood-decaying fungi which are classified further, based on the type of decay caused. While brown-rot and soft-rot fungi mainly depolymerize carbohydrate polymers, white-rot fungi are reported to cause complete degradation of lignin to CO_2 and H_2O [26, 34, 35]. Certain species of white-rot fungi such as *Pleurotus* spp. and *Ceriporiopsis subvermispora* cause selective delignification of biomass

leaving behind cellulose enriched biomass that would have several industrial interests. Certain other white rot species such as *Trametes versicolor*, cause simultaneous degradation of all cell wall components with the formation of radial cavities [28]. While white rot fungi generally grow on hardwood angiosperms, brown rot fungal species grow primarily on soft-woods and degrade wood polymers while partially oxidizing lignin. Soft rot fungi belonging to Ascomycota are known to mainly degrade hardwood lignin but to a limited extent. Compared to white rot fungi, research on lignocellulosic degradation by soft rot fungi is limited [26, 35].

Lignin degradation by fungi is a complex, multi-enzymatic process involving cooperative action between lignin-modifying enzymes and lignin-degrading auxiliary enzymes [26, 28]. Lignin-modifying groups broadly fall under two categories, copper containing laccases and heme-containing peroxidases namely lignin peroxidases, manganese peroxidases and versatile peroxidases [26]. Laccases are a group of enzymes found in numerous fungi, plants, and bacteria that oxidize certain lignin substructures and utilizes oxygen as the final electron acceptor [28, 36]. These enzymes are capable of oxidizing low redox potential phenolic substrates of lignin such as structures formed from phenolic β-O-4 and β-1 dimers. However, in the presence of low molecular weight mediator systems such as 2,2'-azino-bis (3-ethylbenzothiazoline- 6-sulfonate (ABTS), the enzyme was found to depolymerize non-phenolic macromolecular lignin structures [35, 37]. Peroxidase enzymes are mainly found in more evolved white rot fungi that have the ability to cleave even non-phenolic substructures of lignin and hydrogen peroxide serves as the final electron acceptor. In general, peroxidase enzymes have a higher redox potential and therefore can catalyse a broader range of substrates in the lignin structure [8, 28].

2.5 Hydrolysis & fermentation of pre-treated biomass

Based on the pre-treatment method employed, the sequence of subsequent steps employed differ. For instance, in chemical pre-treatment methods, certain necessary steps such as neutralization and detoxification steps are performed to neutralize pH and remove any fermentation inhibitor compounds such as HMF, furfural or acetic acid [38]. Bioethanol production from pre-treated biomass majorly employs biological routes and involves two major steps namely hydrolysis (saccharification), in which breakdown of cellulose and hemicellulose polymers yields corresponding sugars and fermentation process, which converts released sugars to bio-ethanol. While hemicellulose fraction gets easily hydrolyzed and in most cases

gets degraded in pre-treatment step, cellulose hydrolysis is slower and is considered to be the rate-determining step due to the crystalline structure of cellulose as discussed in section 2.2.1 [25].

Conventionally, cellulose hydrolysis was carried out using externally produced cellulase enzymes [39]. Cellulases are a group of enzymes that includes endo-1,4-β-glucanases, exo-1,4-β-glucanases (cellobiohydrolases) and, β - glucosidases which synergistically act and degrade highly ordered cellulose chains releasing glucose sugars. While endo-glucanases randomly cleave internal bonds in the cellulose chains, exo-glucanases attack the reducing or non-reducing end of the cellulose polymer and produce cellobiose oligomers. β-glucosidases then convert cellobiose units to glucose monomers [22]. Hemicellulose degradation to its monomeric pentose sugars is relatively easy and can be achieved by enzymatic hydrolysis by hemicellulase enzyme system. Hemicellulose, mainly composed of xylans can be efficiently degraded by cooperative action of hydrolytic enzymes such as endo-1,4-β-xylanases, xylan 1,4-β-xylosidases and accessory enzymes such as acetyl esterases, ferulic and p-coumaric esterases, α-l-arabinofuranosidases etc. [7].

The major constraint of using externally produced cellulase enzymes for hydrolysis is its high cost which reportedly constitutes 30 to 50% of the total cost [40]. Therefore, solid state fermentation employing cellulolytic fungi was developed to produce enzymes and simultaneously degrade biomass which proved to be a vastly improved cost-effective option [41]. However, such processes suffer from severe mass transfer limitations arising from poor mixing of fungi with biomass and low rates of diffusion through the solid-liquid phases. Certain species belonging to ascomycetes group such as *Trichoderma reesei, Aspergillus niger, Neurospora* species are known to secrete high titre values of cellulase enzymes [42–44]. Certain bacterial strains such as *Clostridium thermocellum* are also found to be effective cellulase producing strains [13]. For the production of xylanase enzymes required to degrade hemicellulose, *P. chrysosporium, Aspergillus niger,* and *Trichoderma* spp., have been reported to be efficient [7, 42].

Fermentation of hexose sugars to ethanol is straightforward and well-studied. Yeast strains such as *Saccharomyces cerevisiae* and *Zymomonas mobilis,* are the conventional choice which are capable of fermenting glucose released from cellulose hydrolysis to ethanol [45]. However, hemicellulose hydrolysis releases a mixture of pentosans and the wild type *S. cerevisiae* and *Z. mobilis* strains lack the enzymatic machinery to ferment pentose sugars. While recombinant *S. cerevisiae* strains have been developed, yeast species capable of pentose fermentation have also been identified, such as *Candida shehatea, Pichia stipitis, and Pachysolen tannophilus* [10].

However, the efficiency of conversion is reportedly lower and further optimization of this step is required to increase the final ethanol yields.

On an average, the conversion of 'de-lignified' biomass to bioethanol with current technologies is still reportedly as low as 30-50%. Research is being conducted to improve the overall yields by developing genetically engineered strains, or by operational modifications that would increase hydrolysis rates and yields and reduce the formation of fermentation inhibitors [46]. Biological delignification can easily be integrated with subsequent hydrolysis and fermentation processes without the additional steps of neutralization or detoxification processes. Moreover, biological processes using fungal strains have advantages such as broader tolerance to process conditions, secretion of high titre values of hydrolytic enzymes and also higher ethanol tolerance as compared to yeast or bacteria [47]. An integration of biological delignification with solid state fermentation setup using lignocellulosic biomass is reported to be a low-cost option for production of otherwise expensive cellulase enzymes required in consecutive steps [48]. The following chapter describes the screening of three ligninolytic fungi based on their ability to grow on solid biomass substrates as well as degrade lignin effectively.

CHAPTER 3

SCREENING OF MICROORGANISMS AND PROCESS OPTIMIZATION

3.1 Introduction

Pretreatment of biomass is the first step in the biomass to biofuels process, where the biomass is delignified so as to increase cellulose accessibility in the subsequest hydrolysis step. As discussed in chapter 2, biological pretreatment methods commonly involve the use of wood-decaying fungi, that are known to cause selective de-lignification of biomass. Fungi have been found to grow in a variety of conditions and are one of the few species that can survive in environments with very low moisture content. Their filamentous nature also allows them to penetrate solid substrates and their ability to form spores enables them to disperse throughout the biomass. Such properties of fungi make them ideal candidates for microbial delignification compared to bacteria and other species that have the ability to degrade lignin. White rot fungi are known to secrete several enzymes such as laccases and more evolved peroxidase enzymes such as manganese peroxidase, lignin peroxidase and versatile peroxidase enzymes that efficiently degrade and even mineralize the most recalcitrant lignin polymer[35]. Several other fungal species, belonging to soft-rot and brown-rot fungal groups have been extensively used as a source of cellulases and hemicellulases that have the ability to degrade cellulose and hemicellulose polymers of lignocellulosic biomass [35]. Development of an efficient biological pretreatment step requires (1) selection of a fungal strain capable of growing rapidly on a variety of lignocellulosic biomass substrates and degrading lignin effectively and (2) identification of optimal parameters for growth and delignification by the fungus.

This chapter reports the initial screening of three different fungi for delignification of lignocellulosic biomass based on their growth characteristics and biomass degradation capabilities. The studies were conducted using solid-state fermentations setup using sugarcane bagasse as biomass substrate. Sugarcane bagasse was chosen as a substrate due to its abundant availability and also as it is reported to be an ideal substrate for microbial processes for the production of value-added products [1]. Solid state fermentations are reportedly more suitable for the production of enzymes from filamentous fungi [41]. Additionally, solid-state fermentations require lower energy inputs and lower reactor volumes [49]. The fungal strains chosen for the initial screening studies were: white rot fungi - *Phanerochaete chrysosporium* and *Pleurotus ostreatus*, which are well known for their ability to breakdown lignin in wood and biomass residues as well as an indigenous fungal isolate, which was found growing on the

bark of a Subabul wood tree. The two white rot fungi are known to secrete specialized oxidative enzymes that are capable of degrading several recalcitrant bonds in lignin [26, 50, 51].

One of the main drawbacks of solid state fermentation is mass transfer related diffusional limitations which causes severe inhomogeneities in overall biomass degradation. Filamentous fungi having the ability to penetrate the biomass layers better would ensure more uniform growth of the fungi which would, in turn, cause uniform degradation of all the biomass particles. Moreover, fungal strains that are capable of producing abundant spores will have a better dispersibility to distant locations, which would, in turn, increase the accessibility to inner biomass layers in a solid state fermentation setup. Also, fungal micro-organisms that can rapidly grow on biomass would hydrolyze and degrade the biomass at a faster rate and therefore can decrease the overall processing times. Therefore, the selection of the fungi based on growth characteristics was evaluated on the basis of how rapidly they grew on biomass, uniformity of growth across the substrate, the number of spores produced, and overall weight reduction which expresses the fungal strain's ability to degrade the biomass.

This chapter further reports phylogenetic identification of the new isolated indigenous fungus using 18s rRNA technique as well as the optimization of the growth conditions for this fungus using the design of experiments approach. The two white rot fungi, *P. chrysosporium* and *P. ostreatus* are well-studied microorganisms and literature on their optimum growth conditions for secreting high titer values of ligninolytic enzymes is available [52]. Therefore, a study based on the statistical design of experiments was conducted for the locally isolated fungal strain and several process parameters such as temperature, pH, initial spore count, moisture content and enzyme modulators were tested to optimize the growth conditions of the fungus to effectively degrade lignin fraction in biomass residues.

3.2 Materials and Methods

3.2.1 Fungal strains

Pure cultures of the different fungi namely, *Phanerochaete chrysosporium* (NCIM 1197), and *Pleurotus ostreatus* (NCIM 1200), were obtained from National Collection of Industrial Microorganisms (NCIM), Pune and stored at 4^{o}C. These strains were sub-cultured in potato dextrose agar (PDA) slants and Petri-dishes and stored at 4^{o}C. The working stock cultures were used for inoculating the solid state fermentations. Also, a novel fungal strain from the bark of a locally growing Subabul wood tree was isolated and grown in the research lab in PDA slants and Petri-dishes and stored at 4^{o}C.

3.2.2 Fermentation setup

For studying fungal growth characteristics and initial screening of fungi, solid state fermentations were set up using 3 g of biomass and 20 ml of deionized water in 250-ml Erlenmeyer flasks. The flasks were covered with non-absorbent cotton plugs and autoclaved at 121°C for 15 min for sterilization. The fungal filaments and spores were dislodged from respective agar plates via gentle scraping using a sterile spatula and the cells were added to the flask under aseptic conditions. The flasks were incubated at 30°C for 30 days. For each fermentation flask, a corresponding control containing the same quantity of substrate and deionized water was also set up without any cells. (Figure 3.1)

Figure 3.1: Solid state fermentations of sugarcane bagasse in stationary incubator

In the design of experiments study conducted for optimizing growth parameters of the indigenous fungus, Vogel's minimal media was used in the place of de-ionized water. Vogel's minimal media [53], was prepared by dissolving 2.5 g of $Na_3C_6H_5O_7.2H_2O$, 5 g of KH_2PO_4, 2 g of NH_4NO_3, 0.2 g of $MgSO_4.7\ H_2O$, 0.1 g of $CaCl_2.\ 2H_2O$, 0.1 ml of trace element solution in 1000 ml of de-ionized water by adding the constituents successively and vigorously stirring until complete dissolution. The minimal media was autoclaved at 121°C for 15 min and 1 ml of filter-sterilized biotin stock solution was added to the media at the time of inoculation. Trace element solution was prepared by adding 5 g of citric acid monohydrate, 5 g of $ZnSO_4.7H_2O$,

1 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.25 g of $CuSO_4.5H_2O$, 0.05 g of $MnSO_4.1H_2O$, 0.05 g of H_3BO_3 (anhydrous) and 0.05 g $Na_2MoO_4.2H_2O$ in 100 ml of de-ionized water by dissolving the constituents successively with stirring. Biotin stock solution was prepared by dissolving 5 mg biotin in 50 ml water and the solution was stored at 4°C [53]. All the chemicals used in this medium were purchased from Himedia Labs. Solid state fermentations were set up using 3 g of biomass and 20 ml of minimal in 250-ml Erlenmeyer flasks. For the inoculation of flasks, a conidial suspension of indigenous strain was prepared by dislodging the filaments from agar via gentle scraping and adding the cells to a known quantity of minimal media under aseptic conditions. The fungal suspension was gently mixed and filtered through a sterile muslin cloth to obtain a homogeneous spore suspension. The final spore count was adjusted to 3.7×10^7 cells/ml, and based on the initial spore count required; 1, 1.5 or 2 ml suspension volumes were used to inoculate each flask. The flasks were incubated at 30°C for 30 days. For each fermentation flask, a corresponding control containing the same quantity of substrate and minimal media was also set up without any cells.

3.2.3 Sample Preparation

At the end of the fermentation, the contents of the flask were filtered using a cotton cloth to separate the liquid and solid fractions. The solid fractions were thoroughly washed with deionized water, filtered, and dried at 105°C till constant weight. The liquid fractions were centrifuged at 10,000 rpm for 20 min, and the clear supernatant was stored at 4 °C for further analysis.

3.2.4 Imaging of the fungi

To study the microscopic structure of the fungi, the specimens were stained with lactophenol cotton blue (Make: Himedia) and observed at 50 X magnification using a phase contrast microscope attached with a digital CMOS camera with Toupview. (Make: ToupTek Photonics).

3.2.5 Overall weight reduction

The dried solid biomass fractions obtained at the end of the fermentations were weighed to calculate the overall weight reduction using the formula given below:

$$Overall\ weight\ reduction\ (\%) = \frac{(dry\ weight \text{ of the biomass in the control} - \text{ dry weight of the biomass in the fermented flask})}{\text{dry weight of the biomass in the fermented flask}}$$

3.2.6 Lignin content

The lignin content in the solid biomass samples was measured using a standard Klason's method [54]. The method contains taking 0.25 g of biomass in Erlenmeyer flask and adding 5 ml of 72% H_2SO_4. The sample was kept for 2 hours at 25^oC and was stirred every 15-20 min. The solution was diluted to 3% H_2SO_4 by adding distilled water and autoclaved at 121^oC for 1 hr. The sample was filtered and the solid residue was thoroughly washed with hot water to remove traces of sulphuric acid. Finally, the solid residue, which is 'acid insoluble lignin' was oven dried at 105^oC overnight and its weight was noted. A correction for ash content was made. The absorbance of the filtrate is noted at 205 nm after suitable dilution and converted to 'acid soluble lignin' using suitable absorptivity constant[54, 55]. Total lignin content was obtained by adding the acid-insoluble lignin measured gravimetrically and acid soluble lignin measured spectrophotometrically. Lignin content was measured before and after the fermentation to calculate percentage lignin degradation as follows,

$$Percentage\ lignin\ degradation = \ 100\ X\ \frac{(Initial\ lignin\ content - Final\ lignin\ content)}{(Initial\ lignin\ content)}$$

3.2.7 Cellulose content

The cellulose content in the solid fraction was analyzed gravimetrically using nitric acid and acetic acid. The protocol involves adding 15 ml acetic acid and 1.5 ml concentrated nitric acid to 3 g of oven dried biomass and refluxing the mixture for 20 minutes. The samples are then washed with ethanol and filtered using a cotton cloth followed by overnight oven drying. The sample is then weighed and the difference between the initial and final weights is noted as cellulose content [56]. A correction for ash content is made. Cellulose content was measured before and after the fermentation to calculate percentage cellulose degradation as follows,

$$Percentage\ cellulose\ degradation = \ 100\ X\ \frac{(Initial\ cellulose\ content - Final\ cellulose\ content)}{(Initial\ cellulose\ content)}$$

3.2.8 Ash content

The ash content in the solid biomass samples was estimated by keeping the samples at 575^{o}C for 6 hours in a muffle furnace and noting down the difference in weight [54].

3.2.9 Cellulase activity

Liquid samples of the fermentation were centrifuged, and the supernatants were sparged with CO_2 to adjust the pH between 6 and 6.5 to maintain uniform pH conditions. The total cellulase activity in day 30 liquid supernatant samples was measured by the 'Filter paper assay' using rolled Whatman no. 1 filter paper strip [57]. To 50 mg of rolled filter paper strip (1 x 6cm), 1ml of 50 mM pH 4.8 citrate buffer was added and the mixture was equilibrated to 37^{o}C in glass test tubes. To this mixture, 0.5 ml of the sample supernatant was added and incubated at 50^{o}C for 1 h. A test-tube containing 1.5 ml citrate buffer along with the filter paper strip was set aside as substrate control incubated under similar conditions. At the end of incubation, 3 ml of DNSA reagent is added and the mixture is placed in a boiling water bath for another 5 min. The solution is then cooled and suitably diluted by water before taking the absorbance readings at 540 nm. The absorbance of substrate control was also noted and subtracted from enzyme sample. The cellulase activity is reported in 'Filter paper units' (FPU) per g of biomass [57].

3.2.10 Laccase activity

Laccase activity was determined by measuring the oxidation kinetics of 2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), which was obtained from Sigma-Aldrich. To 0.6 ml of the liquid supernatant fraction, 0.6 ml of 0.1 M pH 5 citrate buffer was added and the mixture was equilibrated at room temperature. The spectral time scans at 420 nm were started as soon as 0.6 ml of 3mM ABTS was added to the sample making the total volume to 1.8 ml. The concentration of the green-blue cation radical formed from oxidation of ABTS was correlated to the enzyme activity using the extinction coefficient (ε_{420}) 36000$M^{-1}cm^{-1}$. One unit of enzyme activity was defined as the amount of product formed per unit volume per unit time at assay conditions ($\mu M\text{-}sec^{-1}$) [58, 59].

3.3 Results and discussion

3.3.1 Selection of fungal micro-organism

For the initial screening of the fungal strains, solid state fermentations were set up as detailed in materials and method section and incubated in a stationary incubator at 28^{o}C for 30 days (Figure 3.1). The different fungi whose growth characteristics in the fermentations were studied

were *Phanerochaete chrysosporium, Pleurotus ostreatus* and an indigenous isolated fungus from wood. The final selection of the fungi was based on the growth characteristics of the micro-organisms and percentage degradation of the biomass.

3.3.2 Growth characteristics of the fungal microorganisms

The screening of the fungi was based on growth characteristics evaluated on the basis of how rapidly they grew on biomass, uniformity of growth across the substrate, number of spores produced, and the extent of shrinkage of the biomass. The extent of shrinkage of biomass determines the efficiency of the fungi in secreting enzymes responsible for the degradation of biomass and therefore the ease of degradability of the biomass can be established. The images of the fungal growth on the sugarcane bagasse are shown in Figure 3.2 while Table 3.1 summarizes the growth characteristics of the three fungi in solid state culture conditions.

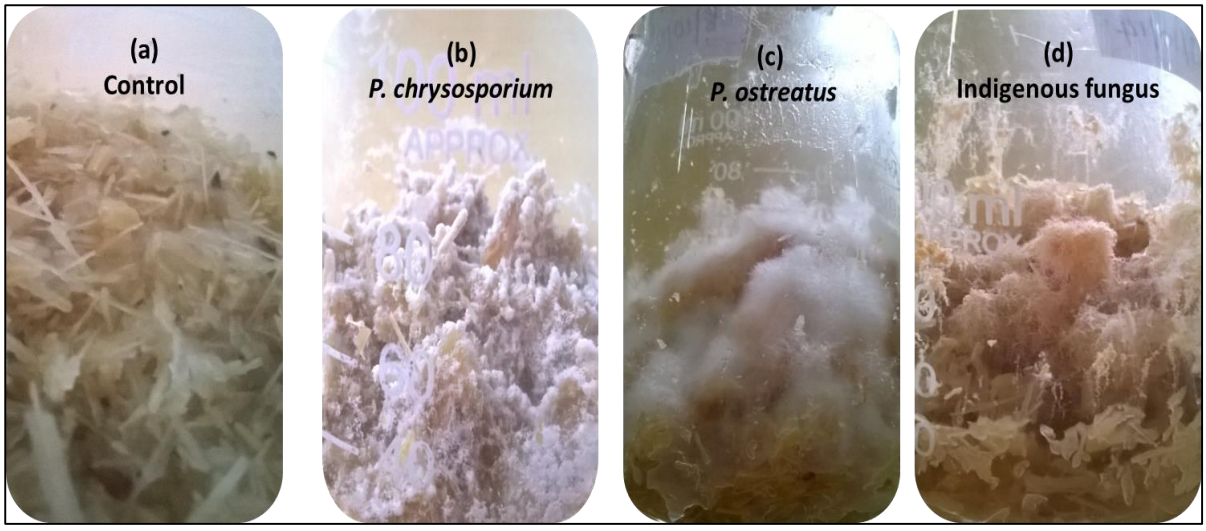


Figure 3.2: Growth of the different fungi in solid state fermentations of sugarcane bagasse a) Control b) *P.chrysosporium* c) *P.ostreatus* and d) Indigenous fungus

Table 3.1: Growth characteristics of the fungi

Sr. No	Growth characterization	*Phanerochaete chrysosporium* (Figure 3.2a)	*Pleurotus ostreatus* (Figure 3.2b)	Indigenous fungus (Figure 3.2c)
1	Growth rate of the fungi	-Fungal growth noticed after 2-3 days, as white patches on biomass surface. -Abundant visible growth seen, characterized by the spread of white filaments across the biomass layers.	- Small, white, discrete colonies seen on biomass surface after 3-4 days. - Developed into strong aggregated network along with the biomass fibers majorly on the surface	- Faint orange colored filaments seen on the surface in 16-24 hours. - Developed as thick long filaments (3-4 inches) covering the biomass surface
2	Uniformity of growth	Few white patches noticed on the bottom side of the flask.	-Growth restricted to topmost biomass layer -Non-uniform growth.	-Mostly non- uniform growth. -Preferred aerial growth along the sides of the flask -A few filaments seen penetrating the innermost biomass layers.
3	Spore formation	Spores in the form of white patches noticed that easily dislodged	No spores seen, only filamentous mat was noticed.	Abundant spores formed along with long filaments
4	Extent of biomass shrinkage	A visible shrinkage noticed in biomass volume	Shrinkage of the biomass was not noticed.	A visible shrinkage noticed in biomass volume
5	Other significant observations	A significant generation of liquid hydrolysate from biomass was noticed	Although biomass particles were engulfed with filamentous fungi, no apparent solubilization of biomass was visible.	Filaments developed a characteristic orange color over time due to the accumulation of carotenoids [60]

From the Figures and the table, while a more rapid growth rate and abundant sporulation is seen by indigenous fungus, better shrinkage of biomass and uniform growth were observed by *P.chrysosporium* fungal strain. In comparison to the two fungal strains, *P.ostreatus* showed non-uniform growth pattern with lower growth rates with no observable sporulation. Certain strains of *P.ostreatus* also known as oyster mushrooms are known to be completely sporeless [61, 62].

3.3.3 Microscopic images of the fungi

From the fermentation studies, *P.chrysosporium* and indigenous fungus showed promising results in terms of growth rate, the extent of shrinkage and spore formation. To study the microscopic structure of the fungi, the cells were scraped from their respective petridishes and stained with lactophenol cotton blue which binds to the chitin in the fungal cell walls. The slides were then observed at 50 X magnification. The thickness of the filaments was calculated from the software. Figure 3.3 shows the microscopic images of the different fungi. *P.chrysosporium* showed thin filamentous network interspersed with a few spores (Figure 3.3a) with the thickness of the filaments varying between 2-3 µm. *P. ostreatus* grew as highly interwoven mass of fungi with the filaments thickness varying between 0.7-1.2 µm (Figure 3.3b). Also, the strain did not show any sporulated growth. Indigenous fungus showed filaments that were significantly thicker than the other two fungi as well as abundant spores, with a characteristic orange color, possibly due to the presence of carotenoids. The thickness of the indigenous fungus varied between 7-12 µm (Figure 3.3c) which was 3-10 times greater than the other fungi. This indicates stronger filaments, which would have a greater capacity to penetrate solid biomass. From its physical characteristics, the indigenous species was identified to be belonging to Ascomycota family which produces abundant spores. Best known examples of filamentous fungi belonging to Ascomycota group that secretes carotenoids are *Neurospora crassa* and *Fusarium fujikuroi* [63].

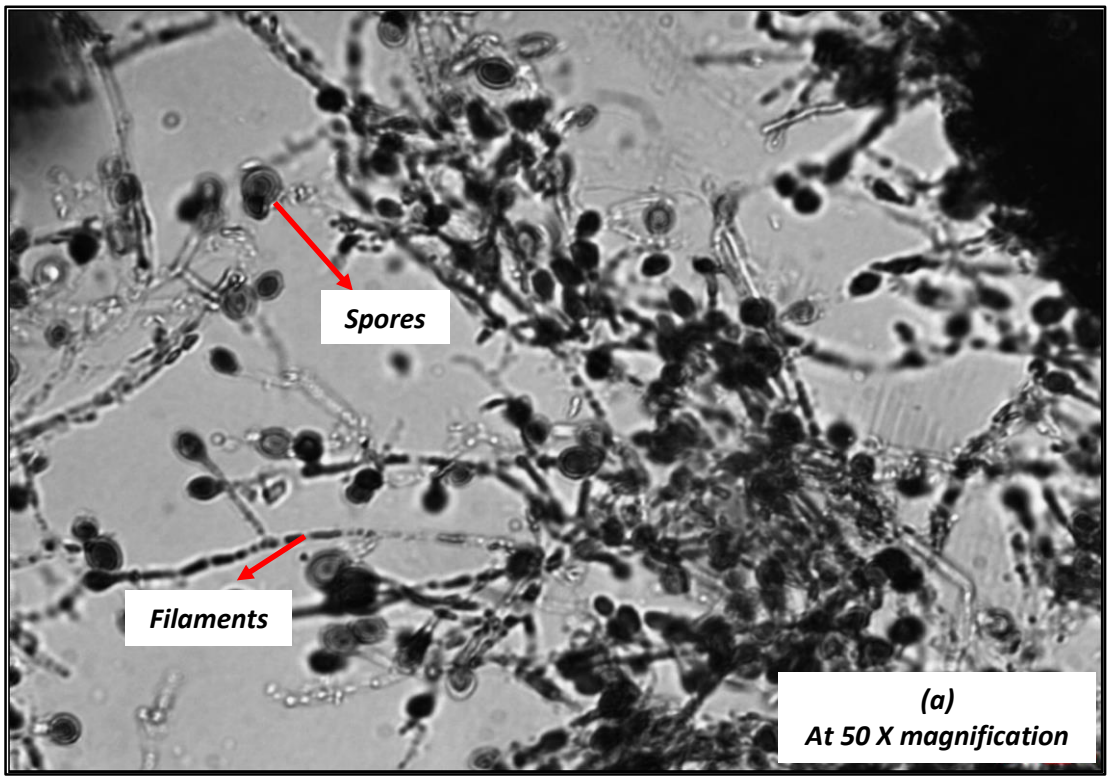


Figure 3.3 a: Microscopic image of *P. chrysosporium*

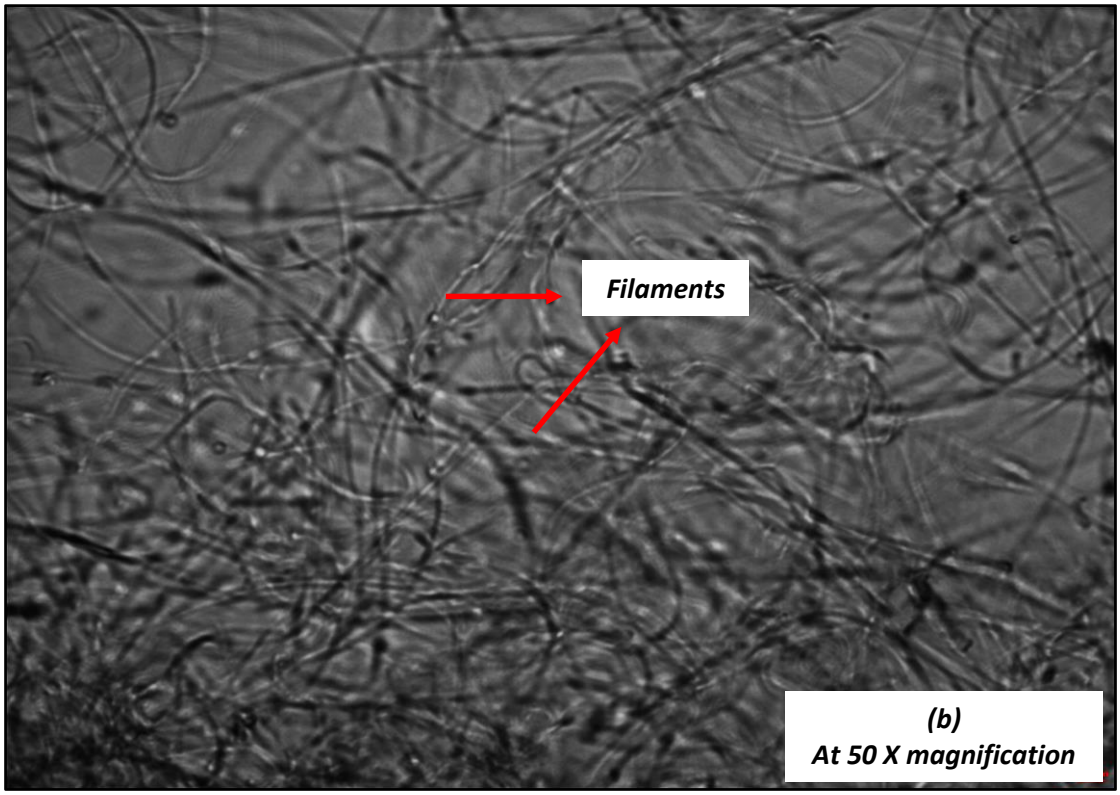


Figure 3.3b: Microscopic image of *P. ostreatus*

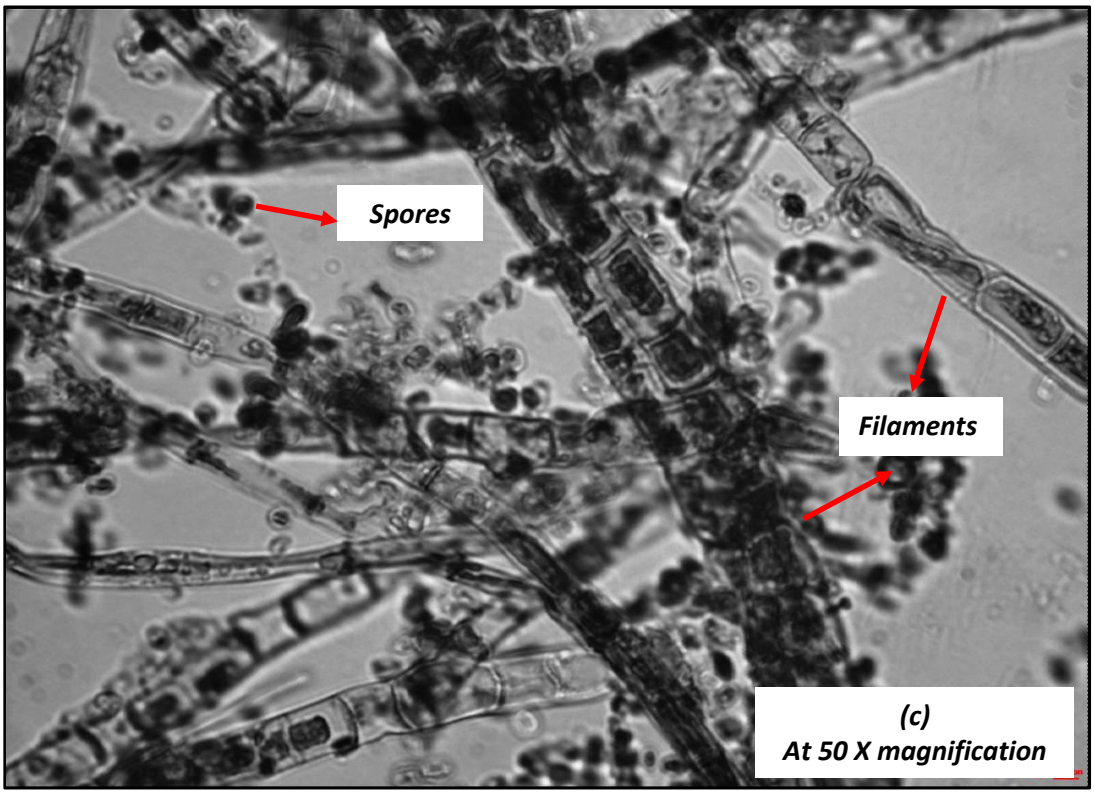


Figure 3.3c: Microscopic image of Indigenous fungus

3.3.4 Degradation of biomass

The degradation capability of the three fungi was studied using gravimetric analysis. At the end of the fermentation, the solid biomass residue was separated from liquid fraction and the overall degradation of biomass was calculated based on its initial and final dry weights as detailed in materials and method section. Also, lignin content in the biomass before and after fermentation was determined using Klason's method and the percentage lignin degradation was calculated. Table 3.2 shows overall weight reduction and percentage lignin degradation for the three fungi grown on sugarcane bagasse. Weight reduction in the biomass by *P. chrysosporium* was found to be the highest while *P. ostreatus* and indigenous fungus showed a similar reduction in weight. Percentage lignin degradation was found to be the highest for *P. chrysosporium* at approximately 27%. For *P. ostreatus* and indigenous fungus, the values were 23% and 20% respectively. As the growth of the fungus was at times restricted to certain areas of the biomass in solid state fermentation setup, considerable variability was noticed in the two gravimetric based methods. At the end of the fermentation, while a few biomass residues were totally degraded and formed fine particulates, certain biomass residues were found to be intact.

Table 3.2: Percentage overall biomass degradation and lignin degradation in biomass by different fungi in solid state fermentations

Micro-organism	Overall degradation (%)	Lignin degradation (%)
P. chrysosporium	32 ± 3%	27 ± 4%
P. ostreatus	29 ± 5%	23 ± 4%
Indigenous fungus	27 ± 4%	20 ± 5%

3.3.5 Characterization of novel isolated indigenous fungus

From the results discussed above, the indigenous fungus showed a lignin degrading ability which was similar to that of the white rot fungi tested under the same conditions. Moreover, the indigenous fungus showed the most rapid growth among all three and better uniformity in growth compared to *P. ostreatus*, which indicated that it could be a viable microbial candidate for the delignification step. Therefore, the fungus was sent to Yaazh Xenomics, Chennai, India for phylogenetic identification using18s ribosomal RNA (rRNA) sequencing technique. Briefly, the technique involved extracting the fungi genomic DNA from the strain and subjecting the 18S rRNA gene to polymerase chain reaction (PCR) amplifications. The PCR amplifications were done using 18S rRNA Internal Transcribed Spacer (ITR) Region Universal primers. Furthermore, the PCR was purified by removing unincorporated PCR primers with an ethanol precipitation protocol. The samples were then resuspended in distilled water and subjected to electrophoresis. The 18s rRNA gene sequence data were later aligned and analyzed using BLAST search tool made available by the National Centre for Biotechnology Information (NCBI) for the closest match. The phylogenetic tree was developed from the BLAST software (Figure 3.4) and the strain was identified to be *Neurospora discreta*. Since the presence of this species has not been reported earlier in India, the strain was also sent to Microbial Type and Culture Collection and Gene Bank (MTCC), Chandigarh which confirmed the strain to be *Neurospora discreta*. The ITS/5.8s rRNA gene sequence data from MTTC with 99.47% match is given in Figure 3.5.

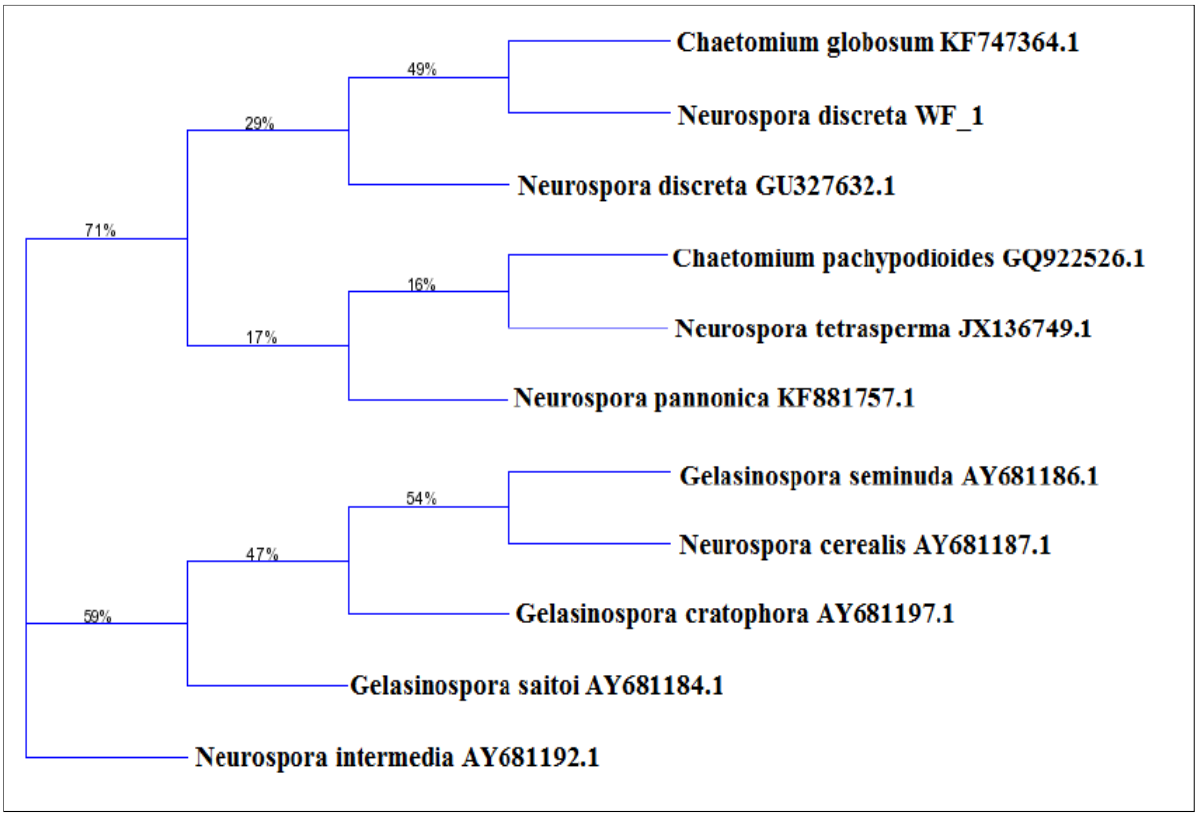


Figure 3.4: Phylogenetic tree for the indigenous fungus showing percentage match

GTAAGGGGTACCTGCGGAGGGATCATTACAGAGTTGCAAAACTCCCACAAACCATCGCGAATCTTACC
CGTACGGTTGCCTCGGCGCTGGCGGTCTGGAATGGCCTTCGGGCCCTCCCGGATCCTTGGGTCTCCCGC
TCGCGGGAGGCTGCCCGCCGGAGTGCCGAAACTAAACTCTTGATATTTTATGTTTCTCTGAGTAAACTT
TTAAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAG
TATTCTGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCATCAAGCTCTGCTTGCGTTGGGGATCCGCG
GCTGCCCGCGGTCCCTCAAAAACAGTGGCGGGCTCGCTAGTCACACCGAGCGTAGTAATTCTACATCG
CTATGGTCGTGCGGCGGGTGCTTGCCGTAAAACCCCCCCATTTCTAAGGTTGACCTCGGATCAGGTAG
GAATACCCGCTGAACTTAAGCATATCAATAAG

Figure 3.5: 18s rRNA sequence results for the indigenous N.discreta strain

Neurospora discreta belongs to the Ascomycota family which is well known for their abundant spore formation, modest nutritional requirements and also for the production of carotenoids[64]. An extensively studied and well-known species of the family, *Neurospora crassa* has served as a model organism for various genetic and molecular studies as well as for its biotechnological applications in the production of ethanol [44, 65, 66]. *Chrysonilia*

sitophilia, a teleomorph of *Neurospora sitophilia* has been reported to effectively cause lignin degradation in softwoods as well as hardwoods [67, 68]. It was also found to cause effective biodegradation of lignosulphonate which is a byproduct of paper and pulp industry, and considered to be more recalcitrant towards biological treatment than lignin [69]. *C. sitophilia* was also used for carrying out degradation studies on β-O-4 lignin model compounds to study the mechanism of lignin degradation [70]. Lignin degradation on softwood *Pinus radiata* was also studied and it was reported that *C. sitophila* caused 20% weight loss of pine wood in 3 months, with the losses of carbohydrate and lignin being 18% and 25%, respectively [67]. However, Neurospora sp. has not been explored for lignin degradation in agricultural residues so far. Moreover, the species *N. discreta* has never been tested for its ability to degrade lignin. In fact, this fungal species is usually found in western North America, Europe and Central Africa and easily found growing on trees affected by forest fires [71]. To our knowledge, the occurrence of *N. discreta* is thus far unrecorded in India.

3.3.6 Design of experiments for optimizing growth parameters of *N. discreta*

P. chrysosporium and *P. ostreatus*, are well known for their lignin degradation capabilities and their growth conditions have been optimized for laccase production as well as for selective de-lignification of biomass [52]. However, *Neurospora discreta* has not been studied in the context of biomass delignification and therefore the optimized growth conditions for this fungus have not yet been developed. Hence, a detailed study was carried out using the Design of Experiments (DOE) to evaluate various growth conditions as well as the presence of certain enzyme modulators on lignin degradation. For the DOE study, solid state fermentations using bagasse were setup using Vogel's minimal media instead of de-ionized water. As the primary objective was to accelerate the growth of the fungus, minimal media consisting of certain nutrients that provides basic support for fungal growth was used [53]. Taguchi optimization, a statistics-based approach to design experiments method was used, in which the parameters of interest are varied over a specified range, typically at two or three levels. The aim of the design of experiments was to accelerate the growth of fungus and induce laccase enzyme which is responsible for lignin degradation. Furthermore, several research studies report contrasting effects on cellulase enzyme by lignin degraded phenolic compounds present in the culture medium. Reports in the literature suggest both inhibition as well as stimulation by the products of lignin solubilization [72, 73]. To test this effect, liquid hydrolysate containing degraded

lignin obtained from previous fermentation experiments was also tested as cellulase enzyme modulators.

The parameters tested were temperature, pH, initial moisture content, initial spore count and biotin concentration, all of which are reported to have a direct effect on the growth of the fungus and also on the production of lignocellulosic enzymes [74]. Temperature and pH are considered two of the most critical process parameters during cell growth as they impact growth rate and enzyme activities. Typical temperature values for fungal growth are reported between 25-35^{o}C and slightly acidic ranges between 3 to 6 are preferred for optimal fungal growth [74]. Initial spore count impacts the initial growth rate of the fungus, while biotin is an essential vitamin that aids fungal growth. Literature reports an optimal Biotin concentration of 1µg/ml of minimal media [53] so two other levels with a ten-fold difference from this value were tested in parallel. Moisture content determines the substrate utilization and water activity levels of the fungal growth, which will eventually determine the overall degradation of the biomass [75] Furthermore, literature reports several laccase inducers which when added to culture medium enhances laccase production and facilitates biomass delignification [76–78]. For this reason, copper sulphate, ferulic acid and veratryl alcohol were chosen as laccase inducers, at three different concentrations each as shown in Table 3.3 [76–78]. The liquid hydrolysate was used as cellulase inhibitor at three different concentrations. Therefore, nine variables at three levels were tested and an L_{27} orthogonal array using MINITAB was created and used to analyze experimental results obtained from the 27 experiments. The overall degradation of biomass, percentage lignin and cellulose degradation were analyzed for each experiment. Moreover, the activity of laccase and cellulase which are enzymes responsible for degrading lignin and cellulose respectively were also measured in the liquid fractions at the end of fermentation.

Table 3.3: Selected parameters and assigned levels in design of experiments for optimizing growth parameters of *Neurospora discreta*

Sr. No	Factors	Low	Middle	High
1	Temperature	28^{o}C	30^{o}C	33^{o}C
2	pH	3	4	6
3	Moisture content	5 ml	10 ml	20 ml
4	Initial spore count	$37*10^{6}$	$56*10^{6}$	$75*10^{6}$
5	Biotin content	0.1 µg/ml	1 µg/ml	10 µg/ml
6	Laccase inducer –Copper sulphate	0.05 mg	1 mg	10 mg
7	Laccase inducer – Veratryl alcohol	0	0.672 mg	6.72 mg
8	Laccase inducer – Ferulic acid	0	0.776 mg	7.76 mg
9	Cellulase stimulator/inhibitor-Liquid hydrolysate	0	0.5 ml	1ml

Figure 3.6 reports the effect of selected variables on the overall degradation of biomass and percentage lignin degradation in the biomass by the fungus. The plots were obtained from the ANOVA analysis by specifying responses and the corresponding factors. The X-axis reports the three levels of the variable and the y-axis shows the responses, i.e., either overall degradation (Figure 3.6a) or percentage lignin degradation (Figure 3.6b). An increase in temperature, moisture content and spore count increased overall biomass degradation, while an increase in biotin content had a negative effect. Also an increase in the concentration of laccase inducer copper sulphate had a negative effect on lignin degradation. Apart from this, laccase enzyme activity was not detected in any of the experimental sets, across all 27 sets although a reduction in lignin content was found as analyzed from Klason's method. Probably, due to the low levels of laccase activity expressed by the fungus, its activity could not be detected by the standard assay conditions used. Based on the trends observed for overall degradation, the optimum temperature for the fungus was fixed at 28^{o}C and as moisture content aided in overall degradation, the future experiments were conducted using submerged type fermentation. The biotin content, copper sulphate were maintained at minimum concentrations, while pH was set at 5.

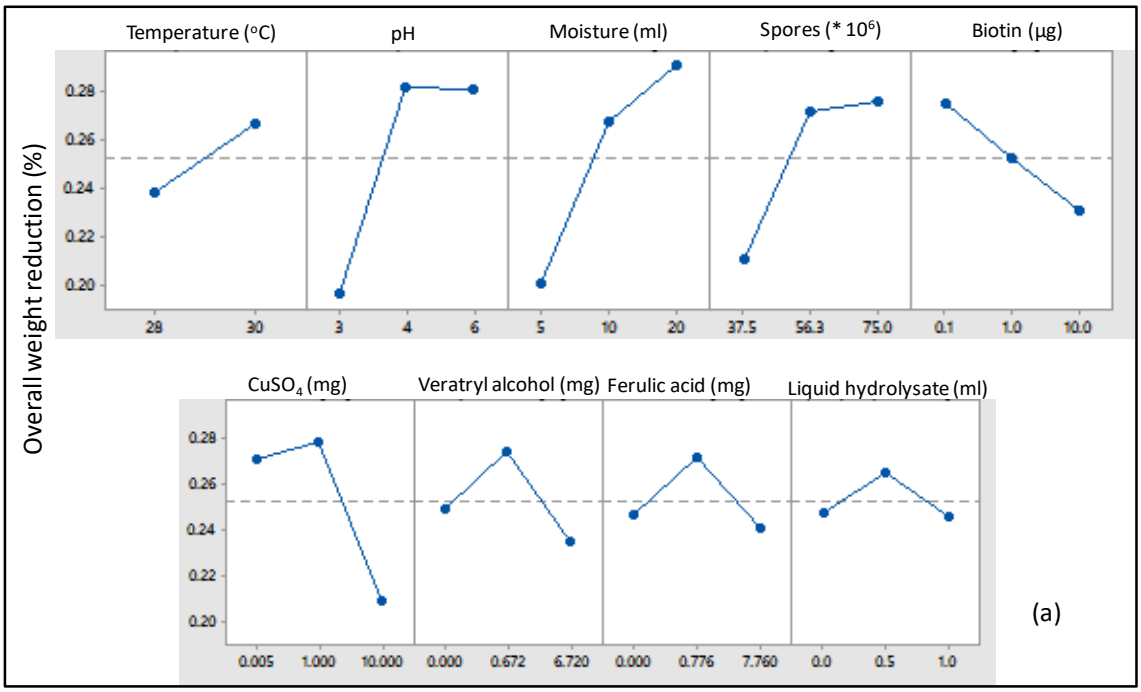


Figure 3.6a: Impact of selected parameters on overall weight degradation (%)

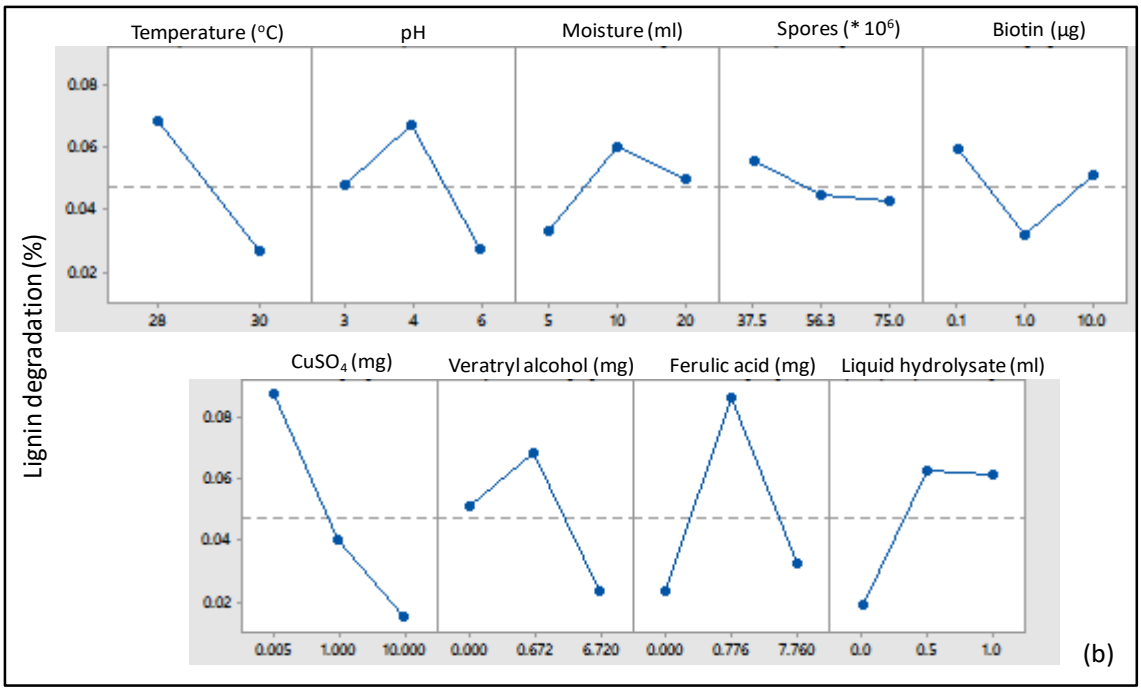


Figure 3.6b: Impact of selected parameters on Lignin degradation (%)

Figure 3.7 reports the effect of nine selected variables on the cellulase activity measured in FPU/ g of biomass and the percentage cellulose degradation. A strong correlation is noticed between percentage of cellulose degradation and cellulase enzyme activity. An increase in temperature, moisture content and spore count enhanced cellulose degradation while cellulase

enzyme activity was found to be optimum at a pH of 4. An increase in biotin had the same effect as overall degradation and was found to negatively impact cellulose degradation. Laccase inducer, veratryl alcohol seemed to negatively impact cellulase activity and a similar trend is reported in the literature [77] Ferulic acid and degraded lignin compounds present in liquid hydrolyzate showed similar effects for both cellulase activity as well as cellulose degradations. In both the cases, a slight stimulation at lower concentrations was seen followed by inhibition, which is also supported by literature which reports the activity of cellulase enzyme to be strongly dependent on the concentration of the phenolic compounds released during lignin degradation [72].

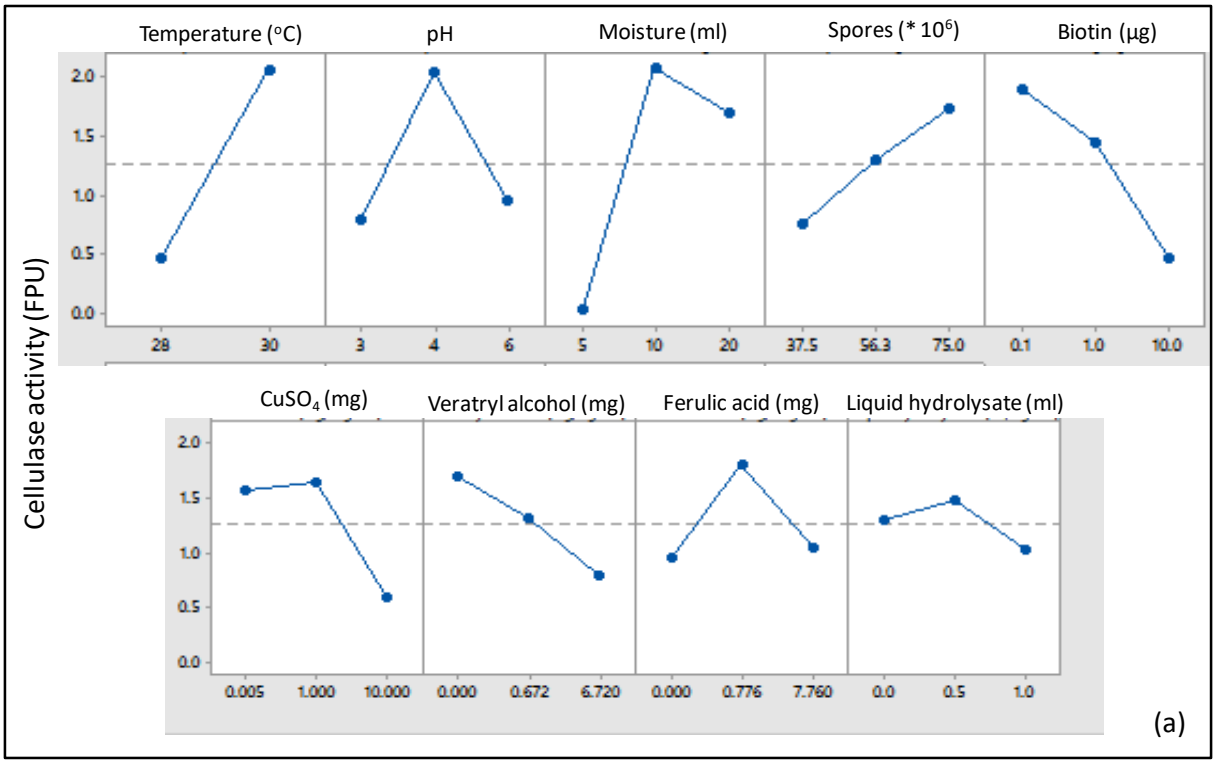


Figure 3.7a: Impact of selected parameters on Cellulase activity (FPU)

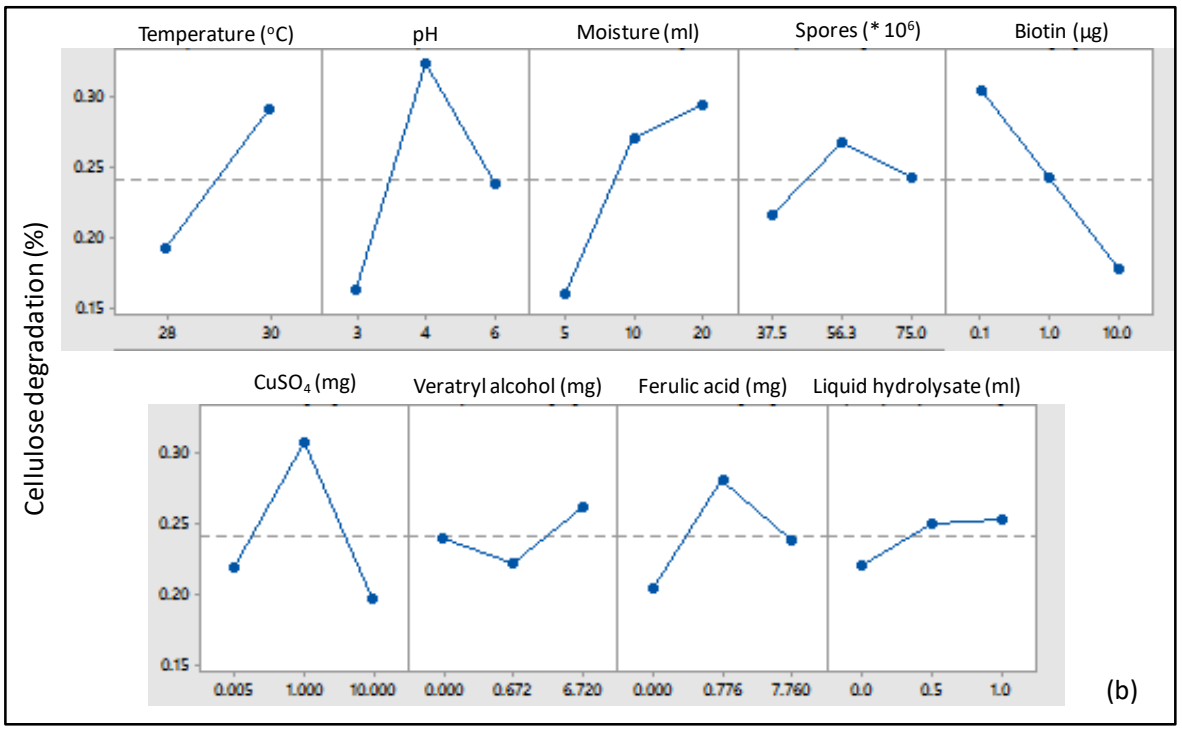


Figure 3.7b: Impact of selected parameters on Cellulose degradation (%)

3.4 Conclusion

Screening of three fungi, i.e., *P. chrysosporium*, *P. ostreatus* and an indigenous fungus using solid-state fermentation of sugarcane bagasse was carried out. Based on their growth characteristics and biomass degrading capabilities, *P. chrysosporium* and the indigenous fungus were selected for further studies. The indigenous fungus was identified to be *Neurospora discreta* using 18srRNA technique, and its occurrence is reported for the first time in India. The optimum growth parameters for *N. discreta* were identified from the design of experiments study. Based on the DOE study, the temperature, pH, moisture content, biotin and copper sulphate concentrations in growth media were fixed and used in all further studies. However, laccase activity was not detected in any of the fermentation experiments. From the solid state fermentations and the design of experiments study, it was found that *N. discreta* was capable of degrading lignin and cellulose components of biomass. In all experiments, the growth of fungi in solid state fermentations was found to be highly heterogeneous, which also caused a huge variability in gravimetric analysis of lignin and cellulose. To overcome heterogeneity and mass transfer limitations and also since higher moisture content aided the overall degradation of biomass, submerged fermentations were used for further studies instead of solid state fermentations.

CHAPTER 4

SELECTION OF FUNGUS FOR DELIGNIFICATION

4.1 Introduction

Conventional methods of pretreatment such as chemical, thermo-chemical or physico-chemical methods are frequently employed for removal of lignin in the pretreatment step [12] but they have drawbacks such as high energy requirements and detrimental impacts on the environment. Microbial degradation of lignin using fungal strains overcomes many of these drawbacks, although lower yields and longer processing times are potential roadblocks for a successful process [29, 48]. Basidiomycetes, in particular, white rot fungi are known to produce ligninolytic enzymes such as lignin peroxidase (LiPs), manganese peroxidase (MnPs), versatile peroxidase (VP) and laccases that can effectively breakdown the macrostructure of lignin, along with several accessory enzymes [8, 28]. However, the use of white rot fungi as a delignification step is still not considered a viable alternative to chemical and thermochemical routes due to the lower rates of delignification seen.

In the previous chapter, an indigenous strain of *Neurospora discreta* was evaluated along with well known white rot fungi for biomass delignification. Based on growth rate and growth characteristics as well as delignification capability, *N. discreta* and *P. chrysosporium* were selected for further studies. This chapter reports a more detailed evaluation carried out to compare these two fungi and select the better candidate for delignification of biomass.

The extent of biomass degradation is heavily dependant on the composition of the biomass. Generally, it is accepted that higher lignin in the biomass makes it more difficult to degrade and its efficient removal is considered one of the central aspects of cellulosic ethanol production at industrial scale [29]. Lignin polymerization from the three monolignols results in a complex three-dimensional structure due to both chain branching as well as inter and intrachain coupling during the polymerization process. The three monolignol structures, give rise to three types of lignin units, namely p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (Figure 4.1) and its relative composition in the lignin macromolecular influences the de-lignification process [29, 79]. The absence of methoxy group at the C5 position in the aromatic ring makes guaiacyl lignin unit more resistant to cleavage due to the formation of more C-C linkages that contributes to condensed recalcitrant lignins [79, 80]. The S-units present in the lignin are considered to be having lower redox potentials due to the presence of two electron-donor methoxy groups. Also, this leads to the formation of easily cleavable C4 ether linkages

as C3 and C5 are both occupied by methoxy groups [80]. S/G ratio has therefore been used as an important indicator of degradability of the biomass species [80–82]. Thus, the overall lignin content, as well as the relative composition of the lignin units within the polymer, determines its degradability.

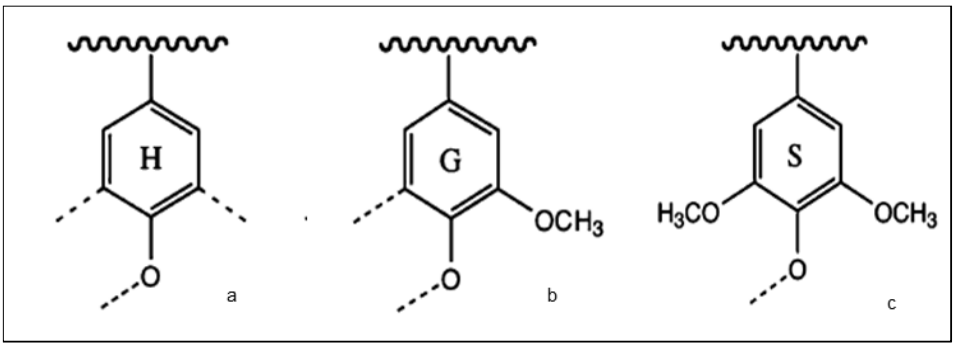


Figure 4.1: Structural units in lignin: (a) p-hydroxyphenyl unit, (b) Guaiacyl unit and (c) Syringyl unit. Adapted from [28]

An ideal microbial catalyst for delignification should be able to handle a variety of biomass residues having a range of lignin concentrations as well as varying structures. Therefore, it is important to select the fungus based on different biomass residues. This chapter describes the study carried out to compare *N. discreta* with the well-known white rot fungus *P. chrysosporium* using two widely different biomass residues: sugarcane bagasse and cocopeat or coir pith (Figure 4.2). Sugarcane bagasse, a well-studied substrate reportedly contains lignin (10±20%), cellulose (40±41.3) and hemicellulose (27±37.5)[83]. Cocopeat, on the other hand, is a much less studied residue although it is known to contain significantly higher levels of lignin. Israel et al., [19] conducted a detailed characterization of cocopeat and reported that it contains a high percentage of lignin (53.5%), cellulose (36%), and ash (9%). The two residues were selected for three reasons: (1) abundant availability of these residues in India and other tropical and sub-tropical regions, (2) a wide difference in lignin concentration and (3) difference in syringyl to guaiacyl (S/G) ratios. Literature reports an S/G ratio of 0.23 for coconut coir [84] and 0.82 - 0.85 for sugarcane bagasse [85, 86], making cocopeat harder to degrade compared to sugarcane bagasse.

Figure 4.2: Biomass substrates with varying lignin content (a) Sugarcane bagasse and (b) Cocopeat

4.2 Materials required

4.2.1 Biomass residues

Sugarcane bagasse and coco-peat were both obtained from local vendors (Figure 4.2). Sugarcane bagasse was thoroughly washed with water and then dried at 80 - 90 °C in a hot air oven till constant weight was achieved. It was chopped to 15–20 mm size and stored in airtight containers at 2–4 °C till further use. It should be noted that both the substrates were heterogeneous in nature and while sugarcane bagasse contained 'pith' fiber [87], cocopeat had some fibrous material apart from the pith.

4.2.2 Fungal strains

A pure culture of *Phanerochaete chrysosporium* (NCIM 1197) was obtained from National Collection of Industrial Microorganisms, Pune and stored at 2–4 °C. *Neurospora discreta* was previously isolated from the bark of a locally growing Subabul wood tree by streaking cells scraped from the bark on to the agar plates. Both cultures were maintained by periodic sub-culturing on agar plates and slants as described below and incubating at 28-30^{o}C until the plates were fully grown. Thereafter, the cells were refrigerated at 2-4^{o}C until further use.

4.2.3 Media for fungal growth and maintenance

Potato dextrose agar (PDA) was procured from Himedia. PDA plates were prepared by pouring sterile molten agar into Petri plates and allowing it to solidify. Vogel's minimal media [53], which was used in all studies, was prepared by dissolving 2.5 g of $Na_3C_6H_5O_7.2H_2O$, 5 g of KH_2PO_4, 2 g of NH_4NO_3, 0.2 g of $MgSO_4.7\ H_2O$, 0.1 g of $CaCl_2.\ 2H_2O$, 0.1 ml of trace element solution in 1000 ml of de-ionized water by adding the constituents successively and vigorously

stirring until complete dissolution. The minimal media was autoclaved and 1 ml of filter-sterilized biotin stock solution was added to the media at the time of inoculation. Trace element solution was prepared by adding 5 g of citric acid monohydrate, 5 g of $ZnSO_4.7H_2O$, 1 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.25 g of $CuSO_4.5H_2O$, 0.05 g of $MnSO_4.1H_2O$, 0.05 g of H_3BO_3 (anhydrous) and 0.05 g $Na_2MoO_4.2H_2O$ in 100 ml of de-ionized water by dissolving the constituents successively with stirring. Biotin stock solution was prepared by dissolving 5 mg biotin in 50 ml water and the solution was stored at 4^{o}C [53]. All the chemicals used in this medium were purchased from Himedia Labs.

4.3 Fermentation setup

4.3.1 Submerged fermentations

Submerged fermentations were set up in 250-ml Erlenmeyer flasks. Each flask contained 1 g of the substrate (coco peat or bagasse) and 0.5 g of sucrose in 100 ml Vogel's minimal media. The flasks were covered with non-absorbent cotton plugs and autoclaved at 121°C for 15 min.

4.3.2 Inoculation and incubation of flasks

A conidial suspension of each of the fungi was prepared by dislodging the filaments from agar via gentle scraping and adding the cells to a known quantity of minimal media under aseptic conditions. The fungal suspension was gently mixed and filtered through a sterile muslin cloth to obtain a homogeneous spore suspension. The final spore count was adjusted to approximately 10^7 cells/ml, and 1 ml suspension was used to inoculate each flask. The flasks were incubated at 30°C for 30 days. For each fermentation flask, a corresponding control containing the same quantity of substrate and minimal media was also set up without any cells.

4.3.3 Sample Preparation

At each time point, one flask of each fungal strain was removed, and the contents were filtered using a cloth to separate the liquid and solid fractions. The solid fractions were thoroughly washed with deionized water, filtered, and dried at 105°C till constant weight was attained. The liquid fractions were centrifuged at 10,000 rpm for 20 min using refrigerated centrifuge, and the clear supernatant was stored at 4 °C for further analysis.

4.4 Analytical Methods

4.4.1 Sugar Estimation by DNSA method

The concentration of total reducing sugars was measured by Di-Nitro Salicylic Acid (DNSA) method in the clear supernatant liquid sample at regular time intervals [88]. The concentration

of reducing sugars in the samples was calculated using a standard curve plotted between the optical density at 540 nm and the concentration of the standard glucose solution in mg/ml. The method involves adding 20µl of concentrated HCl to 1 ml supernatant sample in a clean glass test-tube and heating the solution in boiling water bath for 5 min. After cooling, 50 µl of 5N KOH was added to neutralize the sample. Furthermore, to 1 ml of above sample, 2 ml of DNSA reagent was added and the mixture is placed in a boiling water bath for another 10 min. The solution is then cooled and suitably diluted by water before taking the absorbance readings at 540 nm. The DNSA reagent was prepared by dissolving 300g of sodium potassium tartrate in 500 ml distilled water. To 200 ml of 2N NaOH solution, 10 g of 3,5-dinitrosalicylic acid (Make: Merck) was added and vigorously stirred. The two solutions were mixed and the final volume was adjusted to 1000 ml by distilled water. The reagent was stored in amber bottles at room temperature.

4.4.2 High-Performance Liquid Chromatography

The clear supernatant obtained from fermentation sets were analyzed for lignin degradation products using RP-HPLC method. Chromatographic separation was performed on Thermo-Scientific BDS Hypersil C-18 column (250 × 4.6 mm ID, 5 µm particle size). The flow rate was 1 ml/min, the injection volume was 25 µL, and the column temperature was set at 27 °C. The mobile phase used was a mixture of two solvents: A, 1% acetic acid in deionized water, and B, 100% acetonitrile. A gradient elution with the following concentrations of solvent B was used: at 0 min— 0 %; 5 min— 3 %; 10 min—6 %; 15 min—10% and 20 min—20 %. The baseline was monitored for 20 min before the next injection, and the elute was continuously monitored by a photodiode array detector at 254 nm. The data was integrated and analyzed using the Shimadzu Automated Software system. Also, a calibration curve was plotted using 1000 mg L^{-1} - 5000 mg L^{-1} water soluble Kraft lignin (Make: Sigma-Aldrich, Catalogue No: 4710003), which was used to quantify the lignin degradation products. Kraft lignin is obtained by treating lignocellulosic biomass residues with high alkaline sulphide solutions [89], that degrades the lignin fraction to water soluble lignin intermediates was procured from Sigma-Aldrich.

4.4.3 Lignin content

The lignin content in the solid biomass samples was measured using standard Klason's method [54]. The method contains taking 0.25 g of biomass in Erlenmeyer flask and adding 5 ml of 72% H_2SO_4. The sample was kept for 2 hours at 25^oC and was stirred every 15-20 min. The solution was diluted to 3% H_2SO_4 by adding distilled water and autoclaved at 121^oC for 1 hr. The sample was filtered and the solid residue was thoroughly washed with hot water to remove

traces of sulphuric acid. Finally, the solid residue, which is 'acid insoluble lignin' was oven dried at 105ºC overnight and its weight was noted. A correction for ash content was made. The absorbance of the filtrate is noted at 205 nm after suitable dilution and converted to 'acid soluble lignin' using suitable absorptivity constant [54, 55]. Total lignin content was obtained by adding the acid-insoluble lignin measured gravimetrically and acid soluble lignin measured spectrophotometrically. Lignin content was measured before and after the fermentation to calculate percentage lignin degradation as follows,

$$Lignin\ degradation\ (\%) = \ 100\ X\ \frac{(Initial\ lignin\ content - Final\ lignin\ content)}{(Initial\ lignin\ content)}$$

4.4.4 Ash content

The ash content in the solid biomass samples was estimated by keeping the samples at 575ºC for 6 hours in a muffle furnace and noting down the difference in weight [54].

4.4.5 Fourier transform infrared (FTIR) spectra

The FTIR spectra of the solid samples before and after fungal treatment were acquired on FTIR 4200 spectrophotometer of JASCO Make. Sample pellets were prepared using 1 mg of sample and 100 mg of oven dried potassium bromide. For each spectrum, 64 scans in the range of 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} were conducted in the absorbance mode and averaged.

4.5 Results and Discussion

4.5.1 Sugar estimation

Figure 4.3 shows the sugar concentration at different time points during fermentation, assayed by DNSA method. The total reducing sugar content was measured as 5 mg ml^{-1} for cocopeat fermentation setups on day zero, while it was slightly higher (around 6 mg ml^{-1}) for sugarcane bagasse fermentations because of the free sugars present in the biomass itself. *N. discreta* rapidly utilized the sugar content in both cocopeat and bagasse fermentations. In the case of *P. chrysosporium,* an initial increase in sugar content was seen until day 8 and then a gradual decrease with time was noted. In *N. discreta*, the sugar content rapidly decreased by day 5 and remained at a minimum value for the rest of the fermentation. This could be attributed to the higher growth rate of *N. discreta* compared to *P. chrysosporium*, which was also observed visually.

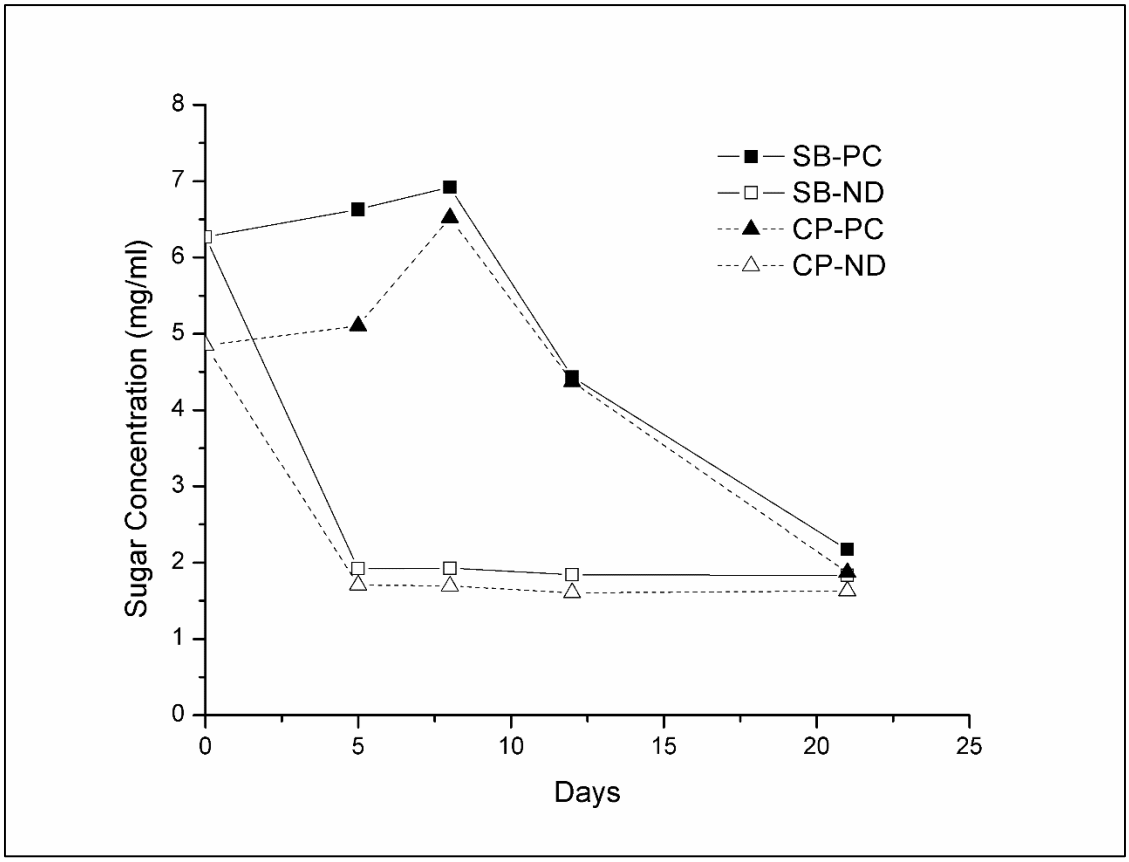


Figure 4.3: Sugar concentration measured by DNSA method on different days

(SB-PC: Sugarcane bagasse treated with *P. chrysosporium*), SB-ND: Sugarcane bagasse treated with *N. discreta*, CP-PC: Cocopeat treated with *P. chrysosporium*, CP-ND: Cocopeat treated with *N. discreta*)

4.5.2 High-Performance Liquid Chromatography

The liquid supernatant from the submerged fermentation flasks was subjected to RP-HPLC to analyze the lignin degradation products. Soluble alkali lignin was used as a standard for comparison. A chromatogram of the standard alkali lignin analyzed using RP-HPLC was shown in Figure 4.4. The RP-HPLC analysis of the standard sample indicated the presence of a major peak at retention time of 2.9 ± 0.2 min along with other minor peaks.

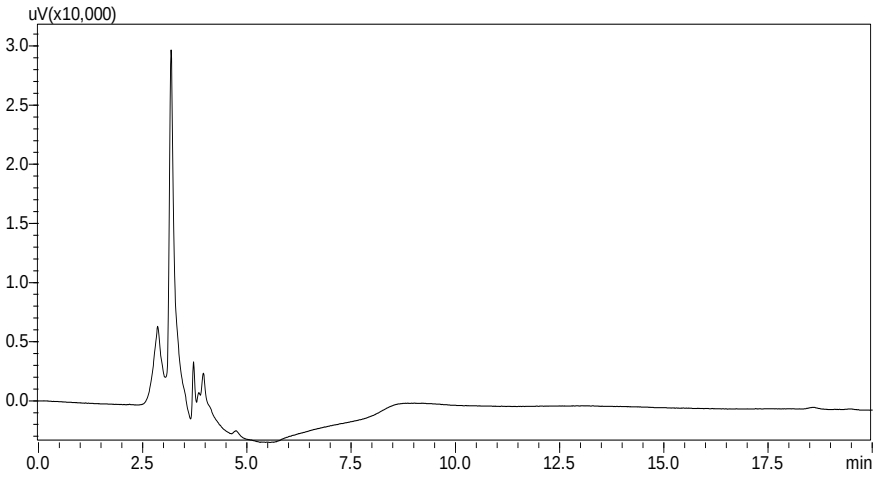


Figure 4.4: Chromatogram of standard alkali lignin (0.5 mg/ml)

In the case of the *N. discreta* a major single peak is seen at a retention time of 2.9 ± 0.015 minutes was seen for both the substrates, cocopeat (Figure 4.5a) and sugarcane bagasse (Figure 4.5b) and an increase in peak area and peak height were noted as fermentation progressed. The peaks exactly corresponded to the standard alkali lignin peak at RT 2.9.

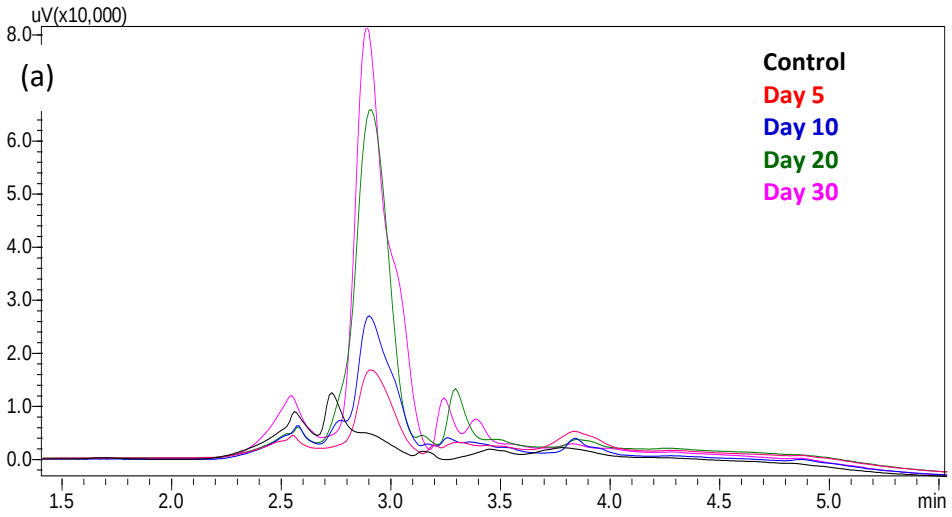


Figure 4.5a: Chromatograms of liquid supernatant samples of cocopeat treated with *N. discreta*

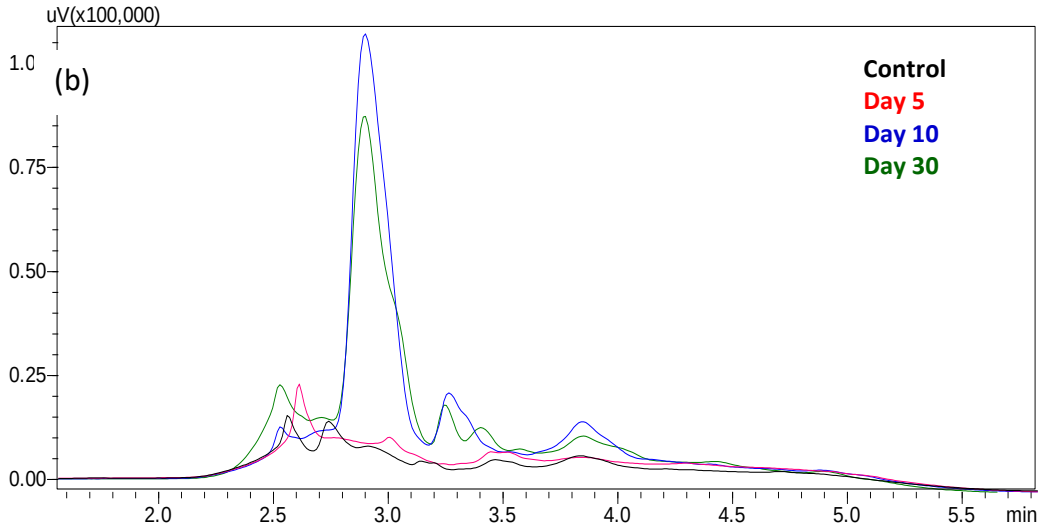


Figure 4.5b: Chromatograms of liquid supernatant samples of sugarcane bagasse treated with *N. discreta*

In the case of *P.chrysosporium* samples, three smaller peaks were seen between retention time: 2.0 and 3.2 minutes which changed with time (Figure 4.6). The additional lignin-degrading enzymes (manganese, lignin and versatile peroxidases) in *P. chrysosporium* could be the reason for the more heterogeneous profile.

While comparing the chromatograms of *N.discreta* with *P.chrysosporium* (Figures 4.5 and 4.6), the solubilized lignin content in the case of *N.discreta* was found to be higher than *P.chrysosporium* for both cocopeat as well as sugarcane bagasse substrates. The concentration of the solubilized lignin (in mg/ml) in liquid supernatant samples was calculated from the calibration curve obtained from standard alkali lignin plotted at different dilutions. For day 30 samples, 180% increase was seen using *N.discreta* over *P.chrysosporium* for cocopeat biomass and a 130% increase in sugarcane bagasse.

Degradation of lignin by *N.discreta* caused the formation of low molecular weight water-soluble products. Enzymes such as laccases and lignin peroxidases were found in certain strains of Neurospora [90]. These enzymes are known to degrade lignin to low molecular weight soluble compounds. However, in white rot fungi such as *P.chrysosporium*, the water-soluble degraded lignin further undergoes mineralization to form CO_2 gas. It is reported that while lignin peroxidase enzymes plays an important role in lignin solubilization, more evolved manganese peroxidase enzyme in *P.chrysosporium* regulates the mineralization of water-soluble lignin to CO_2.

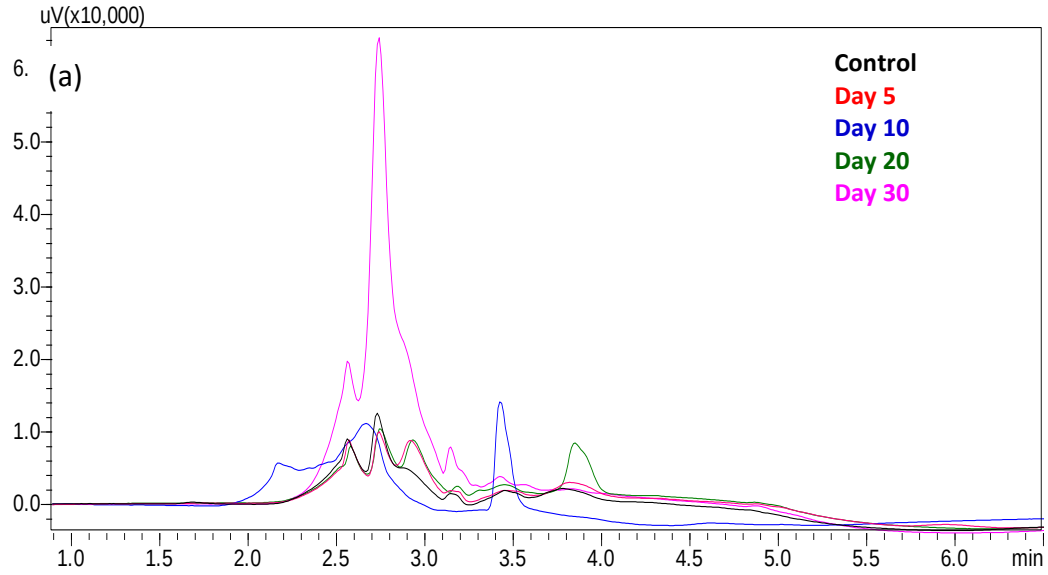


Figure 4.6a: Chromatograms of liquid supernatant samples of cocopeat treated with *P. chrysosporium*

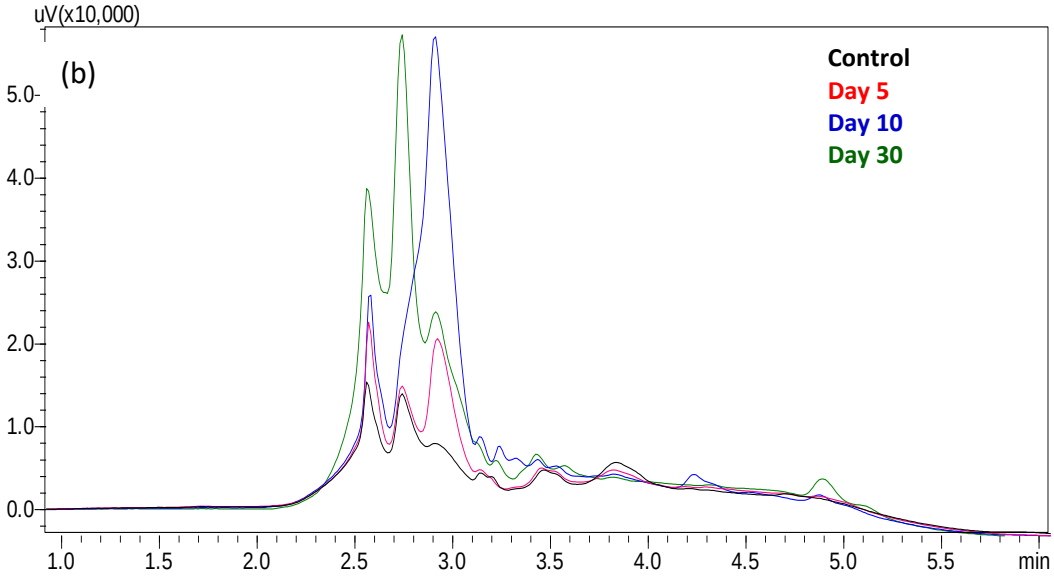


Figure 4.6b: Chromatograms of liquid supernatant samples of sugarcane bagasse treated with *P. chrysosporium*

4.5.3 Fourier Transform Infrared Spectroscopy

Figure 4.7 shows the FTIR spectra of the solid substrates: sugarcane bagasse and cocopeat before and after fungal treatment as well as the S/G ratios for each sample. S/G ratios were calculated using absorbances at 1335 cm^{-1} (S) and 1275 cm^{-1} (G) as suggested in the literature [80]. These wavelengths represent the aromatic ring breathing of syringyl and guaiacyl units

respectively. As seen from Figure 4.7 sugarcane bagasse had a significantly higher S/G ratio (1.0) compared to cocopeat (0.43). This agreed with the trend in S/G ratios reported in the literature and supports the hypothesis that sugarcane bagasse is easier to degrade compared to cocopeat owing to the structure of lignin. Also, the S/G ratio of bagasse stayed nearly the same with *P.chrysosporium* (0.94) while it decreased after treatment with *N. discreta* (0.8). This could be due to the fact that *P.chrysosporium* degraded guaiacyl units along with syringyl units due to the presence of more evolved peroxidase enzymes. With *N.discreta* the syringyl units may have been degraded to a greater extent compared to the guaiacyl units resulting in a decrease in S/G ratio. In the case of cocopeat, the S/G ratios increased significantly after treatment with *P.chrysosporium* (0.85) and slightly with *N.discreta* (0.53). This could be due to the unusually high guaiacyl content in cocopeat which might have altered the mechanism of fungal action. Further analysis is necessary to investigate this phenomenon.

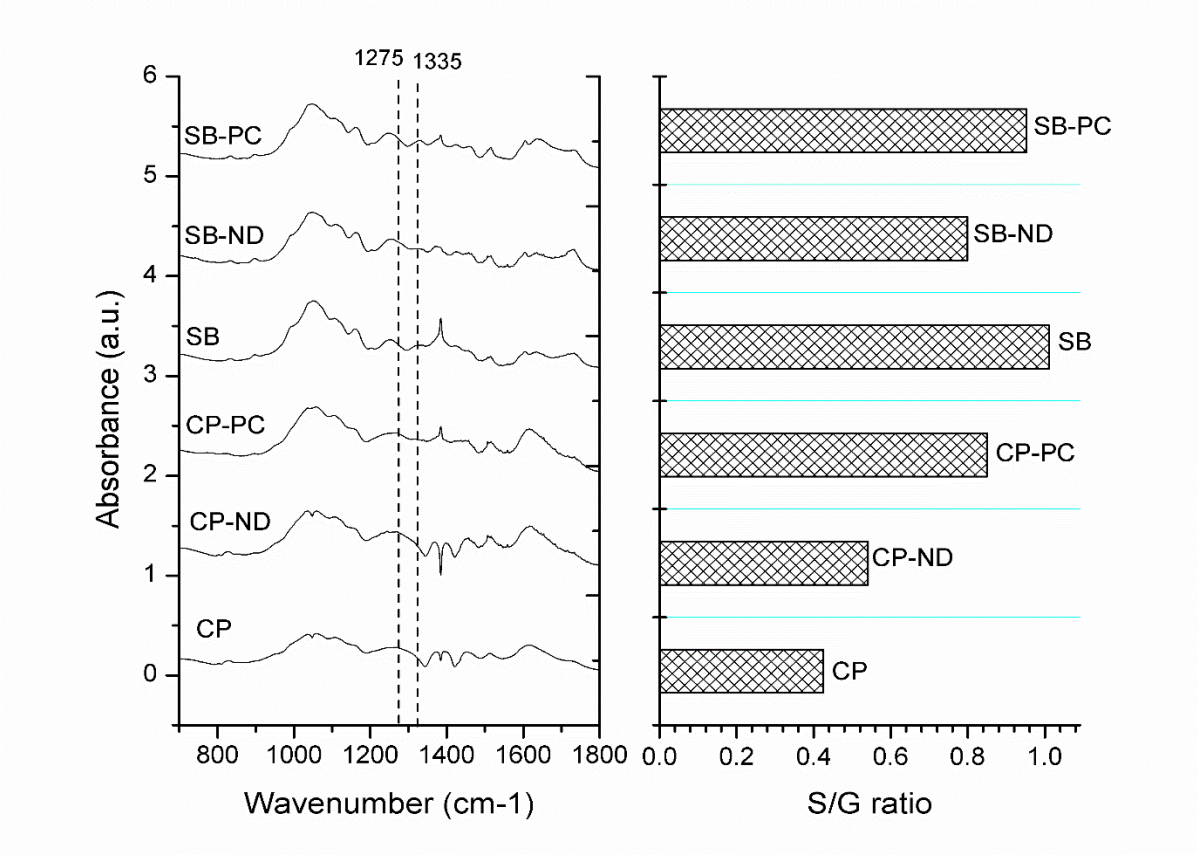


Figure 4.7: FTIR spectra and S/G ratios

(SB-PC: Sugarcane bagasse treated with *P. chrysosporium*, SB-ND: Sugarcane bagasse treated with *N. discreta*, SB: Untreated Sugarcane bagasse, CP-PC: Cocopeat treated with *P. chrysosporium*, CP-ND: Cocopeat treated with *N.discreta*, CP: Untreated Cocopeat)

4.5.4 Lignin content by Klason's method

The lignin content in the biomass samples at the beginning and end of the fermentations was estimated using Klason's method as mentioned in section 4.4.3. The Klason's lignin content of cocopeat before fungal treatment was 49±1% and that of sugarcane bagasse was 21±2%. Figure 4.8 shows the percentage lignin degraded by both fungi. In the case of sugarcane bagasse, the percent lignin degradation by *N.discreta* was nearly twice that by *P. chrysosporium*. In cocopeat samples, the lignin degradation was around 16% with both the fungal strains after 30 days. A comparison of delignification of cocopeat and bagasse shows that bagasse is easier to degrade compared to coco peat. Lignin in sugarcane bagasse has a higher S/G ratio as discussed in section 4.1 and is mainly composed of β-O-4 ether bonds, indicating principally linear chains, which makes it easy to degrade. On the other hand, lignin in cocopeat consists predominantly of guaiacyl units (lower S/G ratio). The main substructure present in the lignin of cocopeat is the β−O−4′ aryl ether, followed by β−5′ phenylcoumaran substructures and β−β′ resinol substructures. While β−O−4′ aryl ether linkages can be broken down by low redox potential enzymes such as laccases, for breaking the more condensed lignin linkages such as β−5′ phenylcoumaran structures, more evolved ligninases such as lignin peroxidase, manganese peroxidase or versatile peroxidases are required [28]. *P.chrysosporium* and other basidiomycetes are known to produce laccases, lignin peroxidases, manganese peroxidases and versatile peroxidases which would all contribute to the degradation of the various lignin linkages in cocopeat. Although ascomycetes such as Neurospora primarily produce laccases and lignin peroxidases [67, 69, 70, 91], the extremely rapid growth of *N. discreta* seems to compensate, thus resulting in lignin degradation in cocopeat similar to that by *P.chrysosporium.*

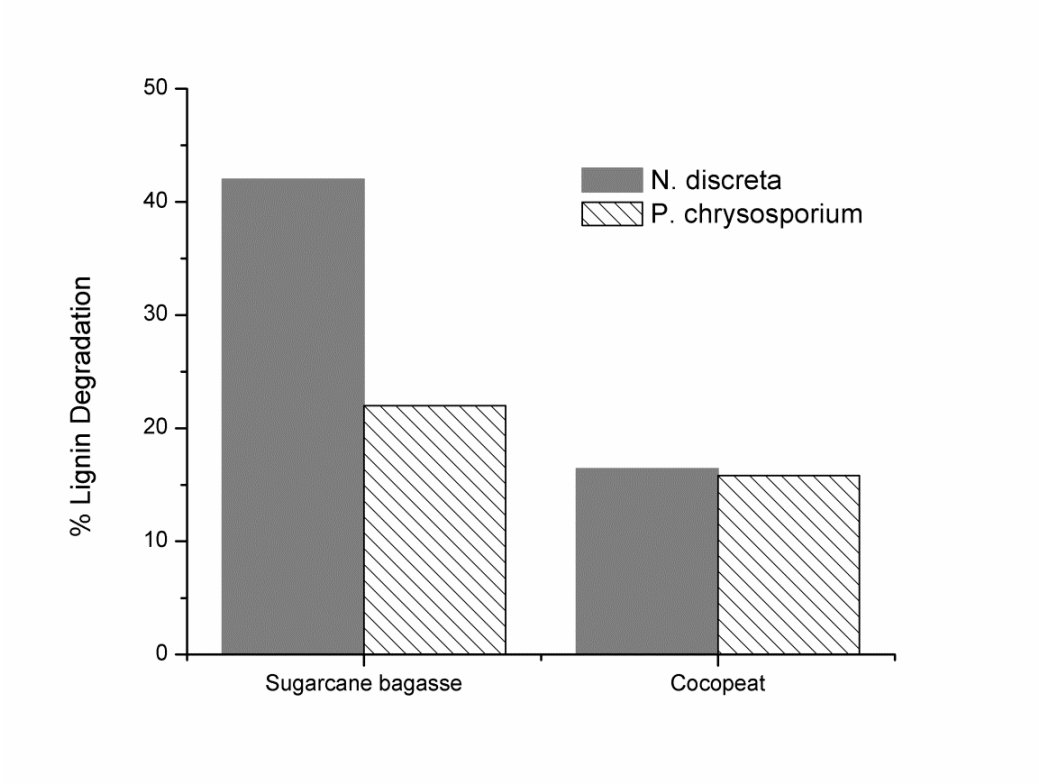


Figure 4.8: Percent lignin degradation measured by Klason's lignin content after 30 days of fermentation (n=2)

4.6 Conclusion

This study demonstrated for the first time, that the indigenous fungal strain *Neurospora discreta* has the ability to degrade lignin effectively in widely contrasting agricultural residues, cocopeat and sugarcane bagasse. Sugarcane bagasse was easier to degrade compared to the more recalcitrant cocopeat, owing to the difference in S/G ratios. The extent of lignin degradation in sugarcane bagasse by *N.discreta* was found to be significantly higher compared to that by traditionally used white rot fungus *P. chrysosporium*. The longer and thicker filaments of *N.discreta* possibly aided in better penetration of fungal filaments to biomass residues and its significantly higher spore count aided in rapid growth rate as well as in better dispersibility to distant biomass fibers. For this reason, *N.discreta* was used for further studies carried out in this work. For a deeper understanding of the process, it was imperative to understand the effect of nature and amount of biomass present on the growth, delignification as well as enzyme activities of the fungus, which will be discussed in the following chapters.

CHAPTER 5

EFFECT OF BIOMASS LOADING ON LIGNIN-DEGRADING ENZYME ACTIVITIES OF *NEUROSPORA DISCRETA*

5.1 Introduction

Due to the heterogeneous and complex nature of lignin, only a few classes of microrganisms are capable of causing efficient degradation of lignin in nature. The degradation of lignin is a complex process involving different classes of enzymes namely, laccases and more specialized enzymes such as peroxidases [92, 93]. Laccases (EC 1.10.3.2) are a group of multi-copper oxidase enzymes, produced by higher plants and fungi, as well as by certain eubacteria and actinomycetes strains [94]. Laccases are known to catalyze the oxidation of only low redox potential phenolic substrates with simultaneous reduction of molecular oxygen to water. Peroxidase enzymes, namely manganese peroxidase, lignin peroxidase, versatile peroxidases and other polyphenol oxidases are more evolved oxidative enzymes with higher redox potentials which are produced majorly by white rot basidiomycetes and certain ascomycetes fungi [28, 90, 93]. These enzymes, which are relatively less studied as compared to laccase enzyme, have the unique ability to catalyze oxidative cleavages of C–C bonds and ether (C–O–C) bonds in high redox potential non-phenolic aromatic substrates. On the other hand, laccases reportedly do not have the ability to catalyze oxidative cleavage of non-phenolic sub-units[28]. However, the redox potential of laccase enzymes can be modulated by adding certain smaller molecular weight phenolic compounds, which enables the enzyme to oxidize high redox non-phenolic lignin units [28, 95]. Also, literature reports that certain strains of fungi such as *Trametes versicolor* and *Neurospora crassa* secrete laccases having higher redox potential as compared to *Pleurotus ostreatus* and other fungal strains [36]. In such strains, a broader range of substrates could be catalyzed by the laccase enzyme.

The major challenges in microbial delignification processes that have prevented its scale-up and commercialization are the lower rates of de-lignification and considerably longer processing times. Studies with white rot fungi have shown that laccase activities tend to decrease as the degradation proceeds [50, 92]. Others have reported higher activities of laccase enzymes in solid state fermentation compared to submerged fermentation [96, 97]. Several studies have also been conducted on the effect of inducers or cofactors on activities of lignin-degrading enzymes, which could be used as strategies to improve the degradation rates [59, 76]. However, little is known on the reasons for lower enzyme activities during biomass

degradation. An understanding of the factors that impact enzyme activities during delignification is therefore critical in the design of effective processes. More specifically, it is important to understand whether any of the process conditions or in-process intermediates were responsible for the inhibition of lignin-degrading enzymes.

In the previous chapter, the indigenous strain of *Neurospora discreta* was found to be a promising alternative to well-known white rot fungi such as *P.chrysosporium* for the degradation of lignin in agricultural residues [98]. Its rapid growth rate and abundant spore formation caused similar lignin degradation as compared to *P.chrysosporium.* Although it has been reported that laccases and polyphenol oxidases were produced by certain strains of Neurospora [93, 99], its activity during biomass degradation has not been studied so far. In the current chapter, the activities of two lignin-degrading enzymes namely, laccase and lignin peroxidase enzymes of *N. discreta* are studied. Submerged fermentation of cocopeat and sugarcane bagasse at two biomass loadings were tested. These two substrates were selected for the wide variation in their composition as well as structure as discussed in the previous chapter.

5.2 Materials and Methods

5.2.1 Microorganism

Neurospora discreta, an indigenous isolated fungal strain reported in previous chapters, was used for this study. The strain was stored at 2-4 ^{o}C on potato dextrose agar (PDA) slant and was regularly sub-cultured on PDA plates by incubating them at 28^{o}C for 2-4 days.

5.2.2 Lignocellulosic biomass substrates

Sugarcane bagasse and cocopeat were obtained from local vendors. The substrates were thoroughly washed, dried and reduced to 15-20 mm in size. They were stored in airtight containers at 2-4^{o}C.

5.2.3 Fermentation set-up

Submerged fermentations were set up in Erlenmeyer flasks, using sub-cultures of *N. discreta*. Vogel's minimal medium with 0.5% sucrose was used in all cases. Two loadings were tested for each biomass: 1 g of biomass in 100 ml medium (1% biomass loading) and 5 g of biomass in 150 ml medium (~3.3% biomass loading). The additional medium in the 5 g loading was added to ensure submerged conditions. It is to be noted that the additional medium does not impact the amount of lignocellulosic biomass available to the cells. 0.5% sucrose was added to the media to initiate cell growth. The flasks containing the biomass and medium were

autoclaved at 121°C for 20 min and then cooled to room temperature before inoculation. For inoculation, a spore suspension was prepared by gently scraping the fungal cells from subcultured PDA plates and adding them to a known quantity of sterile minimal media, which was then filtered through double-layered muslin cloth [98]. To each flask, 1 ml of the spore suspension with a spore count of approximately 10^7cells ml^{-1} has added aseptically. The flasks were mixed well and incubated at 30°C for 30 days. At the end of fermentation, the contents of each flask were filtered. The solid fractions were thoroughly washed, filtered and dried till a constant weight was achieved. Liquid fractions were centrifuged at 10,000 rpm for 20 min and the clear supernatant samples were analyzed for laccase and lignin peroxidase activities.

5.2.4 HPLC analysis of lignin degraded phenolic intermediates

The phenolic compounds released during lignin degradation were analyzed in the liquid fraction obtained at the end of fermentation on a C-18 HPLC column using the acetonitrile-water solvent system as detailed in the previous chapter, section 4.4.2.

5.2.5 Laccase enzyme activity in fermentation samples

Laccase activity was determined by measuring the oxidation kinetics of 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), which was obtained from Sigma-Aldrich (Product No-10102946001). To 0.6 ml of the liquid supernatant fraction, 0.6 ml of 0.1 M pH 5 citrate buffer was added and the mixture was equilibrated at room temperature. The spectral time scans at 420 nm were started as soon as 0.6 ml of 0.3mM ABTS was added to the sample making the total volume to 1.8 ml against a suitable blank. The concentration of the intensely colored, green-blue cation radical was calculated (µM) using $\varepsilon_{420} = 36000 M^{-1}cm^{-1}$ [58].

5.2.6 Lignin peroxidase enzyme activity in fermentation samples

Lignin peroxidase activity was determined by the formation of veratraldehyde from veratryl alcohol (Make: Sigma Aldrich) in the presence of H_2O_2. To 0.5 ml of liquid supernatant sample, 1 ml of 125mM sodium tartarate buffer (pH ~ 3) and 0.5 ml of 10 mM veratryl alcohol was added. After the addition of 1 ml of 10 mM H_2O_2 to the mixture, the spectral scans at 310 nm were monitored and the increase in absorbance was noted (ε = 9300 $M^{-1}cm^{-1}$) [100]. The increase in product concentration was calculated and reported in µM.

5.2.7 Study of laccase activity in presence of water-soluble lignin intermediates

The effect of lignin degradation intermediates released during submerged fermentations of biomass substrates (fungal solubilized lignin) was studied on pure laccase enzyme purchased from Sigma-Aldrich (Source: *Trametes versicolor*). The centrifuged liquid supernatant

fractions obtained from the 1% sugarcane bagasse and cocopeat fermentations were heat treated to deactivate inherently present enzymes. To 0.6 ml of 15 μg ml^{-1} pure laccase enzyme made in 100 mM pH 5 citrate buffer, 0.3 ml of liquid supernatant sample was added and equilibrated at room temperature for 20 minutes. For controls, 0.3 ml of deionized water was added instead of the liquid supernatant sample. To this mixture, 0.3 ml of pH 5 citrate buffer and 0.6 ml of 0.3mM ABTS substrate was added to have a final volume of 1.8 ml and the increase in absorbance was noted at 420 nm. In another set, to increase the concentration of lignin degradation intermediates, the volume of the liquid supernatant sample was increased from 0.3 ml to 0.6 ml and 0.3 ml of 0.6 mM ABTS was added to have a final volume of 1.8 ml as in the previous case.

5.3 Results and Discussion

5.3.1 Lignin degradation

Figures 5.1a shows the amount of lignin that was solubilized by *N. discreta* at the end of the fermentation while Figure 5.1b shows the percentage of lignin degraded in each case, based on Klason's lignin estimation. In chapter 4 [98], the structural differences in lignin were studied by measuring S/G ratio that corresponds to the absorbance at 1335 cm^{-1} and 1275 cm^{-1} in FTIR spectroscopy [80, 98]. As seen in Figure 5.1b, the extent of degradation of lignin in cocopeat was lower than that in sugarcane bagasse, which correlates well with previous results and the S/G ratios reported [98].

Although the extent of degradation was higher in sugarcane bagasse, the amount of solubilized lignin released into the medium was higher in cocopeat samples (Figure 5.1a), as the amount of lignin present is more than double in cocopeat (50%) as compared to sugarcane bagasse (21%).

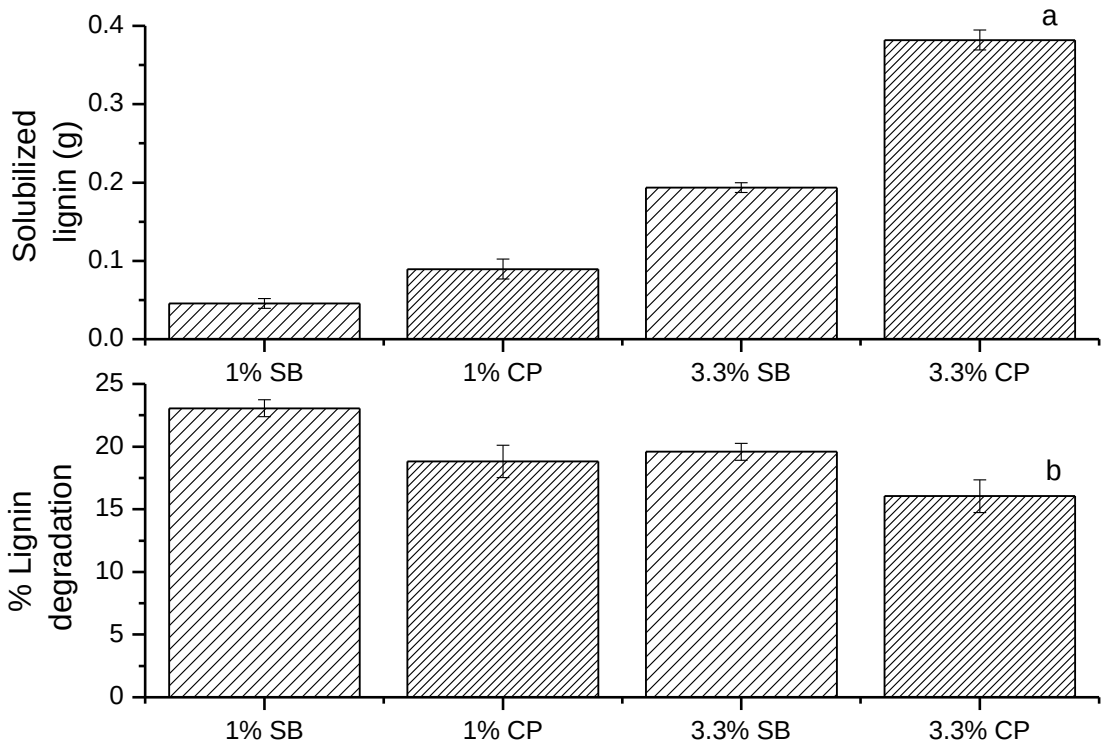


Figure 5.1a: Solubilized lignin (g) measured from Klason's method using solid substrates of sugarcane bagasse (SB) and cocopeat (CP) at the beginning and at the end of the fermentation. Figure 5.1b: Percentage lignin degradation

Figure 5.2 shows the liquid chromatograms of the day 30 liquid supernatant samples and the standard alkali lignin. The solubilized lignin measured by RP-HPLC shows the same trend as seen from the Klason's lignin estimation shown in the previous section, with 3.3% cocopeat showing the highest amount and 1% sugarcane bagasse samples showing the lowest amount of solubilized lignin.

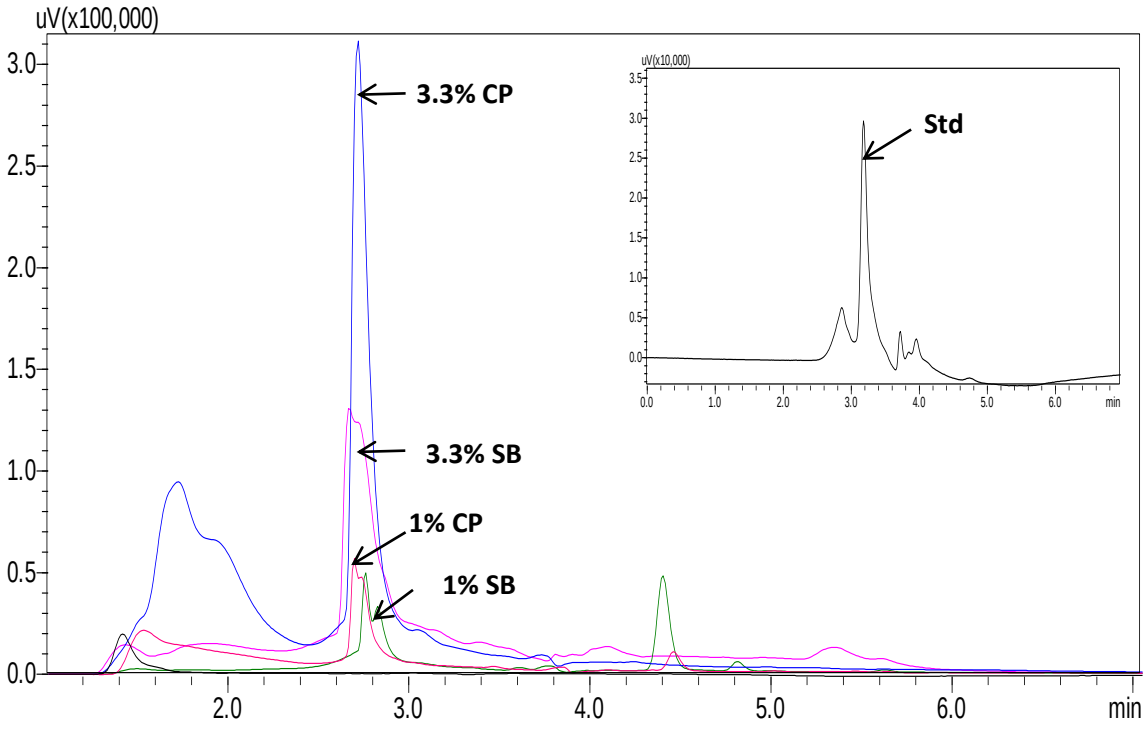


Figure 5.2: RP-HPLC chromatograms of liquid supernatant samples of 1% and 3.3% SB (sugarcane bagasse), 1% and 3.3% CP (cocopeat) & standard alkali lignin (std.)

Figure 5.3 shows the change in concentration of solubilized lignin with time, based on the alkali lignin standard curve. As expected, 3.3% substrate loadings showed higher concentrations of solubilized lignin compared to 1% loadings. Moreover, lignin solubilization increased gradually in case of cocopeat samples from day 10 to day 30, while in sugarcane bagasse, the degradation of lignin did not change after day 10 as reported in our earlier paper [98].

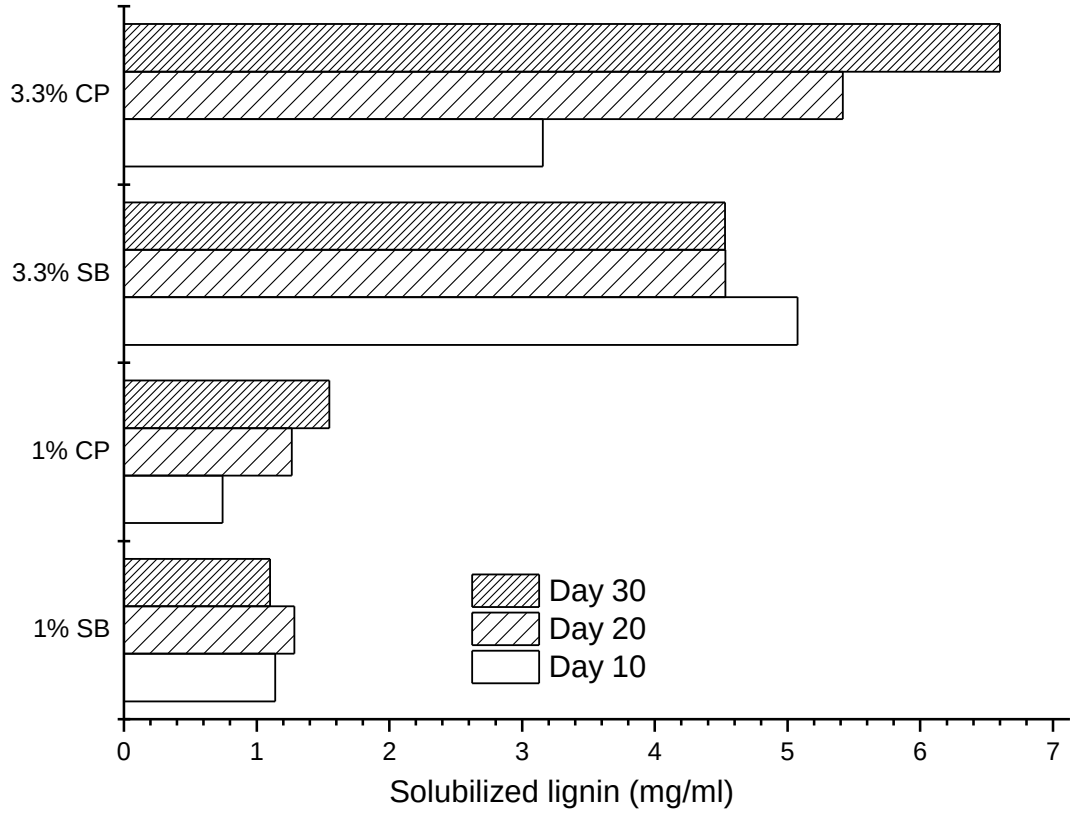


Figure 5.3: Degraded lignin measured by RP-HPLC in liquid supernatant samples of sugarcane bagasse (SB) and cocopeat (CP)

5.3.2 Enzyme activities in Fermentation studies

Figure 5.4 (a and b) shows the rate plots of laccase enzyme activity and lignin peroxidase enzyme activity for 1% and 3.3% biomass loadings in day 30 supernatant samples. Laccase and lignin peroxidase activities were found to be higher in 1% substrate loading as compared to 3.3%, despite the higher concentrations of lignin degradation products seen in the latter case. Moreover, the activity in sugarcane bagasse was higher as compared to cocopeat in 1% biomass loading, although cocopeat showed higher lignin solubilization. This led to the hypothesis that there was a product inhibition of the enzymes by the in-process lignin degradation products. The extent of inhibition in laccase was more pronounced as compared to lignin peroxidase enzyme. To test this hypothesis, the effect of liquid supernatant samples obtained from the biomass fermentation studies on commercial laccase enzyme activity was studied.

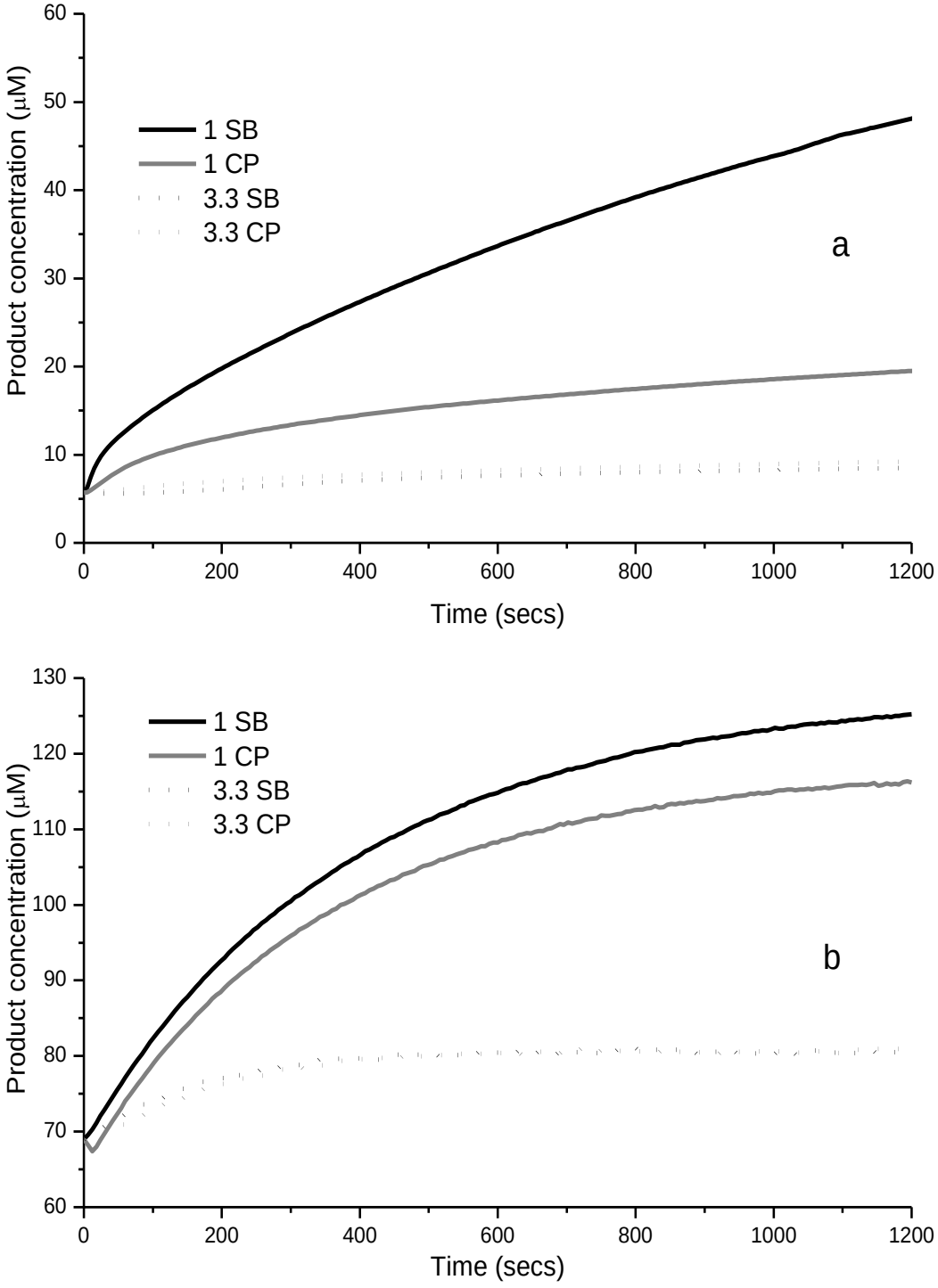


Figure 5.4: Laccase activity (a) and Lignin peroxidase activity (b) in 1% and 3.3% biomass loadings (1 SB-1% sugarcane bagasse, 1 CP- 1% cocopeat, 3.3 SB- 3.3% sugarcane bagasse, 3.3 CP- 3.3% cocopeat)

5.3.3 Effect of fungal solubilized lignin intermediates on commercial laccase enzyme

The effect of fungal solubilized lignin intermediates (FSL) on commercial laccase enzyme was studied by incubating the pure laccase samples with the liquid supernatant samples obtained from biomass fermentation of cocopeat and sugarcane bagasse. Two samples of sugarcane bagasse (1 mg/ml and 2 mg/ml) and two samples of cocopeat (1.5 mg/ml and 3 mg/ml) were used for the study. As seen from Figure 5.5, as the lignin degradation intermediate concentration increased, a clear decrease in the initial slopes is noticed. Moreover, the extent of inhibition was found to be dependent on the final concentrations of lignin intermediates and not on the initial biomass source. Therefore, the in-process generated lignin intermediates had negatively impacted the activity of the laccase enzyme. Recently, studies in the literature have reported possible inhibition of the laccase enzyme in the presence of certain bulky organic compounds, that causes steric hindrances and therefore substrate-enzyme binding is impacted[101, 102].

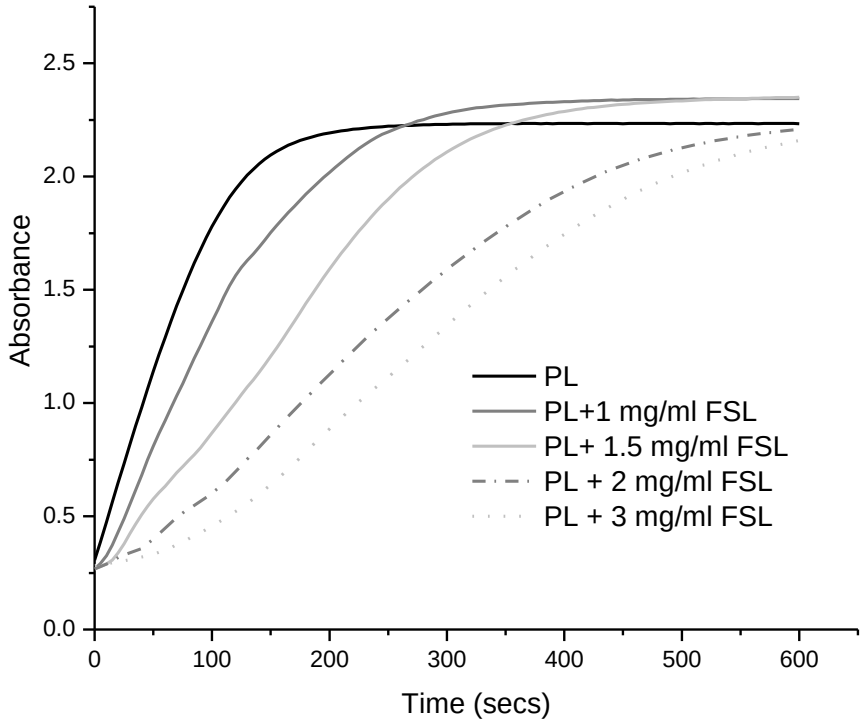


Figure 5.5: Effect of fungal solubilized lignin on commercial laccase enzyme (PL stands for pure laccase)

5.5 Conclusions

The effect of biomass loading on lignin degradation was studied and as seen in the earlier chapter, sugarcane bagasse was easier to degrade as compared to cocopeat even at increased biomass loadings. The in-process activities of ligninolytic enzymes in *Neurospora discreta* - laccase and lignin peroxidase - were measured and reported for the first time in the study. At increased biomass loadings, the activities of both the enzymes were found to be lower and the effect was more pronounced for the laccase enzyme, indicating the possibility of product inhibition by lignin degradation products. This effect was confirmed by studying the effect of fungal solubilized lignin on pure laccase enzyme. A more detailed study of the kinetics of laccase inhibition could provide insight into the nature of inhibition and possible methods to overcome it, which is reported in the next chapter.

CHAPTER 6

KINETIC STUDIES OF LACCASE INHIBITION BY LIGNIN DEGRADATION INTERMEDIATES

6.1 Introduction

As discussed previously, conversion rates of lignocellulosic biomass to ethanol depend to a large extent on the rate and extent of delignification. In the case of microbial delignification, this, in turn, depends on the activities of ligninolytic enzymes such as laccase and lignin peroxidase. The low rate of delignification has been a major challenge in microbial and enzymatic delignification processes and has led to the pressing need to understand the reasons for this.

In literature, laccase inhibition due to the presence of mercury ions [103], sulfhydryl organic compounds [104], free radicals [105] as well as certain bulky organic compounds such as medicarpin [101, 106] has been reported. However, in-process lignin degradation intermediates have never been explored as potential inhibitors of laccase, despite evidence of decreasing laccase activity as the degradation proceeds, for instance, in the case of white rot fungi [50, 92]. As laccases are extracellular enzymes, the presence of water-soluble lignin degradation intermediates could potentially cause product inhibition, resulting in decreased enzyme activity. Reports of such water-soluble high molecular weight intermediates, called acid precipitable polymeric lignin (APPL) released during delignification of woody biomass by bacterial laccase exist [15, 107, 108]. However, they have not been viewed as potential inhibitors.

In the previous chapter, inhibition of ligninolytic enzyme laccase was seen at higher biomass loading. Further investigation led to the inference that this was due to the presence of soluble lignin degradation intermediates. This chapter describes a detailed kinetic study carried out to understand the nature of this inhibition. In this study, lignin degradation intermediates from two different sources were used: (1) lignin degradation products generated during fungal pre-treatment and (2) commercially available Kraft lignin generated by alkali pre-treatment of lignocellulosic biomass.

Kinetics of enzyme-catalyzed reactions can be depicted in the form of equation 1, where the enzyme (E) and substrate (S) combine to form the ES complex, which then forms the product (P). From Michaelis–Menten kinetics, the reaction rate 'v' is represented by equation 2, which denotes the rate of product formation.

$$E + S \underset{K_m}{\Leftrightarrow} ES \rightarrow E + P \quad ---------(1)$$

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad ----------(2)$$

Here, K_m is the Michaelis constant, which represents the affinity of the enzyme for the substrate and V_{max} is the maximum rate of reaction. In the presence of an inhibitor, enzyme activity reduces due to the binding of inhibitor molecule to the enzyme or to the enzyme-substrate complex or both, based on the type of inhibition. The reaction rate would thus decrease due to changes in either K_m or V_{max} or both. This can be represented by equation 3, where $K_{m,\,app}$ and $V_{max,\,app}$ are the kinetic parameters for the inhibited case.

$$v_{inh} = \frac{V_{max,app}[S]}{K_{m,app} + [S]} \quad ---------(3)$$

In order to establish the nature of inhibition, values of $K_{m,\,app}$ and $V_{max,\,app}$ for inhibited samples were compared with those of K_m and V_{max} respectively for the pure enzyme. The substrate concentration (S) was varied between 0.03 mM – 0.3 mM and initial slopes of rate plots were used for measuring reaction rates for the pure laccase enzyme (v) and the inhibited enzyme (v_{inh}) at varying inhibitor concentrations (I). The Lineweaver–Burk plots of $1/v$ versus 1/[S] for pure laccase enzyme controls and $1/v_{inh}$ v/s 1/[S] for the inhibited enzyme samples were plotted. (Equations 4-5) [109].. Michaelis–Menten parameters K_m and V_{max} for pure laccase enzyme and $K_{m,app}$ and $V_{max,app}$ at varying inhibitor concentrations (I) were calculated from the x and y-intercept of the plots.

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad ----------(4)$$

$$\frac{1}{v_{inh}} = \frac{K_{m,app}}{V_{max,app}}\frac{1}{[S]} + \frac{1}{V_{max,app}} \quad ----(5)$$

6.2 Materials and Methods

6.2.1 RP-HPLC Analysis

Both the fungal solubilized lignin as well as the kraft lignin samples were analyzed on a C-18 reversed phase (RP)-HPLC column using an acetonitrile-water solvent system. The total run time was 35 minutes with a flow rate of 1 ml min^{-1} and a sample injection volume of 25 µL. A gradient elution with the following concentrations of acetonitrile was used to differentiate between polar and non-polar compounds of solubilized lignin: at 0 min— 0 %; 5 min— 3 %; 10 min—6 %; 15 min—10% and 20 min—20 %, 25 min—40% and 35 min – 80%. The compounds were detected at 254 nm using a photodiode array detector and the data was integrated and analyzed using the Shimadzu Automated Software system. Also, a calibration curve was plotted using 1000 mg L^{-1} - 5000 mg L^{-1} water-soluble kraft lignin. The inhibitor concentration was calculated from the chromatogram area corresponding to the peak at 2.7 min retention time. The concentration was further converted from mg L^{-1} to µM by using weight average molecular weight obtained from mass spectroscopy for the peak at RT 2.7 min (Table 6.1).

6.2.2 Laccase Activity

The activity of Laccase from *Trametes versicolor*, purchased from Sigma Aldrich (Product No-51639) was determined by measuring the oxidation kinetics of the substrate ABTS (2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Sigma Aldrich, Product No-10102946001). The spectral time scans were noted at 420 nm. The concentration of the green-blue cation radical formed from oxidation of ABTS was correlated to the enzyme activity using the extinction coefficient (ε_{420}) 36000 $M^{-1}cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme forming 1 µmol of product per minute at standard assay conditions (U l^{-1}) [58, 59].

6.2.3 Laccase Kinetic Studies

Laccase activity was measured in the presence of varying concentrations of lignin degradation intermediates, which included both fungal solubilized lignin and alkali lignin samples described below. The final concentrations of the inhibitors were between 50-250 µM. For each concentration, to 0.6 ml of 15 µg ml^{-1} pure laccase enzyme made in 100 mM pH 5 citrate buffer,

0.3 ml of inhibitor sample was added and equilibrated at room temperature overnight. For controls, 0.3 ml of deionized water was added instead of the inhibitor sample. To this mixture, 0.3 ml of pH 5 citrate buffer and 0.6 ml of varying concentrations of ABTS substrate was added and the increase in absorbance was noted at 420 nm.

6.2.3.1 Fungal solubilized lignin

Neurospora discreta, was used for generating solubilized lignin degradation intermediates from the lignocellulosic biomass, cocopeat. Submerged fermentations were set-up using cocopeat at 1% and 3.33% biomass loading using Vogel's minimal media and flasks were aseptically inoculated and incubated for 30 days as described previously [98]. At the end of the fermentation, liquid fractions were filtered, centrifuged and the clear supernatant sample was used as fungal solubilized lignin. All supernatant samples were heat treated to deactivate any inherently present enzymes. The two fungal solubilized lignin samples with varying lignin intermediate concentrations had the final inhibitor concentrations of 101 µM and 207 µM respectively.

6.2.3.2 Chemically-treated kraft lignin

Commercially available kraft lignin obtained by treating lignocellulosic biomass with high alkaline sulphide solutions [89], was procured from Sigma-Aldrich (Product No-471003). Kraft lignin was dissolved in 100 mM pH 5 citrate buffer to maintain optimum pH for enzyme activity. Three samples of kraft lignin namely, 1000 mg L^{-1}, 2000 mg L^{-1} and 3000 mg L^{-1} were chosen for the study which had final inhibitor concentrations of 57, 132 and 230 µM.

6.2.4 Analysis of lignin intermediates by liquid chromatography-mass spectrometry

LCMS was used to further characterize the compounds present in fungal solubilized lignin and water-soluble kraft lignin. The mass spectra were obtained from the Triple Quadrupole mass spectrometer (Make: Shimadzu, Model: 8040), under electrospray ionization mode. Automated software, Labsolutions^{TM} was used for data integration and mass spectrum analysis. 'Multicharge ion analysis', a deconvolution software was used to predict the molecular weights of the intact parent compounds. The number average molecular weight (M_n) and weight average molecular weight (M_w) of both samples were calculated using equations 6 and 7 [110]. Here 'i' represents the number of different molecular weights present in the lignin sample, N_i and M_i represent the corresponding intensity and molar mass respectively.

$$M_n = \frac{\sum N_i M_i}{\sum N_i} - - - - - - - - (6)$$

$$M_w = \frac{\sum N_i {M_i}^2}{\sum N_i M_i} - - - - - - - - (7)$$

6.5 Results and Discussion

6.5.1 Liquid chromatography and Mass spectroscopy

Analysis of the lignin samples using a C-18 column on reversed-phase high-performance liquid chromatography (RP-HPLC) indicated that both the fungal solubilized lignin and kraft lignin contained a major peak at the retention time (RT) of 2.7 minutes (Figure 6.1), indicating the presence of highly polar lignin degradation intermediates in both samples. In addition to this, kraft lignin showed another peak at RT of 27 minutes which was absent in the fungal solubilized lignin sample.

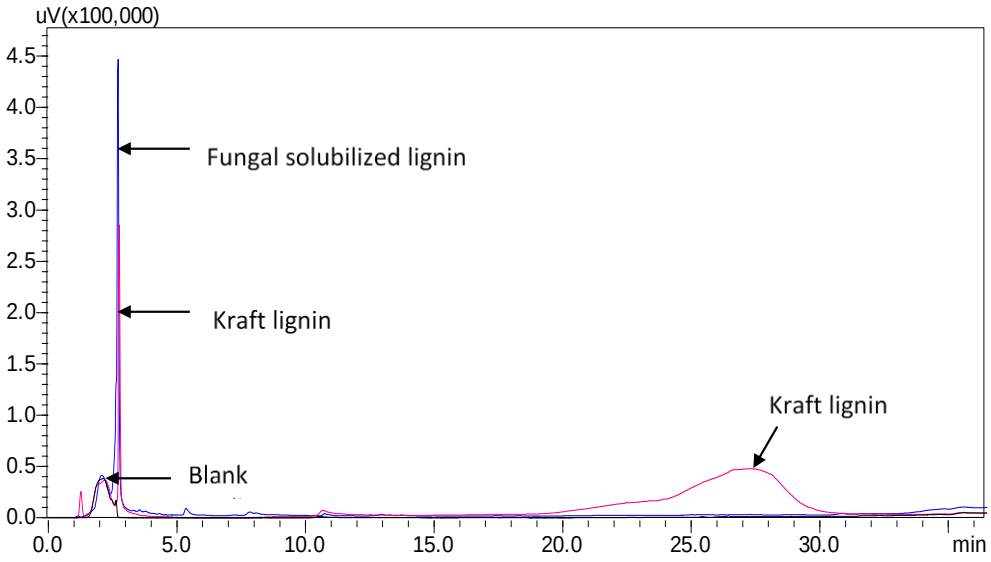


Figure 6.1: RP-HPLC chromatograms of water-soluble Kraft lignin and fungal solubilized lignin. The medium in which both samples were prepared was run as a blank.

Mass spectra of the two distinct peaks were obtained using LC-MS (Figure 6.2) and the average molecular weights were calculated. The average molecular weights of the more polar lignin intermediates eluting at RT 2.7 min in Kraft lignin and in fungal solubilized lignin samples

were 2110 and 2189 g mol^{-1} respectively (Table 6.2). The 27-minute peak which was found only in Kraft lignin had an average molecular weight of 819 g mol^{-1} indicating the presence of lignin intermediates that are lower in molecular weight as well as polarity compared to the intermediates eluting at RT of 2.7 minutes.

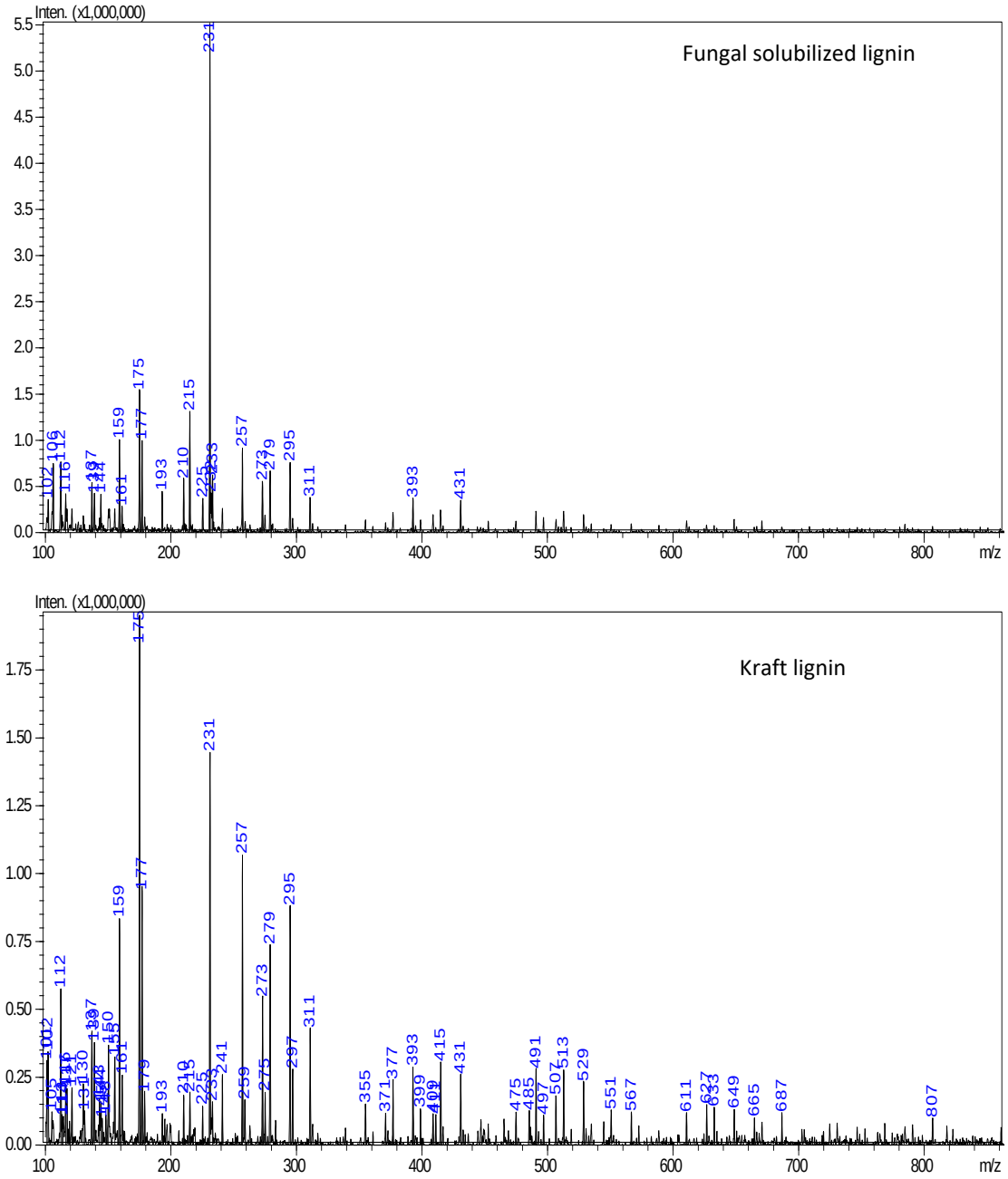


Figure 6.2: Mass spectrums in positive ESI mode of fungal solubilized lignin and Kraft lignin polar intermediates

Table 4.1: Average molecular weights of lignin degradation intermediates

Samples	Number average molecular weight (M_n)	Weight average molecular weight (M_w)
Fungal solubilized lignin	1511 g mol^{-1}	2189 g mol^{-1}
Water-soluble kraft lignin	1491 g mol^{-1}	2110 g mol^{-1}
Literature reported value for Kraft lignin [111]	1500 g mol^{-1}	2500 g mol^{-1}

The effect of these lignin degradation intermediates was evaluated on pure laccase using ABTS as substrate. At 0.03mM ABTS, the reaction rates of laccase were found to be lower in the presence of both fungal solubilized lignin and kraft lignin, compared to that of pure laccase (Figure 6.3), indicating enzyme inhibition.

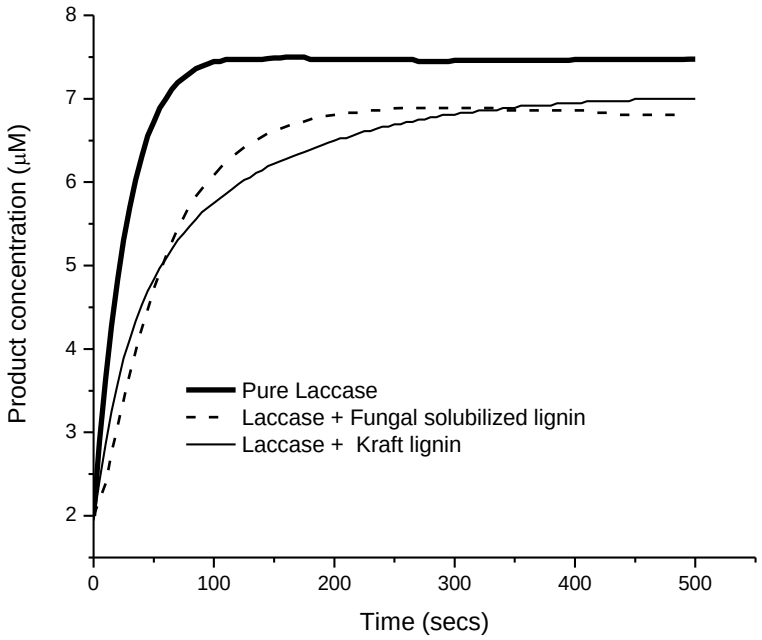


Figure 6.3: Representative rate plot showing laccase activity with and without lignin degradation intermediates

6.5.2 Determination of kinetic parameters

The nature of inhibition was studied from the Lineweaver Burk plots ($1/v$ v/s $1/S$) obtained at the various substrate and inhibitor concentrations (Figure 6.4). For pure laccase, K_m was found to be 80 ± 1µM, similar to the values reported in the literature: K_m was reported to be 59 µM for *T.versicolor* laccase enzyme using ABTS as substrate by [112] and 38 µM by [113]. Maximum reaction rate V_{max} for pure laccase was found to be approximately 62 U l^{-1}. In the presence of lignin degradation intermediates, the values of V_{max} remained nearly the same as that of pure laccase, (within ± 10%). However, as the concentration of lignin degradation intermediates increased, the slope of $1/v_{inh}$ v/s $1/S$ was also found to increase. Therefore, $K_{m,app}$ increased indicating a decrease in affinity of laccase enzyme for the substrate in the presence of the inhibitor, i.e., competitive inhibition.

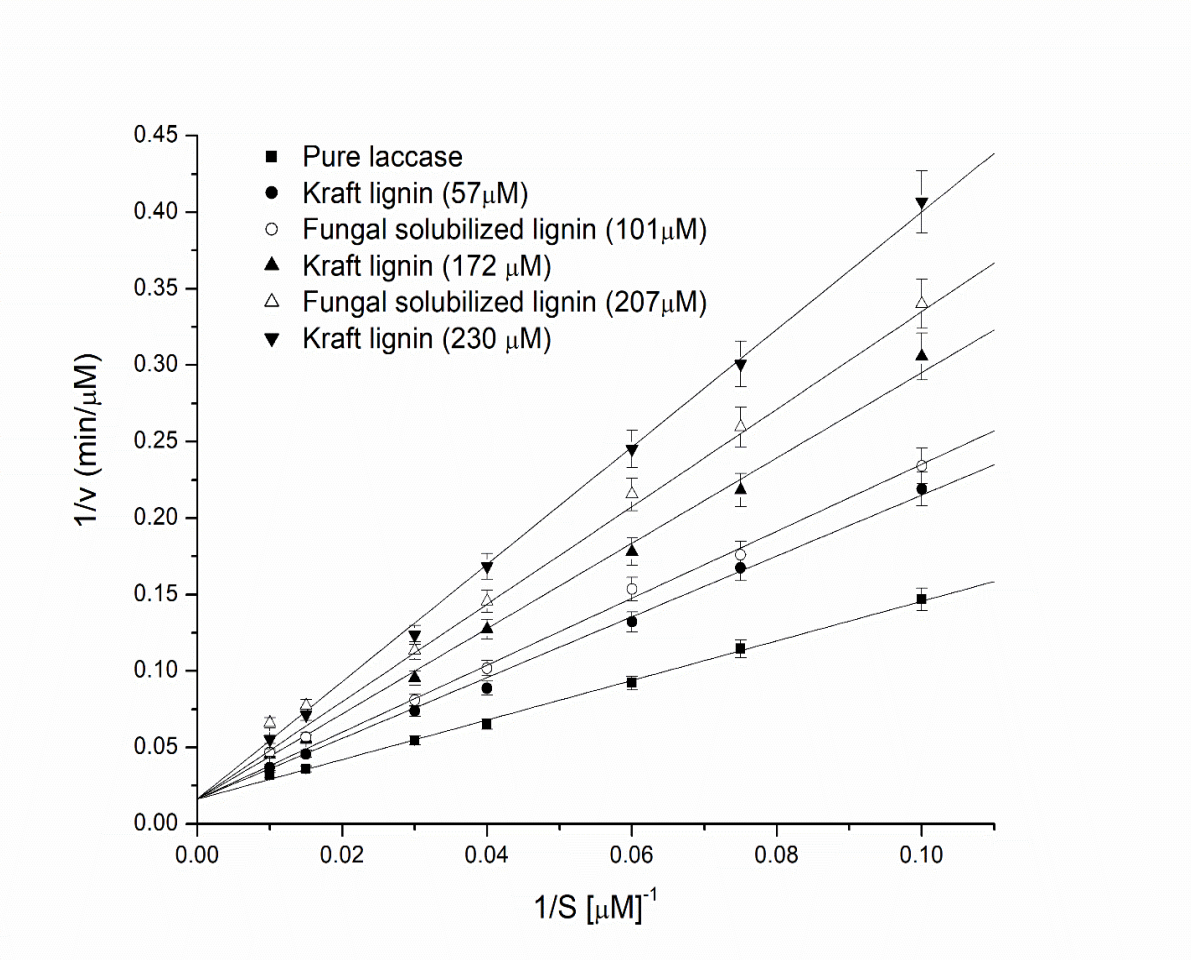


Figure 6.4: Line-weaver–Burk plots of pure laccase enzyme and inhibited laccase in the presence of varying concentrations of Kraft lignin and fungal solubilized lignin.

Interestingly, inhibition by both sources of lignin degradation intermediates i.e., fungal solubilized as well as kraft lignin samples fit the model well. This indicates that the inhibition is mostly due to the polar, higher molecular weight intermediates of lignin (i.e fraction eluting at RT 2.7 min), which are present in both samples. Based on this, the inhibitor concentration was calculated from the calibration curve using the area under the peak at 2.7 RT as detailed in the materials and method section and reported in Table 6.2.

Table 6.2: Inhibitor concentration, Michaelis constant, and % inhibition for lignin degradation intermediates

Samples	Inhibitor concentration (µM)	$K_{m,app}$ (µM)	% Inhibition $[(v-v_{inh})/v]$	
			at S = 10 µM	at S = 100 µM
Laccase + 1000 mg L^{-1} Kraft lignin	57	123 ± 5	33%	14%
Laccase + Fungal solubilized lignin-1	101	135 ± 8	40%	31%
Laccase + 2000 mg L^{-1} Kraft lignin	172	172 ± 2	52%	31%
Laccase + Fungal solubilized lignin-2	207	197 ± 4	57%	52%
Laccase + 3000 mg L^{-1} Kraft lignin	230	237 ± 8	64%	56%

The value of the dissociation constant of the enzyme-inhibitor complex K_I, which represents affinity of the enzyme to the inhibitor molecules was calculated from the plot of $K_{m,app}/K_m$ vs inhibitor concentration (I) (Figure 6.5). The value of K_I was estimated to be 132 µM.

Competitive inhibition is usually overcome by increasing the substrate concentration, thereby increasing the probability of enzyme-substrate binding. However, in delignification processes, increasing the concentration of lignin would increase the concentration of the lignin degradation intermediates, thus compounding the problem of laccase inhibition. In this case, a potential strategy, therefore, could be to maintain low levels of the inhibitor molecules by removing the products or adding fresh medium intermittently. The extent of inhibition shown in Table 6.2 provides an estimate on how low the inhibitor concentration could be maintained, given the concentration of substrate.

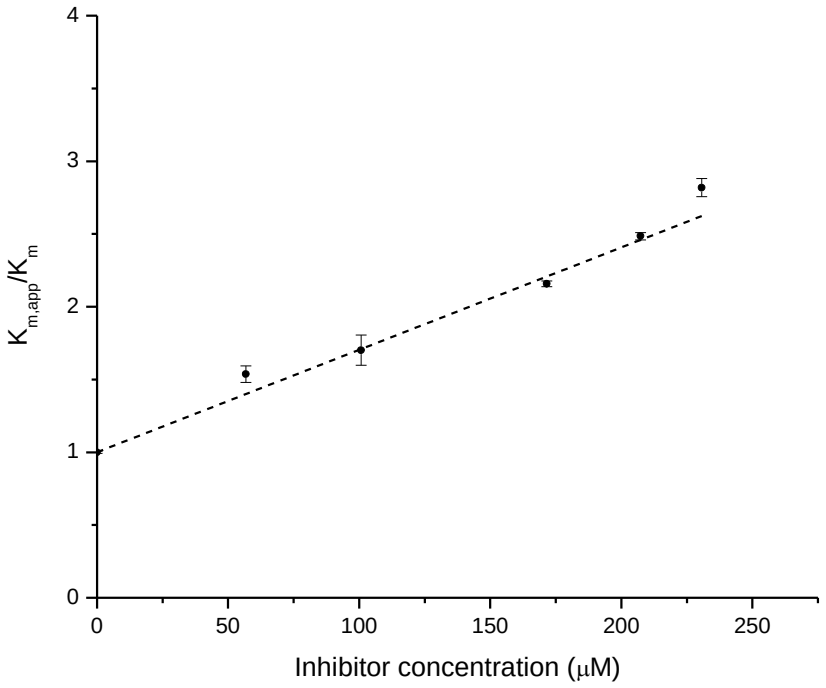


Figure 6.5: Calculation of dissociation constant K_I.

6.6 Conclusion

The current kinetic study using pure laccase enzyme revealed competitive inhibition of the enzyme by highly polar lignin intermediates obtained from two different sources, fungal solubilized as well as chemically treated kraft lignin. This is a highly relevant finding as it explains the low rates of delignification currently seen in microbial processes, and could pave the way for designing more efficient and sustainable microbial delignification processes by overcoming laccase inhibition.

CHAPTER 7

EFFECT OF BIOMASS LOADING ON CELLULASE ACTIVITY AND CELLULOSE DEGRADATION

7.1 Introduction

A major challenge in microbial conversion of biomass to bioethanol is the low rates of de-lignification - and a potential cause for this has been addressed in the previous chapters. In biomass residues, apart from the amount of lignin and cellulose content, the structure of these polymers also affects the biomass 'degradability', thus determining the final ethanol yields. For instance, as crystalline cellulose structures are more difficult to break down as compared to amorphous structures [7], biomass residues with a higher percentage of crystalline cellulose, tend to be more recalcitrant. In such cases, the activity of cellulolytic enzymes (cellulases) has a direct bearing on ethanol yields as it affects the amount of glucose produced from cellulose. Efforts have been directed towards increasing cellulase activity using techniques such as mutagenesis, co-culture techniques [114, 115], the addition of cofactors [116, 117] as well as ultrasound [118] and microwave-based pre-treatment technologies [119].

Cellulase activity is also affected by the presence of lignin degradation products generated during the pre-treatment step. Several studies have reported inhibition of cellulases by lignin degradation products generated during thermal and chemical pre-treatment of biomass [72, 120, 121]. A few reports also exist on the stimulatory effect of model lignin monomers on purified cellulase at specific concentrations [73, 122]. However, to our knowledge, no reports exist on the effects of *in-process* degradation products produced during microbial degradation of lignocellulosic biomass on cellulase activity. In the previous chapters, it was established that lignin degradation products have a pronounced inhibitory effect on laccase. Therefore, it is critical to understand the effect of these products on cellulase activity as well. This chapter reports the effects of in-process lignin degradation intermediates on cellulase activity and cellulose degradation in two biomass residues, cocopeat and sugarcane bagasse. The study described here also includes an analysis of the entire biomass composition before and after fungal treatment.

7.2 Materials

7.2.1 Lignocellulosic biomass substrates

Sugarcane bagasse and cocopeat were obtained from local vendors. The substrates were thoroughly washed, dried and reduced to 15-20 mm in size. They were stored in airtight containers at 2-4^{o}C.

7.2.2 Fermentation set-up

Submerged fermentations were set up using sub-cultures of *N. discreta*, which is a locally isolated fungal strain [98]. Two substrate loadings: 1% and 3.3% were studied using sugarcane bagasse and cocopeat as substrates. Each 250 ml flask contained either 1g or 5g of the substrate with 0.5 g of sucrose in 100 ml and 150 ml Vogel's minimal media to maintain submerged conditions [53]. The inoculation and incubation of the flasks were done as reported in previous chapters.

7.2.3 Sample preparation

At the end of the fermentation, the contents of each flask were filtered through a double-layered muslin cloth to separate the liquid and solid fractions. The solid fractions were thoroughly washed with de-ionized water, filtered and dried at 105^{o}C till constant weight was attained. The weight of dried samples was noted before the final processing and analysis of the residual cellulose and hemicellulose. The liquid fractions were centrifuged at 10,000 rpm for 20 min and the clear supernatant was stored at 4^{o}C for further analysis.

7.3 Analytical methods

A brief overview of the analytical methods used in the current chapter is provided in Figure 7.1. Lignin, cellulose and hemicellulose contents in the biomass samples before and after fungal treatment were determined to calculate percentage degradations and solubilization of each of the fractions.

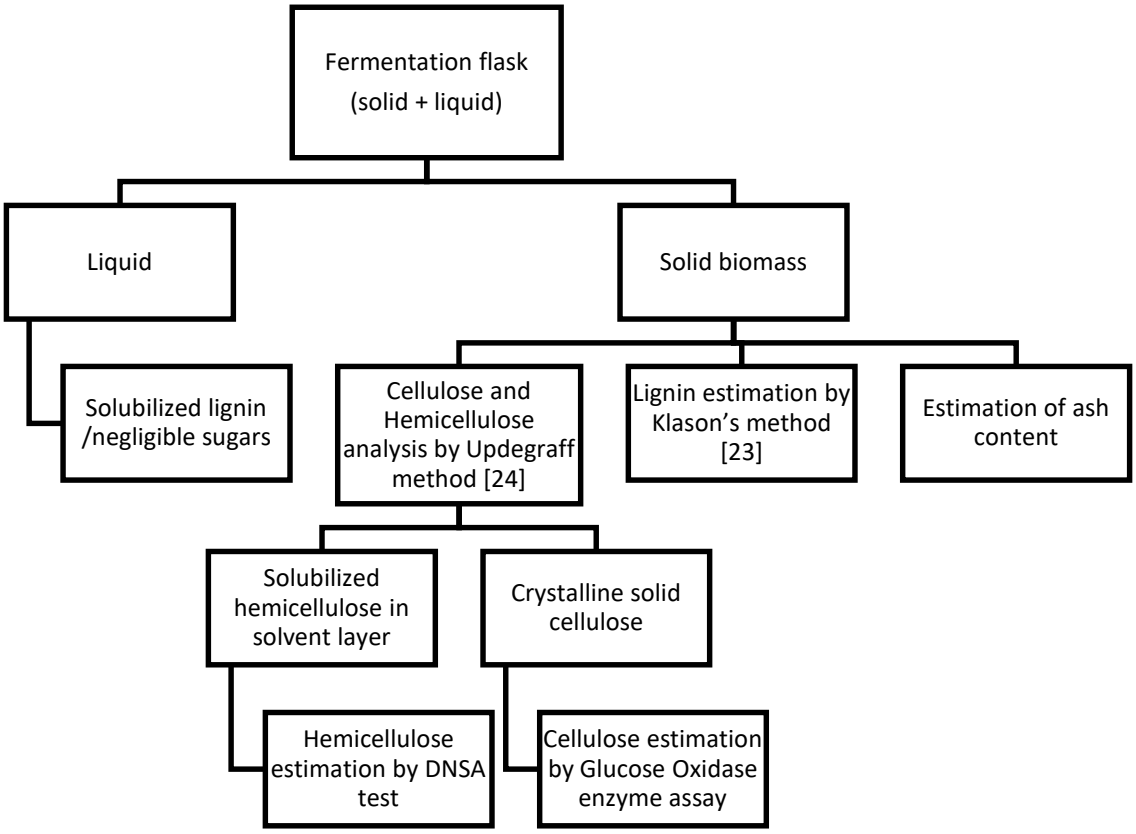


Figure 7.1: Flow diagram representing the comprehensive analysis of biomass samples after fungal treatment

7.3.1 Lignin estimation

The lignin content in the solid biomass samples was measured using a standard Klason's method [54] before and after fungal treatment. The amount of degraded lignin was calculated from the difference between initial and final values.

7.3.2 Cellulose and Hemicellulose estimation by Modified Updegraff method

Modified Updegraff assay [123] was used to analyze the cellulose and hemicellulose in the solid fraction as described in the following sections (Figure 7.1).

7.3.2.1 Sample preparation

30 mg of solid biomass was weighed into 15 ml centrifuge tubes with screw caps. 3 ml of acetic acid/water/nitric acid in 8/2/1, v/v/v ratio was added and the samples were heated in a boiling water bath for 30 min with occasional stirring. The tubes were then centrifuged at 3000 g rpm for 5 mins and 2 ml of the supernatant was removed without disturbing the pellet. The pellet

was resuspended in 7 mL of water, centrifuged and the liquid was discarded. This washing step was repeated once more. Equal amounts were discarded each time, ensuring the solid pellet does not get disturbed. The remaining pellet was then incubated with 72% sulphuric acid for 1 hour with occasional vortexing. Crystalline cellulose gets completely digested to glucose. The solution was then made up to 10 ml using deionized water. 1ml of this sample was taken and its pH was adjusted to around 6.5 to 6.8 using 5N and 0.1N NaOH for further analysis.

7.3.2.2 Cellulose determination by Glucose oxidase assay

A 96-well plate was used to determine glucose content enzymatically in the digested cellulose samples. Glucose oxidase enzyme assay kit purchased from Sigma-Aldrich (Code: GAGO 20) was used. To 50 µl of the pH adjusted samples, the enzyme samples were added and incubated as per the protocol. The end product was measured at 540 nm. A calibration curve was plotted using varying concentrations of glucose solution provided with the kit. An anhydro-correction factor of 0.9 was used for calculating the concentration of cellulose from corresponding monomeric (glucose) sugars [54]. The solubilized cellulose content and percentage cellulose degradation were calculated using the following equations.

$$Solubilized\ cellulose(g) = Initial\ cellulose\ (g) - Final\ cellulose(g)$$

$$\%\ cellulose\ degradation = \left[\frac{Initial\ cellulose\ (g) - Final\ cellulose\ \ (g)}{Initial\ cellulose\ (g)}\right] X\ 100$$

7.3.2.3 Hemicellulose determination by dinitro salicylic assay (DNSA) method

The hemicellulose content in the acetic and nitric acid solvent was determined by estimating the xylose content in the solvent. 0.2 ml of the solvent was taken and neutralized to pH 7 to 7.5 using 5N NaOH. After neutralization, the total reducing sugars was estimated using DNSA method. A calibration curve was plotted using 1mg/ml xylose stock solution prepared in acid/water/nitric acid (8/2/1 v/v/v ratio) after subjecting it to neutralization with 5N NaOH. The following equations were used for calculating solubilized hemicellulose content and percentage degradation of hemicellulose fraction.

$$Solubilized\ hemicellulose(g) = Initial\ hemicellulose\ (g) - Final\ hemicellulose\ (g)$$

$$\%\ hemicellulose\ degradation = \left[\frac{Initial\ hemicellulose\ (g) - Final\ hemicellulose\ \ (g)}{Initial\ hemicellulose\ (g)}\right]$$

7.3.3 Fourier transform infrared (FTIR) spectroscopy for cellulose crystallinity

FTIR spectroscopy of the two biomass substrates was carried out using pellets prepared with 1 mg of the substrate and 100 mg of KBr on FTIR 4200 spectrophotometer (JASCO Make) in the absorbance mode in the range of 400 to 4000 cm^{-1} [98]. The baseline was corrected between 800 to 3500 cm^{-1} and the absorbance values at 897, 1430, 3400 and 1320 cm^{-1} were noted to calculate crystallinity indices of cellulose. Lateral Order Index (LOI) was calculated by taking the ratio of absorbance at 1430 and 897 (A_{1430}/A_{897}) and Hydrogen Bond Intensity (HBI) was calculated as the ratio of A_{3400}/A_{1320} [124, 125].

7.3.4 Ash content

The ash content in the solid biomass samples was estimated by keeping the samples at 575ºC for 6 hours in a muffle furnace and noting down the difference in weight [54].

7.3.5 Percentage total degradation of biomass residues

The solid biomass residues were weighed to calculate the weight reduction and hence the total degradation, using the formula given below,

$$Total\ degradation\ (\%) = \frac{(dry\ weight\ \text{of the initial biomass} - \text{dry weight of the biomass after fermentation})}{\text{dry weight of the biomass after fermentation}}\ X\ 100$$

7.3.6 pH estimation

The pH of the liquid supernatant samples was noted at regular time intervals using thermo-coupled pH meter (Make: Adwa).

7.3.7 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

The soluble products of lignin degradation were analyzed in the liquid fraction obtained at the end of fermentation on a C-18 column using acetonitrile-water solvent system using RP-HPLC as detailed in Chapter 6, section 6.2.1.

7.3.8 Cellulase enzyme activity in fermentation studies

Samples collected on days 10 and 20 of the fermentation were centrifuged and the supernatants were sparged with CO_2 to adjust the pH to between 6 and 6.5 in order to maintain uniform pH conditions. The cellulase activity in these samples was measured using a modified NREL protocol [57]. To 50 mg of cellulose powder (insoluble α-cellulose, Himedia, GRM-126) taken in a test-tube, 1ml of 50 mM pH 4.8 citrate buffer was added and the mixture was equilibrated to 37ºC in glass test tubes. To this mixture, 0.5 ml of the sample supernatant was added and

incubated for 3 hrs at 37^{o}C. At the end of 3 hrs, the sample was centrifuged and the supernatant was analyzed for glucose content using the glucose oxidase enzyme assay described in section 2.5. Cellulase activity was calculated in terms of the amount of glucose produced per unit volume per unit time (µM min^{-1}). Though the original protocol uses the Dinitrosalicylic assay (DNSA) for estimating the glucose produced, in a fermentation study this could lead to an overestimation due to the non-specific binding of the DNSA reagent to other sugars released from the biomass. Therefore, a more specific glucose oxidase assay was used here which would provide a more accurate estimation of cellulase activity.

7.3.9 Study on model system of commercial cellulase and standard soluble lignin

Pure cellulase from *Trichoderma reesei* (Sigma Aldrich, C2730) was used for studying the effect of varying concentrations of soluble standard lignin (Sigma-Aldrich, 471003) on cellulase activity. The concentration range of standard lignin tested was 500- 4000 mg L^{-1}. Cellulase activity was measured using the standard NREL protocol described above. As there was no possibility of interference from other sugars in this experiment, glucose content was measured using Dinitrosalicylic assay (DNSA) method [88] as per the original protocol.

7.4 Results and Discussion

7.4.1 Analysis of biomass composition and cellulose crystallinity

Table 7.1 shows the percentage lignin, cellulose and hemicellulose fractions in the untreated cocopeat and sugarcane bagasse (control) samples analyzed by Klason's method and modified Updegraff method as described in the previous section. This data is consistent with the values reported in the literature. As per literature, sugarcane bagasse consists of approximately 43-50% cellulose and 18-25% each of hemicellulose and lignin [3, 25, 26]. Coco-peat consists of 21-36% cellulose, 48-54% lignin and ash is 9-10% [27, 28].

Table 7.1: Lignin,cellulose and hemicellulose percentage in sugarcane bagasse and cocopeat

Sample	Sugarcane bagasse (n=3)	Cocopeat (n=3)
Lignin (%)	21.00 ± 0.14	49.00 ± 0.05
Cellulose (%)	46.00 ± 1.3	35.00 ± 0.1
Hemicellulose (%)	27.00 ± 0.6	15.00 ± 0.7
Ash (%)	2.30 ± 0.24	7.20 ± 0.50

Crystallinity index is a parameter that is commonly used to describe the degree of crystallinity of polymers and can be measured using FTIR spectroscopy [124–126]. Figure 7.2 shows the FTIR spectra of cocopeat and sugarcane bagasse samples, which were used to calculate the crystallinity indices. Two types of indices, viz Lateral Order Index (LOI) and Hydrogen Bond Intensity (HBI) are used to describe the crystallinity of cellulose. The ratio of absorbances at 1430-1420 cm^{-1} (associated with crystalline cellulose) and 897cm^{-1} (corresponding to amorphous cellulose) is called Lateral Order Index. [124, 125]. Higher the LOI, greater the crystallinity of cellulose. Another parameter that is used to determine the intermolecular regularity and crystallinity of cellulose is called Hydrogen Bond Intensity (HBI), which is determined by the ratio of absorbances at 3400 and 1320 cm^{-1} [125]. Both indices were found to be significantly higher in cocopeat (Table 7.2), indicating that the cellulose in cocopeat was more crystalline compared to that in sugarcane bagasse, making it more difficult to degrade [125]. Thus, cocopeat and sugarcane bagasse not only vary in lignin and cellulose content, but also in the structural linkages and monomeric units present within these polymers.

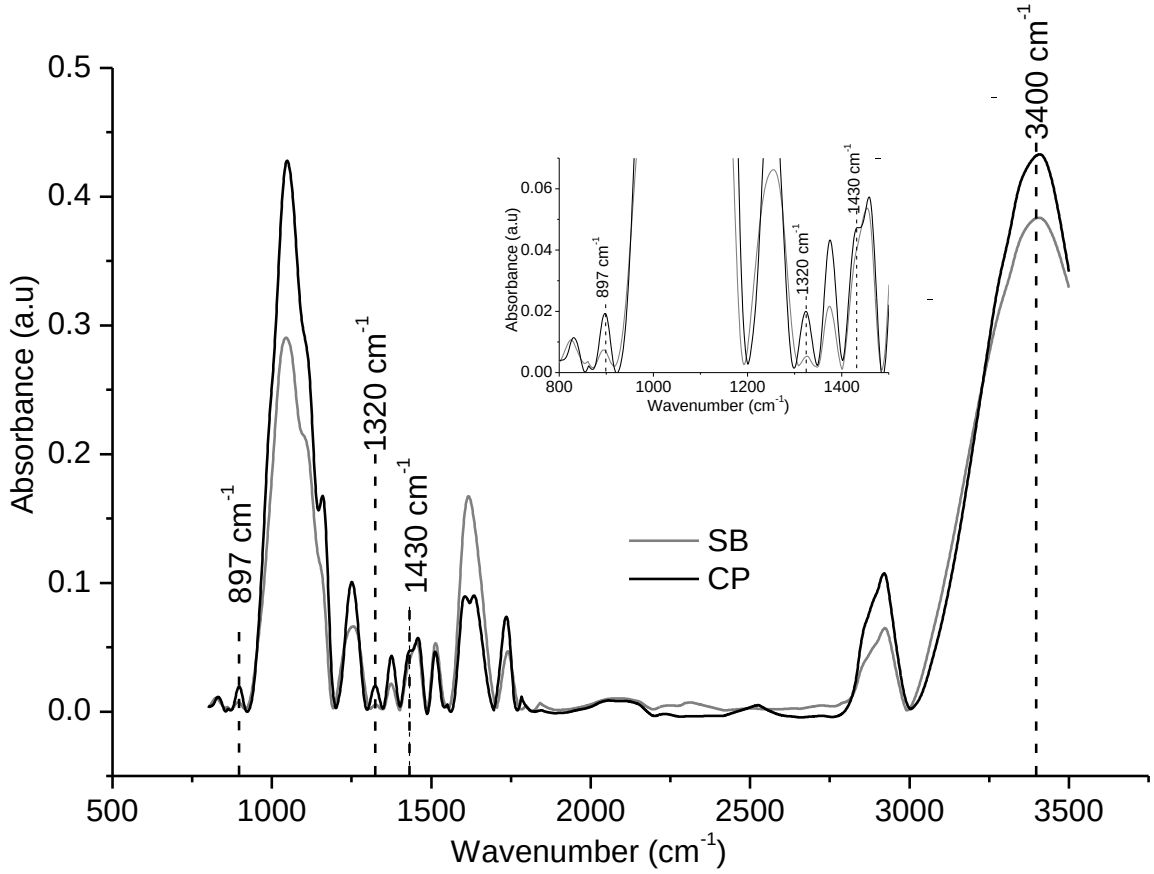


Figure 7.2: FTIR spectra of sugarcane bagasse (SB) and cocopeat (CP)

Table 7.2: Cellulose crystallinity indices for sugarcane bagasse and cocopeat

Sample	Cellulose Crystallinity indices	
	A_{1430}/A_{897} (LOI)	A_{3400}/A_{1320} (HBI)
Sugarcane bagasse (n=3)	2.06 ± 0.34	22.11 ± 2.64
Cocopeat (n=3)	7.71 ± 0.42	34.21 ± 0.22

7.4.2 Cellulose degradation

Figures 7.3a shows the amount of cellulose solubilized during the course of fermentation, while Figure 7.3b shows the percentage cellulose degradation in the samples after fungal treatment.

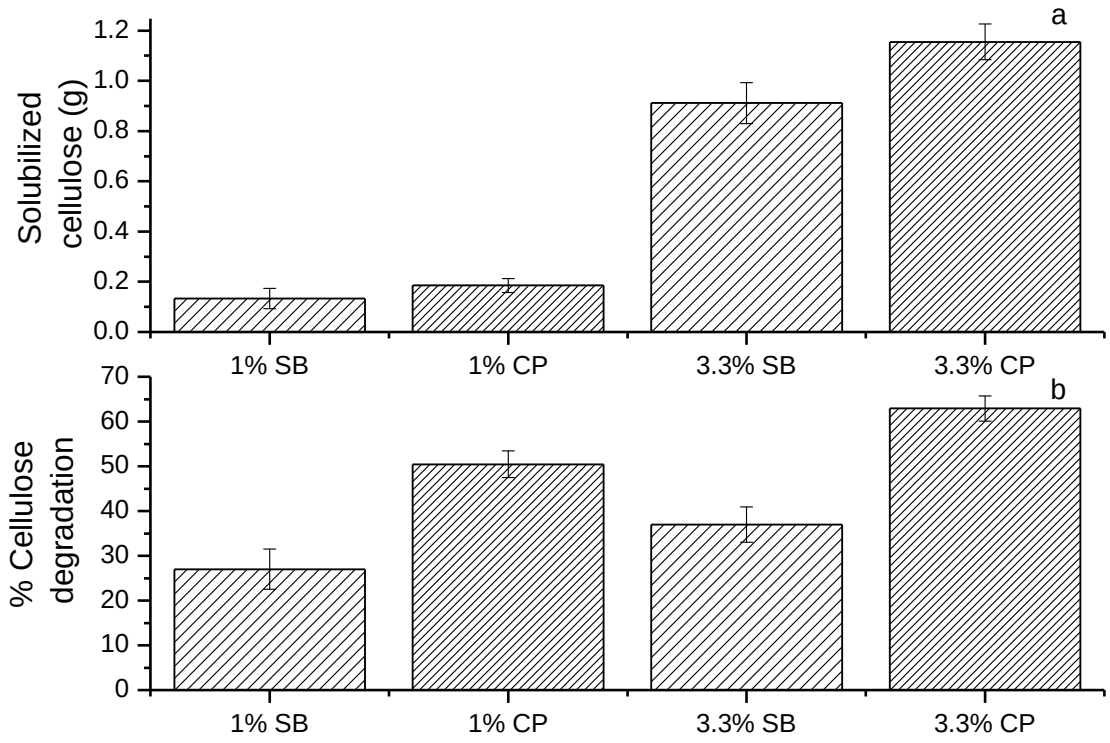


Figure 7.3a: Solubilized cellulose measured in sugarcane bagasse (SB) and cocopeat (CP) at the end of the fermentation. Figure 7.3b: Percentage cellulose degradation.

Overall cellulose degradation in sugarcane bagasse was lower than that in cocopeat, and 3.3% substrate loadings were seen to have more cellulose solubilization than 1% loadings. This seems contradictory to the expected trend from the FTIR data. However, this trend could be explained by the fact that the cocopeat samples had higher amounts of lignin degradation

products, which could impact the cellulase enzyme activity, thereby increasing the extent of cellulose solubilization as discussed in the following section.

7.4.2.1 Effect of degraded lignin on cellulase activity

Based on Klason's lignin estimation, the amount of lignin degraded was found to be higher in cocopeat compared to sugarcane bagasse as expected, owing to the higher initial lignin content in cocopeat. Degraded lignin also increased with an increase in biomass loading, and was the highest in cocopeat at the 3.33% loading. However, despite the lower cellulose content and higher crystallinity indices in cocopeat, the amount of cellulose degraded was nearly 40% higher in cocopeat at the 1% loading and 27% higher at the 3.33% loading compared to sugarcane bagasse. Similarly, the cellulase activities were also higher in cocopeat samples at each biomass loading. Furthermore, the cellulase activities and cellulose degradation also increased with increase in biomass loading for each biomass type. Across all four conditions, cellulase activities and cellulose degradation increased with increase in the amount of lignin degradation products, indicating a stimulatory effect of these products on cellulase, as shown in Figure 7.4.

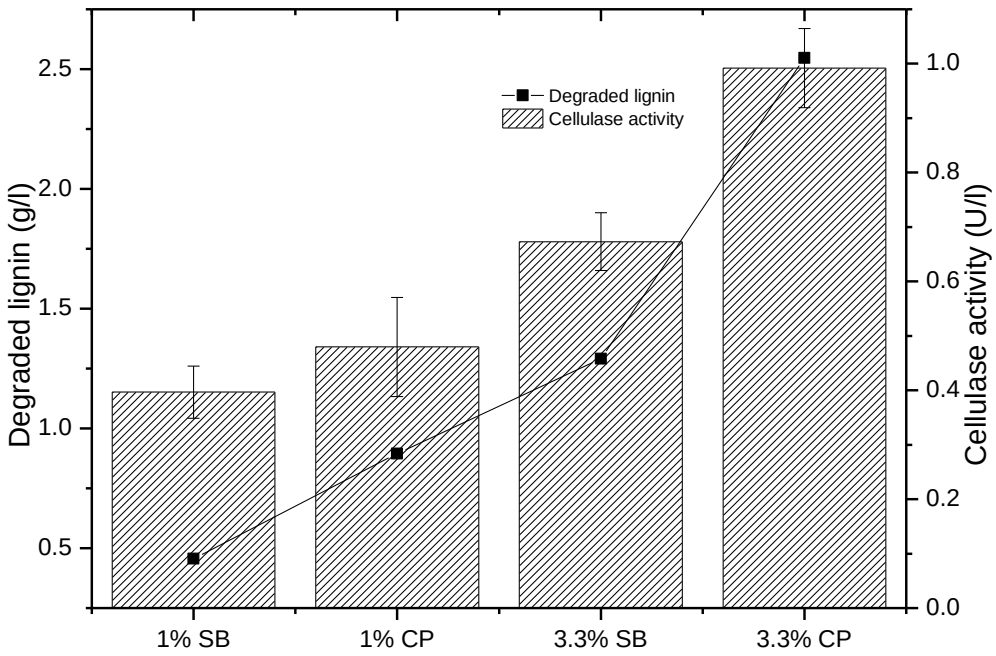


Figure 7.4: Degraded lignin and cellulase activity in sugarcane bagasse (SB) and cocopeat (CP) samples

A strong positive correlation (Pearson's correlation coefficient >0.99) was also seen between the degraded lignin and amount of cellulose hydrolyzed as shown in Figure 7.5, which can be explained by the corresponding cellulase activities. The percentage of cellulose degradation also increased with increase in biomass loading and was the highest for the 3.3% cocopeat sample.

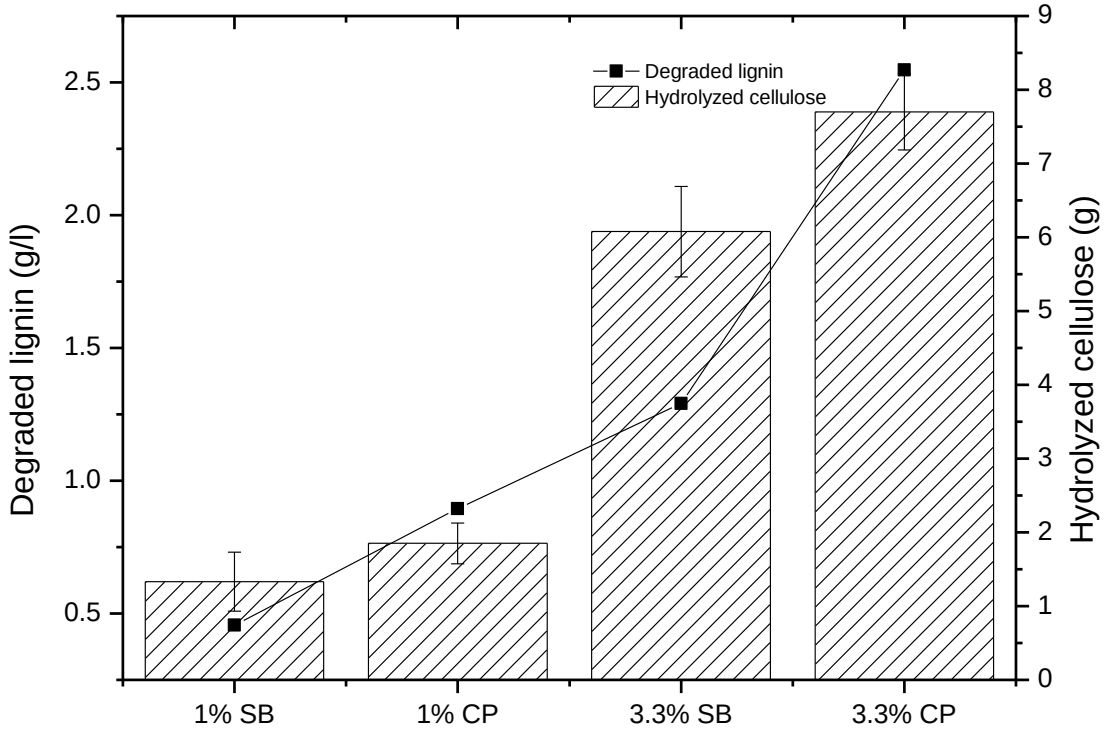


Figure 7.5: Degraded lignin and hydrolyzed cellulose content in sugarcane bagasse (SB) and cocopeat (CP) samples

Several studies have reported that the phenolic compounds released during lignin degradation inhibited cellulase activity [72, 120, 127]. However, these lignin-derived compounds were generated using physical or chemical pretreatment methods such as steam explosion, acid or alkali treatment techniques. Other researchers have reported a concentration-dependent stimulatory effect of certain lignin monomers on cellulase activity [73, 122, 128, 129]. It was hypothesized by Zhao et al., that lignin-derived phenolic compounds at a certain concentration increased the hydrophobicity of cellulase surface just enough to cause better enzyme-substrate connections which lead to a stimulatory effect [73]. The nature of these phenolic compounds also plays a very important role in its action on cellulases [73]. However, these studies were

carried out using synthetic phenolic compounds and not with in-process degraded lignin generated during microbial degradation of biomass.

The stimulation of cellulase by lignin degradation products seen in the present study indicated that biomass loading could be used as a process lever to influence the amounts of lignin degradation products released, which would in turn influence the cellulase activity and cellulose degradation. This also implied that the influence of in-process intermediates on enzyme activities could help overcome the inherent recalcitrance of the biomass, such as in the case of cocopeat.

7.4.2.2 Effect of standard lignin on commercial cellulase activity

While a correlation between degraded lignin and cellulase activity was observed in the fermentation studies, in order to use biomass loading as a process lever, it was important to establish a causal relationship between the two factors. To this end, a model system of commercial cellulase and standard soluble lignin was used. Figure 7.6 shows the effect of varying concentrations of standard soluble lignin on the activity of pure cellulase. The standard lignin was chosen based on its similarity to the lignin degradation products released by the action of *N. discreta* [130]. The concentration range of standard lignin was fixed based on the soluble lignin concentrations seen in the actual fermentation samples from the present study. An increase in cellulase activity was seen with increasing concentrations of standard lignin beyond 1000 mg L^{-1}, which further confirmed the concentration-dependent stimulation of cellulase seen in actual fermentation set-up with increased amounts of degraded lignin in this concentration range. This study not only confirmed the stimulatory effect of soluble lignin on cellulase, but also showed that the stimulation was not specific to the cellulase secreted by *N. discreta* alone.

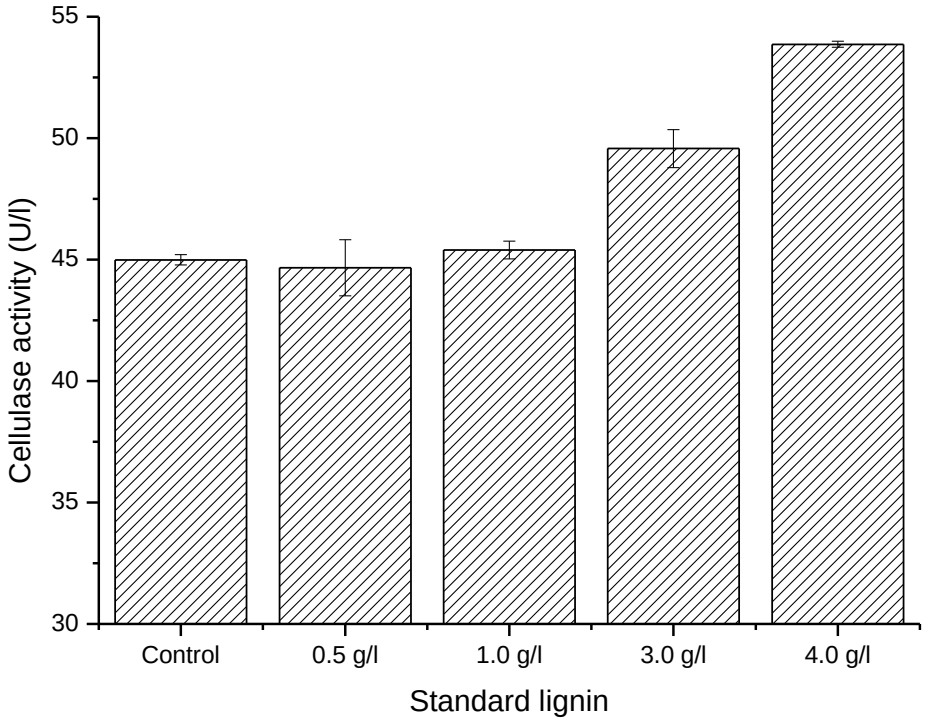


Figure 7.6: Effect of varying concentrations of standard soluble lignin on commercial cellulase activity. The control sample was run in the absence of lignin

7.4.3 Hemicellulose degradation

Figure 7.7a and b show solubilized hemicellulose and percentage hemicellulose degradation in 3.3% sugarcane bagasse and cocopeat samples. In 1% biomass loadings, although hemicellulose content was estimated, the error in measurement was huge, as the analysis was challenging due to the small quantities and therefore was not reported here. Reports have suggested that the hemicellulose removal by various methods is generally not highly selective, but since it is a short-chained polymer, it is easier to hydrolyze as compared to cellulose fraction. In the case of 3.3% loadings, sugarcane bagasse showed higher solubilization of hemicellulose as well as higher percentage degradation of hemicellulose. Although the amount of solubilized lignin and solubilized cellulose was highest in 3.3% cocopeat, the residual cocopeat still contained a higher amount of lignin compared to sugarcane bagasse. Therefore, the trapped hemicellulose fraction in the more condensed lignin linkages might have caused a lower hemicellulose degradation in it. In 3.3% sugarcane bagasse, the loose structure of

biomass and the combined solubilization of cellulose and lignin enhanced the accessibility to hemicellulose fraction causing its rapid degradation.

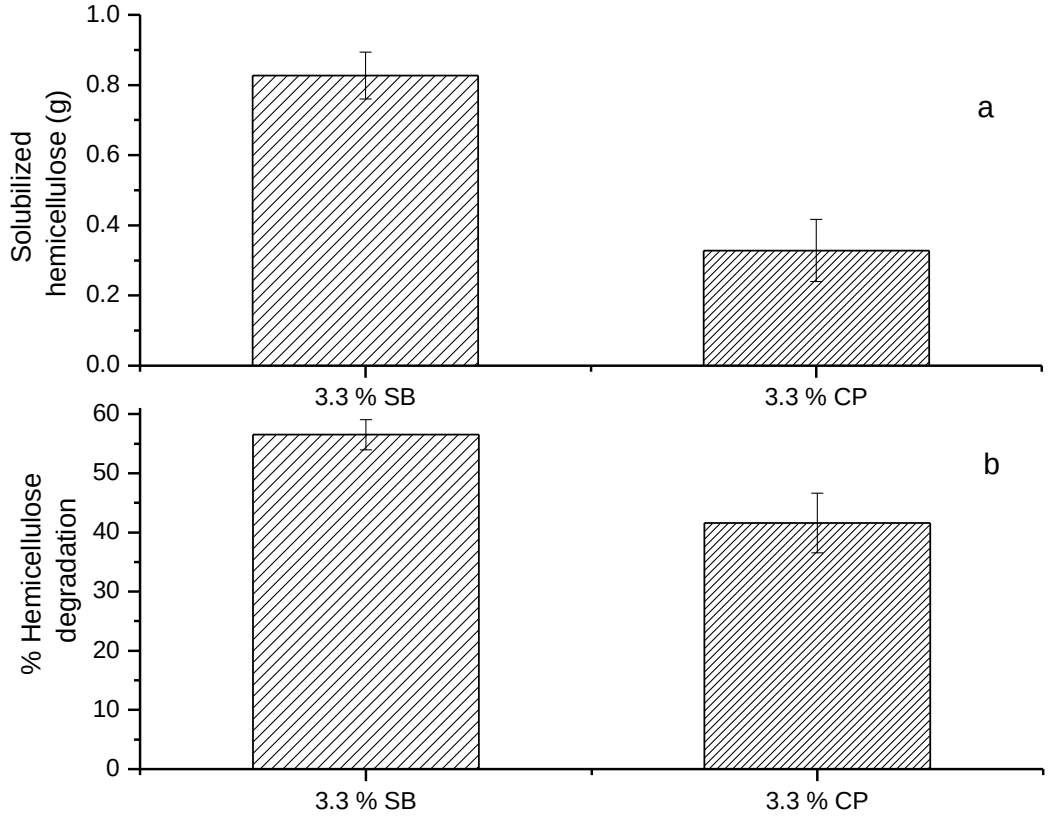


Figure 7.7a: Solubilized hemicellulose (g) measured by DNSA method in sugarcane bagasse (SB) and cocopeat (CP). Figure 7.7b: Percentage hemicellulose degradation

7.4.4 Percentage total degradation

Figure 7.8 shows the pH of the fermentation samples measured during the course of the fermentation. The pH of all the fermentations steadily increased as time progressed. While the pH in the 3.3% sugarcane bagasse sample increased from 5.4 to 8.1 by day 30, in cocopeat sample, the pH increased to 7.2. A similar trend could also be seen in 1% substrate loadings, however, the increase in the pH values was considerably lower. The trends in pH observed correlates exactly with the overall degradation noted in the samples. The highest degradation observed was in 3.3% sugarcane bagasse followed by 3.3% cocopeat. At 1% loading, the degradation was comparatively lower.

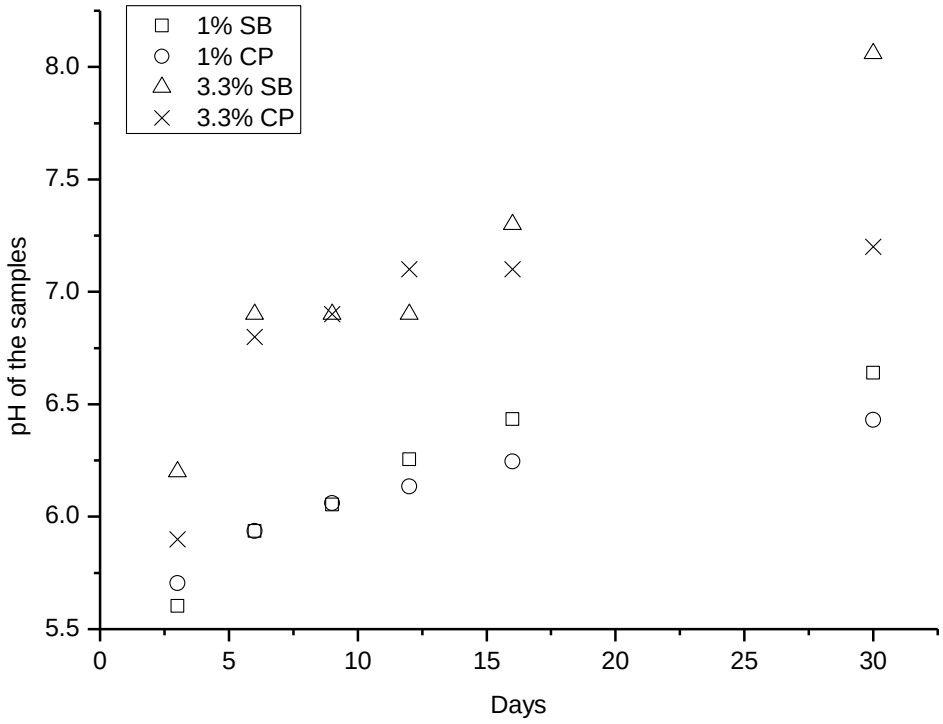


Figure 7.8: pH of the fermentation samples. 1% CP: 1% cocopeat, 1% SB: 1% sugarcane bagasse, 3.3% CP: 3.3% cocopeat, 3.3% SB: 3.3% sugarcane bagasse

From Figure 7.9, an increase in overall percentage degradation of biomass is seen with substrate loading for both cocopeat and sugarcane bagasse. However, in terms of individual components, percentage lignin degradation decreased in 3.3 % loadings, while degradation of cellulose and hemicellulose was higher at 3.3 %. Moreover, percentage cellulose degradation in cocopeat was more than in sugarcane bagasse, because of the stimulation of the cellulase enzyme by lignin degradation intermediates.

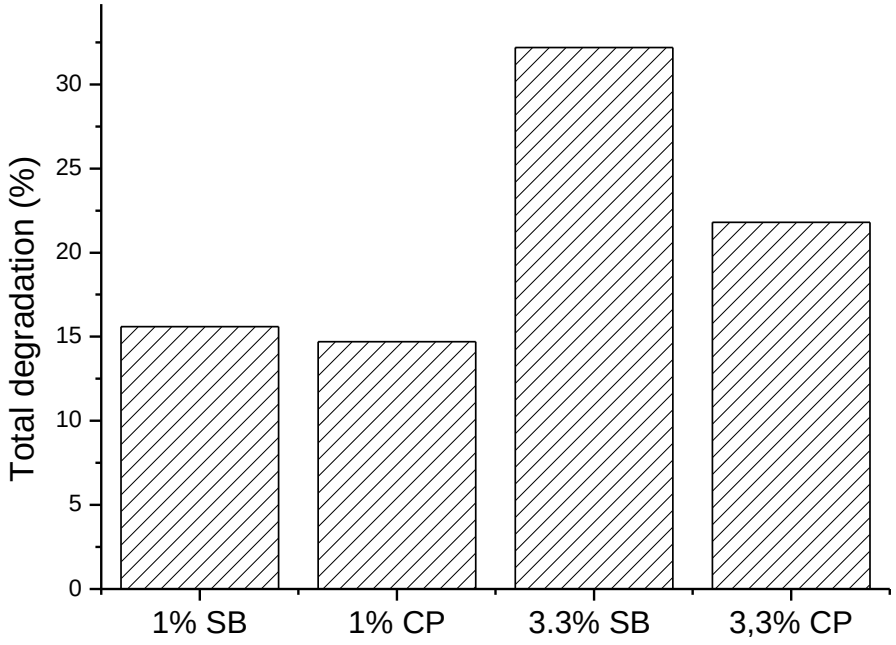


Figure 7.9: Percentage total degradation. 1% CP: 1% cocopeat, 1% SB: 1% sugarcane bagasse, 3.3% CP: 3.3% cocopeat, 3.3% SB: 3.3% sugarcane bagasse (n=2)

From the study of the effect of biomass loadings, it was clear that the overall fungal degradation of biomass had increased because of an increase in substrate loading. The nature and the concentration of phenolics released from lignin degradation seemed to play a pivotal role in cellulase stimulation, which brought about a change in the entire dynamics of degradation of individual polymers. Moreover, increasing the substrate loading of seemingly recalcitrant biomass such as cocopeat, was able to enhance the extent of degradation.

7.5 Conclusion

The ability of *N.discreta* to degrade cellulose and hemicellulose fractions in biomass samples with varying initial percentages was studied and analyzed. Despite the lower cellulose concentrations and higher crystallinity indices of cellulose in cocopeat, an increase in biomass loading resulted in higher cellulose degradation owing to the stimulatory effect of degraded lignin on cellulase. Biomass loading could, therefore, be used as a simple and cost-effective process lever to enhance cellulase activity and cellulose degradation in lignocellulosic residues. Moreover, this study paved the way for utilization of relatively recalcitrant residues such as cocopeat with high lignin content, for the production of biofuels.

CHAPTER 8

A PROOF OF CONCEPT SINGLE STEP CONVERSION OF BIOMASS TO ETHANOL AND VALUE ADDED PRODUCTS

8.1 Introduction

Consolidated bioprocessing of lignocellulosic biomass, involving the microbial conversion of delignified biomass to ethanol has the potential to significantly reduce the overall capital and operating costs in second generation processes [13, 14]. This is because such processes involve an integrated step using suitable microorganisms that are capable of both hydrolysis and fermentation [14, 39]. This results in reduced capital costs, as well as the time required for the overall conversion as it takes place in a single reactor [131]. However, the de-lignification of biomass is still carried out as a separate process, using conventional chemical, thermo-chemical or physico-chemical routes as discussed in chapter 2.

A single step bioprocess would integrate the delignification step into the overall process, resulting in several advantages over existing processes: (1) reduction in cost of capital equipment used in conventional delignification processes (2) reduction in operating costs - including utility costs for steam used in thermochemical routes, cost of chemicals such as acids and alkalis as well as neutralization costs for subsequent pH adjustment [132] (3) prevention of fermentation inhibitors such as furfurals that are typically produced in conventional delignification processes [133]. For such an integrated process, the microbial catalyst used should have the capability to degrade lignin, hydrolyze cellulose and hemicellulose to their monomeric sugars, and also the ability to ferment the sugars to ethanol.

The current chapter reports a proof-of-concept study that demonstrates single step bioprocessing of lignocellulosic biomass using a single fungal strain. Such a study has not been reported in the literature so far. In the previous chapters, the capability of *N.discreta* to degrade lignin, cellulose and hemicellulose fractions of biomass has been demonstrated and therefore could be a potential candidate for single step bio-processing of lignocellulosic biomass residues.

An integration of pretreatment with the subsequent steps has been attempted using a single fungal strain, *Neurospora discreta* (Figure 8.1) in the current study. Hydrolysis of pretreated biomass yields hexose and pentose sugars from glucan and xylan oligomers respectively, which is further converted to ethanol via fermentation. While glucose is easily fermented to ethanol,

the conversion of pentoses requires a set of enzymes that are not present in traditionally used fermentation strains of *S. cerevisiae* etc. The average values of ethanol yields reportedly correspond to about 30 to 50% conversion of biomass to bioethanol [46]. Such low yields cause an increase in the ethanol processing costs as compared to first-generation ethanol plants where the yields are comparatively much higher. As per literature, the production cost of ethanol obtained from lignocellulosic biomass is almost double as that of ethanol produced from sugarcane or corn[31, 134]. However, the increased processing costs in lignocellulosic biorefinery can be compensated by the generation of value-added products from the waste streams such as lignin or by targeting secondary metabolites of fungal growth such as pigments, enzymes or organic acids [60, 63].

Lignin a major component of lignocellulosic biomass is a natural amorphous polymer that has shown huge potential for the production of various aromatic chemicals via biological as well as chemical routes. These chemicals and fuels are currently sourced from petroleum-based sources and there exists a massive potential to produce them from lignin waste streams [135]. Traditionally many large scale processes employing biomass residues such as paper and pulp industries and biorefineries have burnt lignin for power generation [136]. Literature quotes a substantial generation of lignin ranging between 100,000 to 200,000 tons per year, which is way beyond that required for power generation and efforts to transform them to value-added products are underway. Certain potential value-added products are low-cost carbon fibers, plant- derived plastics and composites, resin adhesives, polymeric foams and membranes, and a huge range of commodity chemicals [136, 137]. The major challenges hindering the development of these products are the broad distribution of bond strengths in various C-C and C-O linkages found in lignin, the massive heterogeneity of the low molecular weight products formed and the re-condensation tendency of released low molecular weight compounds to form lignin oligomers [27, 135].

Apart from targeting value-added products from waste lignin streams, fungal biomass and various metabolites such as pigments, citric, gluconic and lactic acids, enzymes etc., generated during microbial degradation of biomass can be used as valuable co-products. Filamentous ascomycetes fungus, in particular, have been regarded as the ‘core of the lignocellulosic biorefineries’ as they have been shown to produce an array of secondary value added co-products [138]. Edible fungal biomass can serve as protein-rich animal feeds and certain strains are a rich source of chitin and chitosan [139]. A few fungal strains of Neurospora have been widely reported as a potential source of pigments [16, 60].

While sub-culturing the filamentous fungi *N. discreta* on potato dextrose agar plates, bright orange colored extracellular waxy like structures were noticed apart from fungal filaments due to the accumulation of carotenoids. Carotenoids are natural color-imparting pigments synthesized by plants and certain micro-organisms. They are also present in animals, which find vast applications in food, cosmetic and pharmaceutical industries. Commercial production of carotenoids from micro-organisms is a new approach as it offers an eco-friendly route over synthetic carotenoid synthesis. Due to the ease of microbial cultivation, with enhanced control over parameters that impact carotenoid synthesis, the fermentation route had gained a lot of interest over the extraction from other natural sources such as plants. In the current study, extraction of carotenoids from the liquid medium during biomass degradation with *Neurospora discreta* was attempted.

In the current chapter, the capability of using *Neurospora discreta* for single step bioprocessing was shown using two lignocellulosic biomass residues, coco-peat and sugarcane bagasse which have widely different lignin content and structures as discussed in earlier chapters. This fungus was capable of producing ethanol directly from untreated biomass in a single step. The fermentative capacity of the fungi to convert hexose and pentose sugars to ethanol was also evaluated. Moreover, the ability of this fungus to produce 'carotenoids' as a by-product of its growth was also shown, which could serve as an important value-added product and further improve the process economics (Figure 8.1). Liquid chromatography-mass spectroscopy of the liquid supernatant sample was conducted to study the products of lignin degradation.

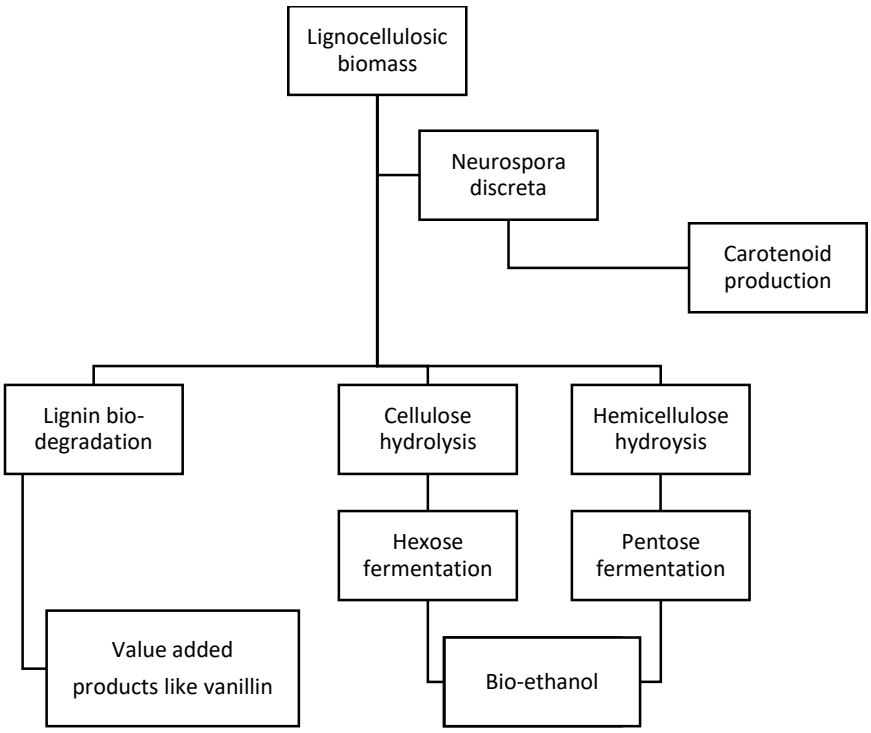


Figure 8.1: Single step bioprocessing of lignocellulosic biomass to bio-ethanol and value-added products

8.2 Materials and methods

8.2.1 Fermentation setup

8.2.1.1 For testing different biomass sources

Submerged fermentations were set up using sub-cultures of the fungus *N. discreta*. Each 250-ml flask contained 1 g of the substrate with 0.5 g of sucrose in 100 ml Vogel's minimal media. The inoculation of the flasks was done as reported in chapter 4 [98], where the final spore count of the cells was adjusted to approximately 10^7cells/ml and 1 ml suspension was used to inoculate each flask. The flasks were incubated at 30 ^oC for 30 days. During the 1st stage, i.e., for 15 days after inoculation, the flasks were aerated every alternate day for accelerating the plant polymer degradation. In the 2nd stage, the flasks were not disturbed to maintain micro-aerobic conditions for maximizing the ethanol production.

8.2.1.2 For testing different carbon sources

Submerged fermentations were set up using different carbons sources such as sucrose and xylose. Each 250-ml flask contained 2 g of carbon source in 100 ml Vogel's minimal media and the inoculation and incubation were done as reported in the earlier paragraph. Approximately 10-12 ml samples on day 5, day 15 and day 25 were periodically removed and

centrifuged at 10,000 rpm for 20 mins to remove cells. The samples were stored at 4^{o}C for further analysis.

8.2.3 Estimation of Ethanol content by Gas chromatography

Gas-chromatography (Shimadzu GC – 2010 Plus) with Flame Ionization detector was used to estimate ethanol and acetic acid in the liquid supernatants. The capillary column had the following dimensions: 30m, 0.25 mm ID and 0.2um thickness (Make: Spinco-tech, Model: EB-1). The operating conditions were as follows: injector, 120 °C; oven, 150 °C; and detector, 160 °C with a carrier gas (Helium gas) flow of 16 mL min $^{-1}$ with a sample injection volume of 25µL.

8.2.4 Chemical identification test for the presence of carotenoids

Carotenoids are considered triterpenoid molecules and the chemical identification test called 'Salkowski' test was performed on the liquid supernatant samples for testing the presence of terpenoid compounds [140]. To 5 ml of the liquid supernatant sample, 5 ml of chloroform was added and vigorously mixed using vortex mixer, for the extraction of carotenoids to chloroform layer. To this mixture, a few drops of 72% sulphuric acid was added and the test tube was allowed to stand for a certain time. The formation of reddish-brown color on the inner surface indicates the presence of terpenoids [141].

8.2.5 Extraction of carotenoids using liquid-liquid extraction

The extraction of carotenoids from the aqueous liquid supernatant samples obtained from 2% sucrose fermentation set using liquid-liquid extraction technique has also been explored. The solvents tested include non- polar solvents such as diethyl ether and hexane, and polar solvents such as acetone and methanol. [63, 142]. The extraction was also tested using mixtures of solvents for varying the polarities. To 5 ml of the liquid sample, 5 ml of the polar organic solvent such as acetone was added and vigorously stirred for 10 minutes. Later, 5ml of di-ethyl ether was added and the solution was again vigorously mixed. Carotenoids, which are generally considered hydrophobic by nature, are extracted into the non-polar ether layer. The two layers were then separated in a separating funnel and subjected to UV visible spectroscopy for carotenoid detection.

Freeze-drying of liquid supernatant samples obtained from biomass fermentation was done using a lyophilizer (Model: LABCONCO). The samples were centrifuged and kept in a

lyophilizer for around 18-24 hours to obtain a powdered sample at the end of the process. The powdered samples were then dissolved in polar solvent methanol and subjected to spectroscopy.

8.2.6 Estimation of carotenoids

Carotenoid content in the organic solvents was estimated using UV-Visible spectrophotometer (HITACHI U-2900) at 450 nm wavelength [63]. A calibration curve was plotted with varying concentrations of pure β-carotene (Make: Sigma Aldrich) solution prepared in respective solvents.

8.2.7 Characterization of solubilized lignin using LC-MS

Liquid Chromatography-Mass Spectrometry (LC-MS) was used for characterization of the compounds present in the liquid supernatant samples obtained from fermentation of lignocellulosic biomass, cocopeat with *N.discreta*. Water-soluble kraft lignin (Sigma-Aldrich, Product No: 471003) was used as the standard. Both the fungal solubilized lignin as well as the kraft lignin samples were analyzed on a C-18 reversed phase (RP)-HPLC column using an acetonitrile-water solvent. The total run time was 35 minutes with a flow rate of 1 ml min^{-1} and a sample injection volume of 25 µL. A gradient elution with the following concentrations of acetonitrile was used to differentiate between polar and non-polar compounds of solubilized lignin: at 0 min— 0 %; 5 min— 3 %; 10 min—6 %; 15 min—10% and 20 min—20 %, 25 min—40% and 35 min – 80%. The compounds were detected at 254 nm using photodiode array detector and the mass spectra of the selected peaks were obtained from the Triple Quadrupole mass spectrometer (Make: Shimadzu, Model: 8040), under electrospray ionization mode. Automated software, Labsolutions^{TM} was used for data integration and mass spectrum analysis. 'Multicharge ion analysis' which is a deconvolution software was used to predict the molecular weights of the intact parent compounds [110].

8.3 Results and discussion

8.3.1 Measurement of ethanol

Lignocellulosic biomass consisting of lignin, cellulose and hemicellulose yields hexoses and pentoses from glucan and xylan oligomers respectively (Equations 1 and 2), which is further converted to ethanol via fermentation (Equations 3 and 4).

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \text{----------(1)}$$

$$(C_5H_8O_4)_n + nH2O \rightarrow nC_5H_{10}O_5 \text{----------(2)}$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \text{----------(3)}$$

$$3C_6H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 \text{----------(4)}$$

Ethanol content was measured in the liquid supernatants of 1% cocopeat and 1% sugarcane bagasse at the end of 30 days fermentation using gas chromatography as detailed in materials and methods section. A chromatogram of pure ethanol of 1% (v/v) concentration and 1%v/v acetic acid shown in Figure 8.2 had a retention time at 4.46 and 9.78 respectively. A calibration curve was plotted using varying concentrations of pure ethanol in water from 0.5% to 2% (v/v) and the ethanol content in the biomass samples was estimated.

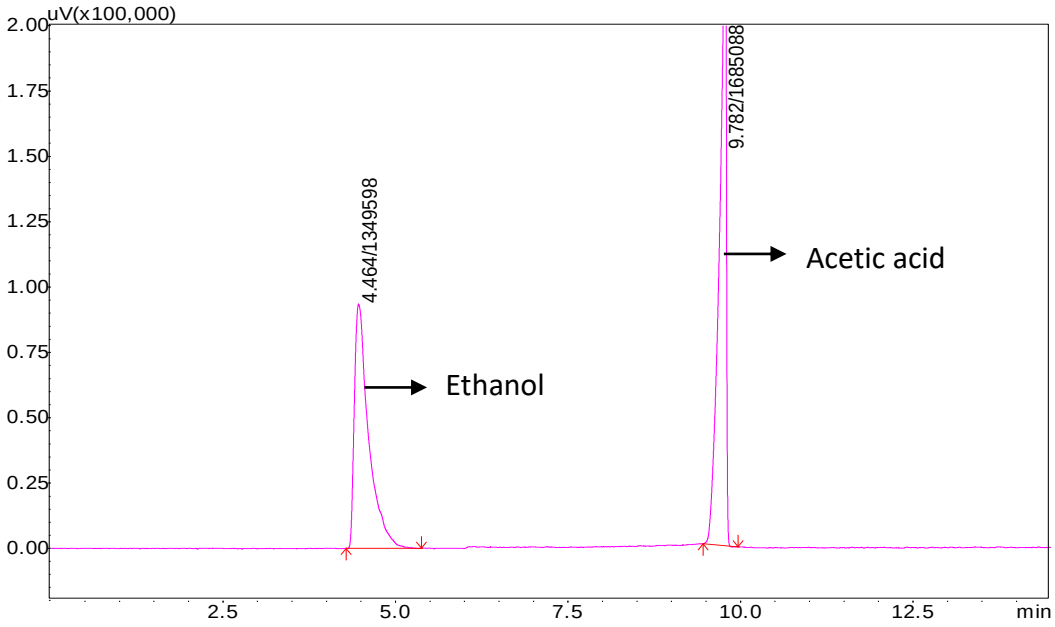


Figure 8.2: Standard chromatogram for 1% (v/v) ethanol and 1% (v/v) acetic acid

Figure 8.3 shows chromatograms of liquid supernatant samples of 1% sugarcane bagasse and 1% cocopeat fermentations which translates to 10 g L^{-1} of initial biomass loading. The liquid supernatant of sugarcane bagasse, as well as cocopeat, showed clear peaks at retention times of 4.48 - 4.58 which corresponds to ethanol and no other peaks were noticed in the samples. The final ethanol content in sugarcane bagasse was found to be approx 1 g L^{-1} while for cocopeat it was 1.23 g L^{-1} . Typical yields of ethanol from lignocellulosic biomass was reported to be around 20 to 50% of the substrate weight [31]. Rao et al., [66] studied direct production of ethanol from pretreated wood and other agricultural residues using *Neurospora crassa* and obtained 4 to 13 g L^{-1} of ethanol from an initial biomass loading of 20 g L^{-1} after 5 days of

fermentation. In the present case, the final ethanol yields were 10% of substrate weight as parameters impacting fermentation were not optimized.

An important criterion for microorganism selection is its ability to ferment pentoses, as this would contribute to the overall ethanol yield. The strains of *Neurospora* reportedly contain pentose fermentation pathway and could therefore convert hemicellulose derived xylose sugars to ethanol [10, 44]. To test this hypothesis, fermentations with pure sugars namely, sucrose and xylose were conducted using the fungal strain, *Neurospora discreta*.

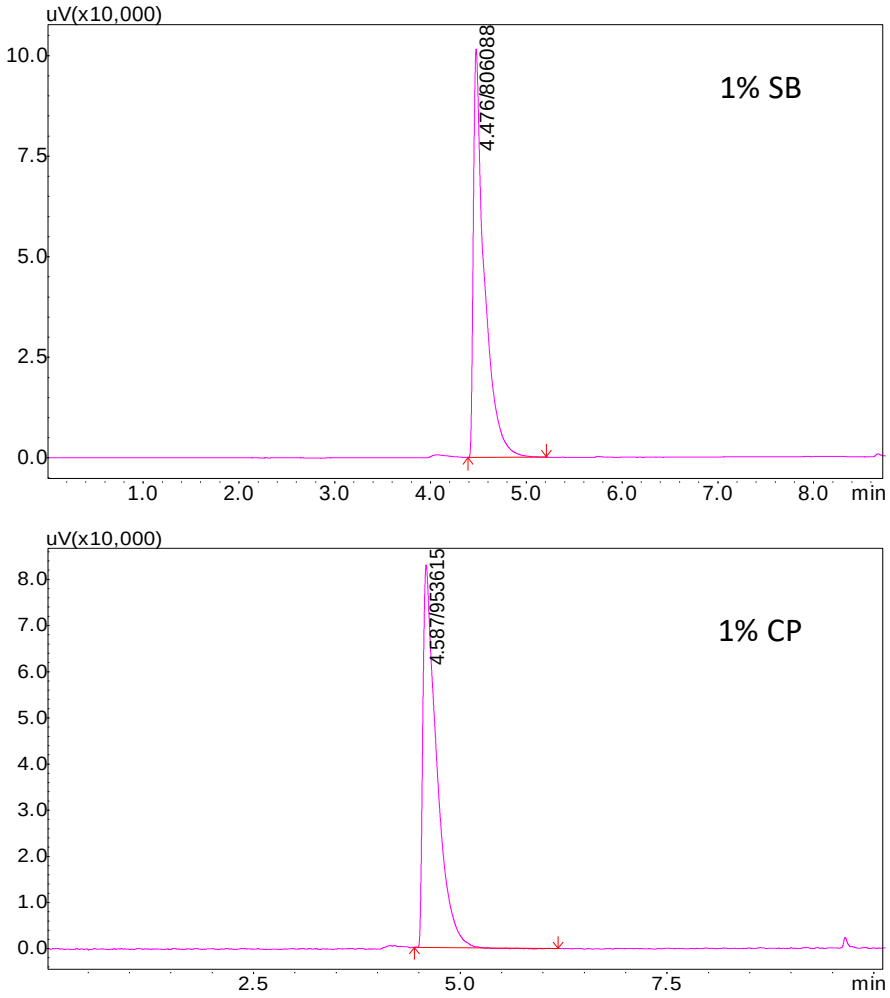


Figure 8.3: Gas chromatograms of liquid supernatant samples of 1% SB (1% sugarcane bagasse and 1% CP (1% cocopeat)

Table 8.1 shows ethanol yields obtained using two different carbon sources, namely sucrose, and xylose, with 20 g L^{-1} initial sugar concentrations. The yield of ethanol was 0.135 g/ g (2.7 g/L) of xylose and 0.356 g/g (7.13 g/L) of sucrose. The maximum theoretical yield of ethanol

from both xylose and sucrose is 0.511 g/g of sugar estimated from equations 3 and 4 [46]. So, around 70% of maximum yield was obtained by sucrose fermentation, while 27% of maximum theoretical yield was obtained via pentose fermentation. Certain yeast strains such as *Pachysolen tannophilus* can reportedly yield 70-75% of maximum theoretical yields of ethanol from pentose sugars [31]. Due to frequent sampling, micro-aerobic conditions could not be maintained during the entire time course of fermentation and therefore optimum ethanol yields may not have been obtained. The above study demonstrated the capability of *N. discreta* to use 5 -carbon sugar as the sole carbon source and produce ethanol which shows that this could be a suitable microbial candidate for a single step conversion of lignocellulosic biomass to ethanol.

Table 8.1: Ethanol content from different carbon sources

Samples	Ethanol content (g/L) with sucrose as carbon source	Ethanol content (g/L) with xylose as carbon source
Day 5	2.59	1.19
Day 15	7.13	2.70
Day 25	5.79	1.65

8.3.2 Carotenoids as a value-added product

8.3.2.1 Confirmatory test for the presence of carotenoids

During fermentation studies conducted using the fungal strain *N. discreta*, the liquid medium turned orange as the fermentation proceeded, due to the accumulation of the pigment in the liquid medium. Figure 8.4 shows the accumulation of the carotenoid pigment in 2% sucrose fermentation samples inoculated with *N. discreta*. Fungi are known to synthesize several important terpenes, by cyclization of the isoprenoids units such as carotenoids, gibberellins, indole-diterpenes etc. [60, 143] A chemical identification test called 'Salkowski' test was therefore performed using chloroform and H_2SO_4 for detecting the presence of terpenoids in the liquid supernatant samples [144].

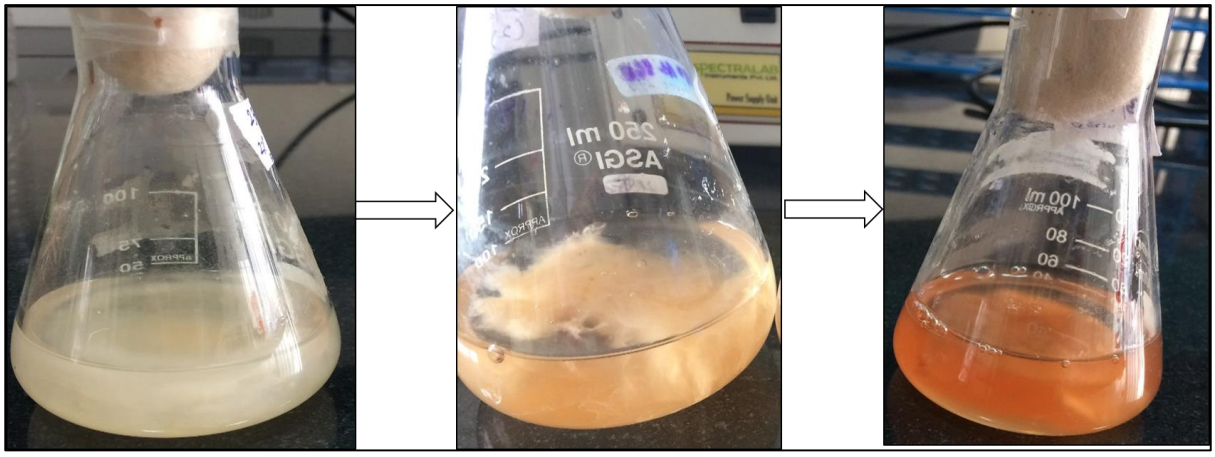


Figure 8.4: Accumulation of carotenoid pigment in fermentation flasks

Two distinct layers of chloroform and acidic liquid layer were seen after the addition of the chemicals to the liquid supernatant sample. (Figure 8.5). Literature reports the formation of a reddish brown coloration at the interface indicates the presence of terpenoids[141, 144]. In this experiment, a reddish brown ring with faint blue tinge was clearly noticed at the surface indicating the presence of carotenoids in dilute quantities. To further confirm the presence of carotenoids, the following solvent extraction procedures were done.

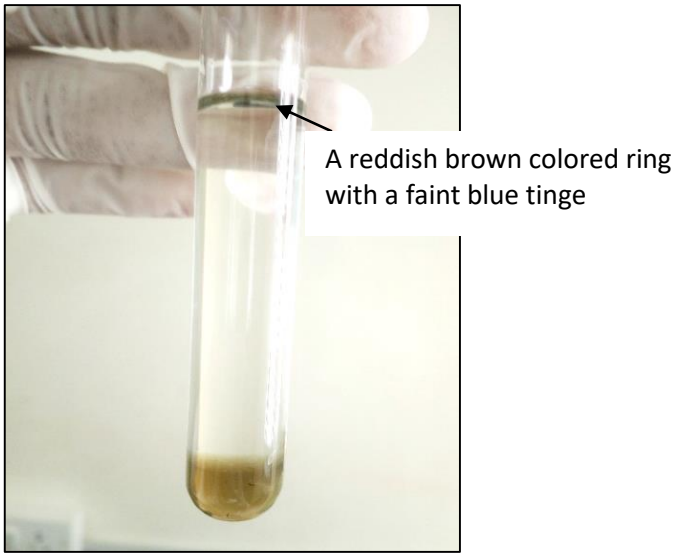


Figure 8.5: Confirmatory test for the presence of carotenoids

8.3.2 2 Solvent extraction and quantification of carotenoids

Solvent extraction of carotenoids using various pure solvents as well as their mixtures was attempted as detailed in materials and methods section. The extraction of carotenoids from the aqueous liquid supernatant samples using nonpolar solvents such as di-ethyl ether, hexane, and

chloroform were conducted. Also, mixtures of acetone-diethyl ether and methanol-diethyl ether were tested to obtain different polarities. The solvent extracts were then subjected to estimation of carotenoids at 450 nm using UV-visible spectrophotometer[63]. However, there was no absorbance seen at 450nm in any of the extracts, and the aqueous liquid supernatant still retained its original yellow-orange color indicating the extraction of the carotenoids from the liquid medium to the non-polar solvent layer was not successful. This indicates that the pigment synthesized by *N.discreta* was highly polar. Carotenoids are reported to broadly fall under two categories: a) β-carotenes or hydrocarbon carotenoids composed of carbon and hydrogen and b) xanthophylls, which are hydroxyl derivatives of carotenes obtained on oxidation of carotenes. While β-carotene is non-polar, xanthophyll pigments are considered to be polar in nature due to the presence of the hydroxyl group [63]. The higher affinity of the pigment towards water indicated the presence of polar groups, which shows that the pigment is xanthophyll and not carotene. Also, the presence of unsaturated bonds in β-carotene structures causes oxidation of carotenes to xanthophyll while in solution, making its extraction from aqueous solutions difficult.

8.3.3.3 Freeze-drying of liquid supernatant samples, extraction & quantification of carotenoids

As solvent extraction of the pigment from the aqueous medium was not possible, 15 ml of the aqueous liquid supernatant sample was freeze-dried using a lyophilizer to remove the water content. The powdered sample was then dissolved in 5ml polar solvent methanol and subjected to spectroscopy. The presence of conjugated double bonds in carotenoids makes them sensitive to heat [60], and therefore high-temperature dehydration could disrupt the native structure of carotenoid molecules. Hence the freeze-drying process was used that removes water at low temperatures and pressures by sublimation process. The freeze-dried sample readily dissolved in the methanol solvent. Carotenoids, consisting of conjugated double bonds can exist in cis- and trans- configurations depending on the relative arrangement of the attached substituent groups. It is reported that generally, carotenoids give a three peak absorption spectrum, with higher values of λ_{max} corresponding to carotenoids with greater number of conjugated double bonds. The various isomers can reportedly absorb anywhere between 400-550 nm. Although absorbance was noted in the entire spectrum, clear peaks were not noticed, which could be either due to dilute concentrations of the pigment or disruption of native form of the structure due to various processing steps. From the calibration curve plotted using β-carotene as standard

at 450 nm, the concentration of the carotenoid pigment was found to be 1.06 mg L^{-1}.

8.3.3 Value-added products from lignin

In order to study the low molecular weight compounds released by fungal solubilization of lignin, liquid chromatography-mass spectroscopy was performed. In the earlier chapters, the presence of highly polar solubilized lignin intermediate was confirmed in liquid supernatant samples obtained by biomass fermentation with *N.discreta* (Fungal solubilized lignin). A similar peak was also observed in water-soluble kraft lignin fraction obtained chemically (Kraft lignin).

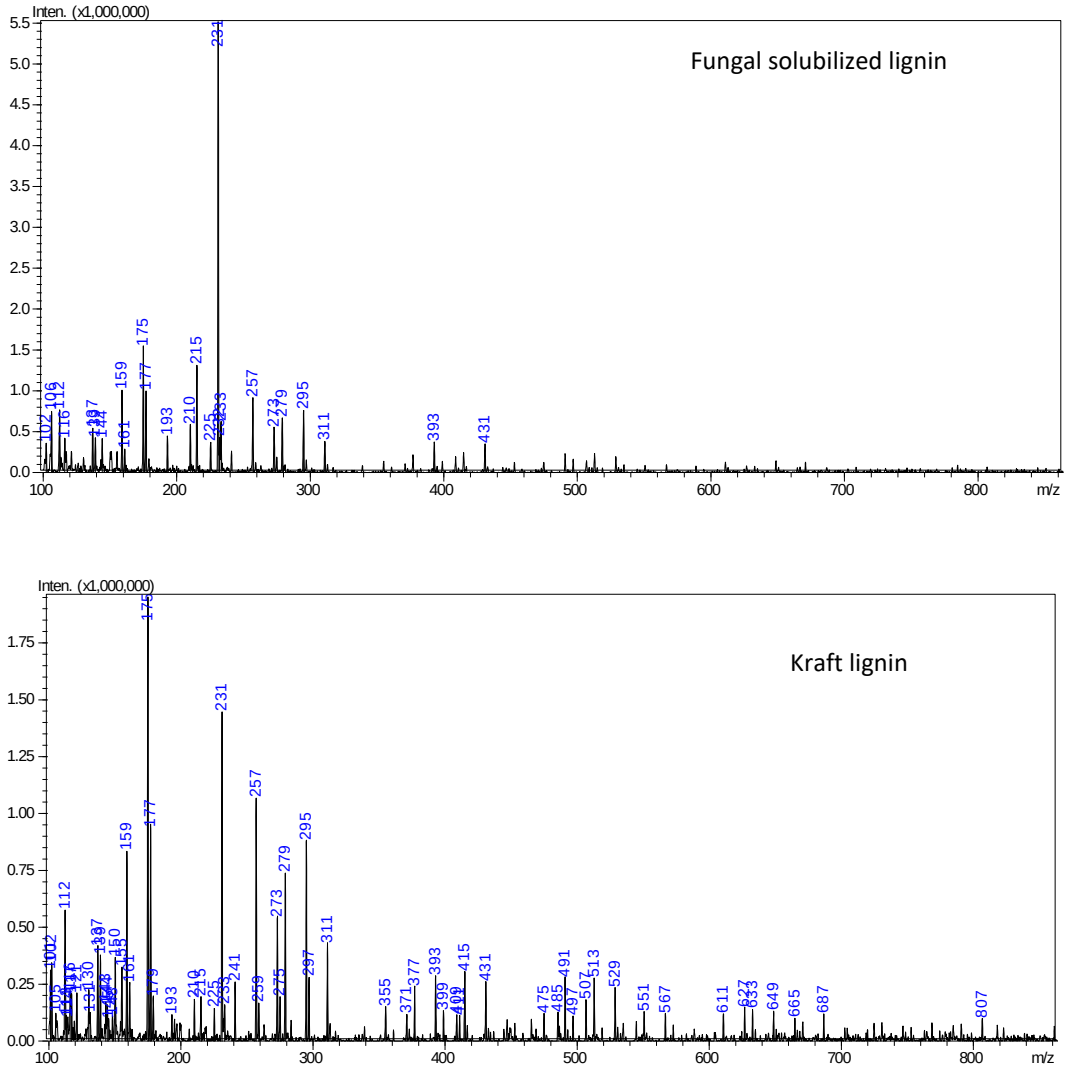
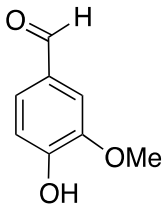
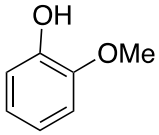


Figure 8.6: Mass spectrums in positive ESI mode of fungal solubilized lignin and Kraft lignin polar intermediates

The mass spectra of fungal solubilized and kraft lignin for the polar intermediates revealed several common m/z peaks and while kraft lignin mass spectrum displayed more mass to charge species, fungal solubilized lignin had fewer m/z peaks (Figure 8.6). All the peaks found in fungal solubilized lignin were also seen in kraft lignin. By running multi-charge ion analysis for the selected peak at 2.7 corresponding to the polar lignin intermediate, the mass spectrum deconvolution calculated a range of higher molecular weight lignin compounds. Mass spectrometry for analyzing soluble lignin fractions for predicting its structure has been the focus area in recent years [145]. Multicharge ion formation and its analysis for kraft lignin have been reported by Andrinova et al. [111]. The media m/z peaks correspond to peaks 215 and 231. Some major common m/z peaks seen were: 175, 140,124, 257,215,177, 142,279, 159, 210, 144, 112,114,151 etc. In literature, m/z peaks at 175, 177, 179, 257, 295, 279, 273, 137, 193, 139 were assigned to lignin model compounds such as vanillin, veratrole, guaiacol, eugenol, coniferyl alcohol and aldehydes, and certain alcohol dimers [111, 145]. Vanillin is a chief constituent of vanilla flavoring and is used as flavoring and fragrance ingredient in the food or cosmetic industries [146]. “Synthetic” vanillin which constitutes to nearly 85% of the world vanillin production is produced from petro-based intermediate guaiacol. Based on the m/z data, the precursor chemical, guaiacol could also be seen in the fungal solubilized lignin stream and also in kraft lignin samples. Zakzeski, J. and Weckhuysen, B.M.[147], studied solubilization and aqueous phase reforming of several lignin sources such as kraft, soda, alcell lignin along with sugarcane bagasse. The author reported disruption of abundant β-O-4 linkages by aqueous phase reforming and formation of aromatic platform chemicals, particularly guaiacol and syringol [147]. Laccase and lignin peroxidase enzymes in *N.discreta* can easily cause disruption of ether-based β-O-4 lignin linkages and release these products as solubilized lignin. From the mass spectroscopy analysis, low molecular weight compounds vanillin and guaiacol were detected in the fungal solubilized fraction (Table 8.2).

Table 8.2: Low molecular weight lignin compounds in solubilized lignin fraction with potential industrial applications

Compounds	Chemical Structure	MW ($g \cdot mol^{-1}$)	Assigned m/z values
Vanillin		152.15	114, 151
Guaiacol		124.24	121,123,255

8.4 Conclusions

This study demonstrates for the first time, the capability of isolated fungal strain *N. discreta* to degrade two widely different biomass substrates -cocopeat and sugarcane bagasse and produce ethanol in a single step process, with no other pre-treatment other than size reduction. This work also demonstrates the ability of *N. discreta* to produce ethanol from pentose sugars such as xylose as the sole carbon source. Finally, the production of value-added products such as carotenoids from the fungus, and low molecular weight aromatic compounds such as vanillin and guaiacol from lignin during the process were shown.

CHAPTER 9

CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

In the current study, production of bio-ethanol from two lignocellulosic biomass residues, sugarcane bagasse and cocopeat using a single robust fungal strain *Neurospora discreta* was studied. The summary of work is presented below:

- ✓ Screening of potential fungi for microbial delignification was carried out using traditionally used white rot fungi, *Phanerochaete chrysosporium* and *Pleurotus ostreatus* along with an isolated indigenous fungus. The indigenous fungus was identified to be *Neurospora discreta,* and was found to grow more rapidly and resulted in higher delignification of biomass compared to the other fungi. Optimal process conditions were then identified for *N. discreta* using a statistical Design of Experiments (DOE) approach. While initial studies were carried out using solid state fermentation, as a result of the DOE, as well as other limitations identified during the experiments, subsequent studies were carried out using submerged fermentation of lignocellulosic residues.

- ✓ Two agricultural residues – cocopeat and sugarcane bagasse were used for the studies and were selected for their wide variation in composition as well as structure. The structural differences in lignin and cellulose among these residues were studied using FTIR spectroscopy, which also explained their 'degradability'. While the extent of lignin degradation was between 25 to 40% for sugarcane bagasse, in the case of cocopeat it was less than 20%. A detailed comparison of the lignin degradation capabilities of *P. chrysosporium* with *N. discreta* showed that the novel strain *N. discreta* could serve as a promising alternative to the conventional white rot fungus.

- ✓ The in-process activities of ligninolytic enzymes in *Neurospora discreta,* laccase, and lignin peroxidase were measured for the first time and inhibition of the enzymes was seen at higher biomass loadings. A detailed kinetic study of laccase inhibition by lignin degradation intermediates obtained from two different sources, fungal solubilized and

chemically treated kraft lignin was conducted. With an increase in the concentration of lignin degradation intermediates, V_{max} remained nearly constant, while K_m increased from 1.5 to 3.0 times that of pure laccase, revealing that the inhibition was competitive in nature. The dissociation constant of the enzyme-inhibitor complex (K_I) was found to have a value similar to K_m of pure laccase, indicating the potential for severe inhibition at high concentrations of lignin degradation intermediates. This is the first report on inhibition of laccase by lignin degradation products, and is a highly relevant finding as it explains the low rates of delignification currently seen in microbial processes, and could pave the way for designing more efficient and sustainable microbial delignification processes by overcoming laccase inhibition.

- ✓ Although the extent of delignification decreased with increase in biomass loadings, cellulose degradation was found to be higher for both the biomass substrates. Owing to the stimulatory effect of degraded lignin on cellulase enzyme activity, despite the lower cellulose concentrations and higher cellulose crystallinity, degradation of cellulose was higher in recalcitrant cocopeat as compared to sugarcane bagasse. At higher biomass loading the extent of cellulose hydrolysis was 63% in cocopeat, while in sugarcane bagasse it was nearly 37%. A study on commercial cellulase and varying concentrations of standard soluble kraft lignin indicated concentration-dependent stimulation of cellulase due to solubilized lignin content.

- ✓ Although percentage delignification was found to be lower at increased biomass loadings, due to inhibition of lignin-degrading enzymes, percentage cellulose degradation was found to be higher at higher biomass loadings. Also, overall biomass degradation was higher at increased biomass loadings. Biomass loading is, therefore, a simple and cost-effective process lever to enhance cellulase activity and cellulose hydrolysis in lignocellulosic residues, due to the effect of lignin degradation intermediates.

- ✓ A proof-of-concept study of single step bioprocessing using *N.discreta* was conducted and the final ethanol yields of approx. 1 g/L was obtained from the two biomass residues. Value addition to the process from fungal secondary metabolites such as carotenoids and low molecular weight phenolic compounds from waste lignin streams was also explored. Mass spectroscopy of solubilized lignin stream revealed low

molecular weight compounds such as vanillin and guaiacol that have important industrial applications. From the study on the extraction of carotenoids from liquid supernatant samples, the pigment synthesized by *N.discreta* was found to be highly polar xanthophyll pigment.

9.2 Future work

The thesis presents details of an efficient microbial pre-treatment process for the breakdown of lignin in lignocellulosic biomass, using indigenous, novel fungal strain *Neurospora discreta*. The strain was also found to be a potential candidate for an integrated process that combines pretreatment, hydrolysis, and fermentation into a single step. For developing a feasible process at large scale, further investigations need to be carried out in the following areas:

- Further optimization of the process parameters of the integrated single step bioprocessing strategy. The major parameters affecting hydrolysis and fermentation of lignocellulosic biomass were identified. However, the final ethanol yields obtained were lower compared to literature values. A detailed study should be conducted to identify the factors impacting the last step fermentation, for increasing the ethanol yields from the hydrolyzed sugars and optimize the fermentation process.
- Optimization of process conditions for increased yields of carotenoids, a potential value-added product and an in-depth characterization of solubilized lignin stream to identify and quantify other potential value-added products with high-value needs to be carried out.
- A detailed cost analysis of the single step bioprocessing strategy with conventional separate hydrolysis and fermentation (SHF) process has to be conducted, for estimating the process profitability and feasibility.

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List of Publications based on Present Research

In Peer−Reviewed Journals:

1. Sirisha Pamidipati and Asma Ahmed. "Degradation of lignin in agricultural residues by locally isolated fungus Neurospora discreta." *Applied Biochemistry and Biotechnology* 181.4 (2017): 1561-1572.

2. Sirisha Pamidipati and Asma Ahmed. "Cellulase stimulation during biodegradation of lignocellulosic residues at increased biomass loading." *Biocatalysis and Biotransformation* (2018): 1-7

3. Sirisha Pamidipati and Asma Ahmed. "Competitive inhibition of laccase by water-soluble lignin degradation intermediates." *Bioresources and Bioprocessing* (Under Review)

Papers Presented in National / International Conferences:

4. Sirisha P., and Asma Ahmed. "A study on solid state fermentation of sugarcane bagasse using different fungi". 11th BRSI convention and international conference on emerging trends in biotechnology, November 2014, New Delhi, India.

5. Sirisha Pamidipati and Asma Ahmed. "Bio-processing of agricultural residues to bio-fuels using Neurospora discreta, Proceedings of the 15th International Conference on Environmental Science and Technology, (CEST 2017- Rhodes, Greece, 31 August to 2 September 2017), CEST2017_00822 (2017)

6. Akshita G., Riya D, Sirisha P., & Asma A*. Extraction and Quantification of carotenoids from Neurospora, National symposium on emerging environmental challenges, an Engineering approach, CSIR-NEERI and BITS-Pilani Hyderabad Campus.

Biography of the Student

Ms. Sirisha Pamidipati has completed B. Tech in Chemical Engineering from Mumbai University, India in 2006. After her bachelor's degrees, she pursued her master's degree, M. Tech in Bioprocess Technology from Institute of Chemical Technology (formerly known as UDCT), Mumbai, India in 2008. After her degree, she worked as a 'Senior Research Associate' in Process R & Development department, in Granules India Limited, Hyderabad for 2 years. Later she moved to Bangalore and worked as a 'Senior Executive' in Kemwell Biopharma Private Limited, Bangalore for two years. Then, she has joined as a 'Lecturer' in Department of Chemical Engineering, Birla Institute of Technology and Science (BITS) Pilani, Hyderabad Campus, in August–2013. Alongside teaching undergraduate students, she has been pursuing her Doctor of Philosophy (Ph.D.) degree and submitted her thesis in second semester of 2018-19.

Biography of the Supervisor

Name of the Supervisor	**Dr. Asma Ahmed**
Designation and address	Assistant Professor, Department of Chemical Engineering, BITS, Pilani, Hyderabad Campus
Education (Ph.D.)	Ph.D. Chemical Engineering, Oklahoma State University, OK, U.S.A
Experience (years)	12 years
Number of publications	Journals - 06
	International and National Conferences - 09
	Book Chapters - 0
	Patents Filed - 3
Sponsored Projects	PI: Production of bio-ethanol via microbial conversion of lignocellulosic waste - Research Initiation Grant, BITS-Pilani
	PI: Biofuels from lignocellulosic biomass waste: Bio-solubilization and bio-methanation of lignin - Department of Science and Technology, Govt. of India
	PI: Treatment of Nitrogeneous waste water using microalgae and its utilization for biofuels and value added products - BITS-Pilani, CORE project, Waste, Water and Energy
	PI: Bioconversion of coal rejects to biogas and humic acid - In collaboration with Ardee Hi-Tech Pvt. Ltd., under Biotechnology Industry Partnership Program, BIRAC, Department of Biotechnology
No. of Ph.D. students guided (in progress)	02
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Biography of the Co-Supervisor

Name of the Supervisor	**Prof I Sreedhar**
Designation and address	Associate Professor, Department of Chemical Engineering, BITS, Pilani, Hyderabad Campus
Education (Ph.D.)	Ph. D. (Chemical Engineering): B.I.T.S, Pilani, Rajasthan M. Tech. (Process Engineering & Design): I.I.T. Delhi B. Tech (Chemical Engineering): N.I.T, Warangal
Experience (years)	22 years
Number of publications	Journals - 31
	International and National Conferences - 34
	Book Chapters - 1
Reviewer to International Journals	More than 20
Sponsored Projects	Submitted: 1. CSIR, Dec 2017 (PI) 2. DST, June, 2018 (PI) 3. DST, June 2018 (Co-PI)
Seminars and Invited Talks	Seminars/Workshops Organized: 1 Invited Talks:5
No. of Ph.D. students guided (in progress)	03
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