CHARACTERIZATION AND PRETREATMENT OF COTTON GIN WASTE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Technology
In
Biotechnology and Medical Engineering

By

GAURAV KUMAR GUPTA

Department of Biotechnology and Medical Engineering
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Under the Guidance of

Prof. KRISHNA PRAMANIK

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Certificate

This is to certify that the thesis entitled **"CHARACTERIZATION AND PRETREATMENT OF COTTON GIN WASTE"** submitted by **Mr. Gaurav Kumar Gupta** in partial fulfillment of the requirements for the award of Masters of Technology in **Biotechnology and Medical Engineering** at National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other university/Institute for the award any Degree or Diploma.

Supervisor

Prof. KRISHNA PRAMANIK

Department of Biotechnology and Medical Engineering

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Date: 25th May 2009 **Gaurav Kumar Gupta**

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Abstract

Bioethanol produced from various lignocellulosic materials, such as wood, agricultural or forest residues, has the potential to be a valuable substitute for, or a complement to gasoline. One of the crucial steps in the ethanol production is the hydrolysis of the hemicellulose and cellulose to monomer sugars. In order to make the raw material accessible to the enzymes some kind of pretreatment is necessary. During the last ten years a large number of pretreatment methods have been developed. White rot fungi has the capability to separate the cellulose and hemicelluloses component from the complex lignin.

In the present work the white rot fungi *Pleuorotus ostreatus* has shown maximum ligninase positive activity, Hence *Pleuorotus ostreatus* has been used to carry out pretreatment experiments for ethanol production and it has shown a maximum lignin degradation capacity of around 56.82% at optimum conditions of 30^{o}C; 5pH; 200rpm; 3weeks age of inoculum and 30ml/10gm moisture content.

Chapter 1

Background History
Objective

INTRODUCTION

India is the third largest cotton producing country in the world and has the large number of cotton mills. A huge quantity of waste is generated during the processing of the cotton in these mills. Due to the stringent environment regulation, the disposal of cotton gin waste is one of the biggest problems faced by cotton industries. The waste generated after the ginning of cotton fibers and recovery of cotton seeds is the lignocellulosic material which can be potentially utilized as feedstock for the production of fuel ethanol since it is rich in cellulose. Production of ethanol by fermentation will provide the cotton ginning industries with the waste management solution and an added bonus of value added product. Therefore the objective of the proposed work is to investigate the chemical composition of cotton gin waste and pre-treatment methods employed for cost effective separation of lignin from cellulosic biomass.

The biomass can be utilized for the production of bio ethanol. However the major technological difficulty for utilizing these biomass materials is the separation of lignin from the cellulose and hemicellulose to make the material susceptible to hydrolysis. No other substantial option for production of transportation fuels can match ethanol made from lignocellulosic biomass with respect to dramatic environmental, economic, strategic and infrastructure advantages. Alcohols, particularly bioethanol, have the potential to revolutionize the supply and use of energy fuel in many parts of the world, particularly in transportation because:

- There is a variety of widely available raw materials from which alcohol can be made.
- Positive environmental advantages, particularly with regard to the low increase of SO_2, CO_2, particulates, unburned hydrocarbons and CO.

- Ethanol has a much higher latent heat of vaporization (855KJ/KG) than petrol (293 KJ/KG).As a result the fuel mixture entering the cylinder is much cooler and hence denser in case of ethanol than in the case of petrol.
- Ethanol has a high octane number (99) than petrol (80-100).As a result pre ignition does not occur when ethanol is used.
- Ethanol is burnt more completely so that hydrocarbon emission is drastically lower as compared to that in case of petrol.
- Ethanol is much less likely to catch fire and explode in cases of fuel leakage, for example during accidents.
- Ethanol can be mixed with petrol; this increases the octane rating of petrol. In USA a 20% ethanol, 80% Petrol mixture being marketed as gasohol.
- Ethanol melts at -114.1°C, boils at 78.5°C, and has a density of 0.789 g/mL at 20°C. Its low freezing point has made it useful as the fluid in thermometers for temperatures below -40°C, the freezing point of mercury, and for other low-temperature purposes, such as for antifreeze in automobile radiators.

Bioconversion of lignocellulosics to ethanol consists of four major unit operations: pretreatment, hydrolysis, fermentation and product separation/ distillation.

Pretreatment:

Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. Pretreatment affects the structure of biomass by solubilizing hemicellulose, reducing crystallinity and increase the available surface area and pore volume of the substrate. Pretreatment has been

considered as one of the most expensive processing steps in biomass to fermentable sugar conversion with cost as high as 30 cents/gallon ethanol produced.

Hydrolysis:

The hydrolysis methods most commonly used are acid (dilute and concentrated) and enzymatic. To improve the enzymatic hydrolytic efficiency, the lignin-hemicellulose net work has to be loosened for the better amenability of cellulases to residual carbohydrate fraction for sugar recovery. Enzymatic hydrolysis offers major advantages over other chemical routes (e.g., acid hydrolysis) such as higher yields, minimal byproduct formation, low energy requirements, mild operating conditions, and low chemical disposal costs.

Fermentation:

The production of ethanol by fermentation involves four major steps

1. The growth, harvest and delivery of raw material to an alcohol plant
2. The pretreatment or conversion of the raw material to a substrate suitable for fermentation to ethanol.
3. Fermentation of the substrate to alcohol, and purification by distillation.
4. Treatment of the fermentation residue to reduce the pollution and to recover by products.

Strain and Inoculation

White rot fungi, the only organisms to biodegrade food, may soon be used to biodegrade toxic chemicals as well. The application of white rot fungus is expected to be relatively economical. The fungi grown on a number of inexpensive agricultural or forest wastes. Fungi and other micro organisms decay wood by releasing enzymes that digest specific components

such as cellulose, hemicellulose and lignin. The white rots are capable of degrading all the major components of food including lignin. Based on laccase production we selected the following fungi. *Pleurotus ostreatus* having the laccase activity (U/L) of 40.02.

Biorenewable resources are usually classified as either wastes or dedicated energy crops. Categories of waste materials that qualify as bio renewable resources include agricultural residues, yard waste, municipal solid waste, food processing waste, and manure. Agricultural residues such as corn stover, rice hulls, wheat straw, cotton stalks, and bagasse, are the portion of the crop discarded after harvest. Municipal solid waste (MSW) is waste discarded as garbage, not all of which is suitable as biomass feedstock. In communities where yard waste is excluded, the important components of MSW are paper (50%), plastics and other fossil fuel derived materials (20%), food wastes (10%), and non-flammable materials including glass and metal (20%).

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1.1 Interest in Biomass and Biobased Products

In the past 10 years, there has been a renewed interest, world-wide, in biomass as an energy source. Technological developments relating to crop production, conversion, etc. promise

the coupling of biomass at lower cost with higher conversion than was previously possible. More advanced options to produce electricity are looking promising and allow a cost-effective use for energy crops in operations such as production of methanol and hydrogen by gasification processes.

Air pollution is an important factor motivating interest in alternative fuels at the global level. Carbon dioxide is responsible for more than half of the projected anthropically mediated climate change. Transportation fuels account for 27% of the 2.2 billion MT of carbon dioxide released annually in the United States from combustion of fossil fuels. Vehicles account for 4.7% of total worldwide carbon dioxide emissions, with U.S. vehicles accounting for 2.5% of total emissions. The use of biomass to produce energy has the potential to reduce the high emission levels of greenhouse gases. When produced by sustainable means, biomass emits roughly the same amount of carbon during conversion as is taken up during plant growth, so the use of biomass does not contribute to a buildup of carbon dioxide in the atmosphere.

1.2 Fuel Ethanol

Ethanol is a high octane, water free alcohol produced from the fermentation of sugar or starch. It is used as a blending ingredient in gasoline or as a raw material to produce high octane fuel ether additives. The use of ethanol as an automobile fuel in the United States dates as far back as 1908, to the Ford Model .T.. Henry Ford was a supporter of homegrown renewable fuels, and his Model T could be modified to run on either gasoline or pure alcohol (Ford Motor Company, 2004). Trillions of miles have been driven on ethanol blended fuel since 1980 and ethanol blended fuels currently account for about 18% of automotive fuels sold in the United States (RFA, 2004). The Clean Air Act of 1990 and the National Energy Policy Act of 1992

created new market opportunities for alternative fuels by phasing in requirements for fleet vehicles to operate on cleaner fuels (NWICC, 2004).

1.3 Stalk Composition

1.3.1 Cell Wall Organization

Most of the carbohydrate content of plants is structural polysaccharides that provide support, strength, and shape for the plant. This complex structural material in the cell wall, known as lignocellulose, is a composite of cellulose fibers embedded in a cross-linked lignin hemicellulose matrix. The three main components of lignocellulosic materials are cellulose, hemicellulose, and lignin, with other minor components being ash, protein, and extractives. The distribution of cellulose, hemicellulose, and lignin in a typical plant cell wall is shown below in Fig 1. Lignin is most abundant in the middle lamella and decreases with increasing distance into the fiber cell wall, with percentages in the primary cell wall and S1 layer of the secondary cell wall higher than in the S2 and S3 sections of the secondary cell wall. Cellulose is most abundant in the secondary cell wall as seen in the diagram below. The cellulose microfibrils in the primary cell wall have no specific orientation, while the microfibrils in the secondary cell wall run parallel to each other, but at a different angle for each of the three layers S1, S2, and S3.

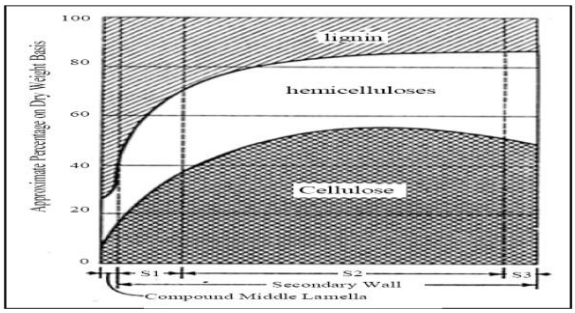


Fig 1: Distribution of cellulose, hemicellulose, and lignin in a typical plant cell wall.

1.3.2 Cellulose

Cellulose is a linear polymer of anhydro D-glucose units connected by β-1,4 glycosidic bonds as shown below in Figure 2. Native cellulose exists in the form of microfibrils, which are paracrystalline assemblies of several dozen (1→ 4) β-D-glucan chains held together by intermolecular hydrogen bonds. Intramolecular hydrogen bonds also form between two glucose units in the same chain. The combined bonding energies of the intermolecular and intramolecular hydrogen bonds increases the rigidity of cellulose and forms the crystalline structure that makes it highly insoluble and recalcitrant to most organic solvents. The cellulose microfibrils are imbedded in a matrix of noncellulosic polysaccharides, mainly hemicellulose and pectic substances, which complicates hydrolysis of cellulose to glucose even further. The cellulose in lignocellulosic biomass feedstocks provides the main source of glucose used during ethanol fermentation.

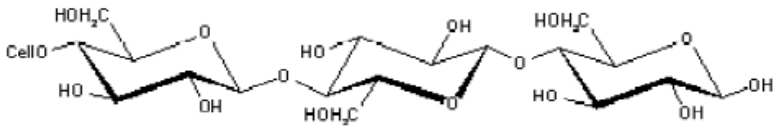


Fig 2: The structure of linear cellulose polymer (HUT, 2004)

1.3.3 Hemicellulose

Hemicelluloses are complex, highly branched polysaccharides that occur in association with cellulose in the cell walls. The monomers that comprise hemicellulose are hexoses (glucose, galactose, and mannose) and pentoses (arabinose and xylose). Hemicellulose can be classified into three groups, namely, xylans, mannans, and galactans based on the polymer backbone that is very often homopolymeric with β-1,4 linkages. In softwoods, the primary hemicellulose

components are galactoglucomannans and arabinoglucuronoxylan, while the principal hemicelluloses in hardwoods are glucomannans and methylglucoronoxylans. Xylan is the most important in terms of the percentage of total hemicellulose found in biomass. The structure of galactoglucomannan is shown in Figure 3. Galactoglucomannan consists of β- 1,4-linked mannose and glucose units in a ratio of 3:1 to which O-acetyl groups and α-1,6- linked galactose side groups are attached.

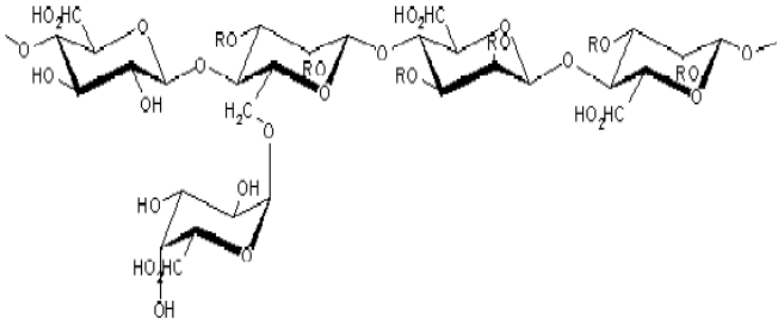


Figure 3: Structure of galactoglucomannan found typically in softwoods

1.3.4 Lignin

Lignin is a three-dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon-carbon bonds. It is constructed of three monomers: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol, each of which has an aromatic ring with different substituents. The dominant monomeric units in the polymers are benzene rings bearing methoxyl, hydroxyl, and propyl groups that can be attached to other units. When the plant is mature and the cell growth ceases, the middle lamella (the cement between the primary walls of adjacent cells) and the secondary wall (inside of primary cell wall) have large amounts of lignin. Lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together.The structure of a small section of a lignin polymer is shown below in Figure 4.

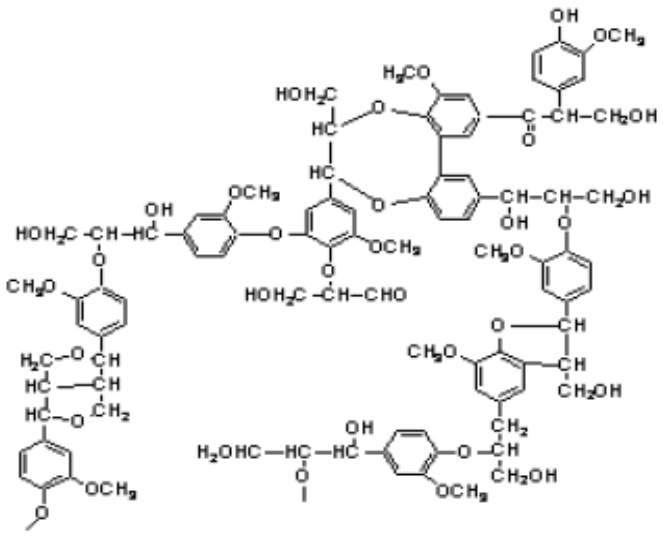


Fig 4: Structure of a section of a lignin polymer

1.4 Pretreatment Lignocellulosic Materials

Pretreatment is the first step required to fractionate lignocellulosic materials into its major plant components of lignin, cellulose and hemicellulose. The mechanisms by which pretreatments improve the digestibility of lignocellulose are however not well understood. An important goal of pretreatment is to increase the surface area of lignocellulosic material, making the polysaccharides more susceptible to hydrolysis. Along with an increase in surface area, pretreatment effectiveness and hydrolysis improvement has been correlated with removal of hemicellulose and lignin and the reduction of cellulose crystallinity.

A successful pretreatment must meet the following requirements:

(1) Improve formation of sugars or the ability to subsequently form sugars by hydrolysis;

(2) avoid the degradation or loss of carbohydrate;

(3) Avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and

(4) Be cost effective. The large number of pretreatments used for lignocellulosic materials can be classified into groups as physical, physico-chemical, chemical, and biological processes.

1.4.1 Physical Pretreatment

Since one of the main goals of pretreatment is to increase the surface area available to cellulase enzymes during hydrolysis, communition, or size reduction, is an integral part of pretreatment. Waste materials can be comminuted by a combination of chipping, grinding, and milling. The size of the materials is usually 10 to 30 mm after chipping and 0.2 to 2 mm after milling or grinding. The process has relatively low energy requirements, ranging from 24,000 kJ/dry ton for wheat straw to 200,000 kJ/dry ton for aspen wood. However, energy consumption increases exponentially with decreasing particle size. For enzymatic hydrolysis, particle size reduction is followed by additional pretreatment methods to further improve hydrolysis. Cellulase enzymes used during enzymatic hydrolysis are large proteins with molecular weights ranging from 30,000 to 60,000 and are thought to be ellipsoidal with major and minor dimensions of 30 and 200 Å. Typically, only 20% of the pore volume of plant tissue is accessible to these large molecules. Thus, without additional pretreatment beyond size reduction, sugar yields from enzymatic hydrolysis are less than 20% of theoretical, whereas further pretreatment can increase yields to 90% or higher.

1.4.2 Physico-Chemical Pretreatment

1.4.2.1 Steam Explosion (auto hydrolysis)

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials. Chipped biomass is treated with high-pressure saturated steam and then the pressure is

quickly reduced, which makes the materials undergo an explosive decomposition. Steam explosion is initiated at a temperature of 160 to 260ºC (corresponding pressure 0.69 to 4.83 Mpa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicelluloses degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Until recently, optimization strategies for the pretreatment of lignocellulosics have focused on the effects of temperature, residence time, and pH, but have not accounted for changes in severity by properties inherent to the starting feedstock.

Consequently, a study was conducted that evaluated the effects of chip properties, feedstock size (40-mesh, 1.5 x 1.5 cm, 5 x 5 cm), and moisture content (12% and 30%) on the overall bioconversion process, and more specifically on the efficacy of removal of recalcitrant lignin from the lignocellulosic substrates following steam explosion. The results indicated that both increased chip size and increased moisture content resulted in improved solids recovery and increased hemicellulose-derived sugar recovery as well as minimized condensation of lignin. Furthermore, a post steam-explosion refining step increased hemicellulose-derived sugar recovery and was most effectively delignified (to as low as 6.5%). The refined substrate could be enzymatically hydrolyzed to very high levels (98%) at relatively fast rates (1.23 g/L/h).

The addition of small amounts of mineral acids, usually sulfuric acid (H_2SO4), improves hydrolysis at reduced temperatures. This process is known as acid-catalyzed steam explosion. Ground biomass is treated with 1 wt-% acid and incubated at 140ºC for 30 minutes or at 160ºC for as little as 10-15 minutes, to achieve complete hemicelluloses removal, which increases enzymatic digestibility of the remaining cellulose to as high as 90%.

Addition of sulfur dioxide (SO_2) gas in steam explosion can also effectively increase sugar yields and improve enzymatic hydrolysis. The maximum sugar yields for steam explosion pretreatment of corn fiber were found when the material was pretreated at 190ºC for 5 minutes after being exposed to 3% SO_2. Sequential SO_2-catalyzed steam explosion and enzymatic hydrolysis resulted in a conversion efficiency of 81% of the combined original hemicellulose and cellulose in the corn fiber to monomeric sugars.

1.4.2.2 Ammonia Fiber Explosion (AFEX)

Ammonia explosion or ammonia fiber explosion (AFEX) is a process in which ground, prewetted lignocellulosic material at a moisture content of 15-30% is placed in a pressure vessel with liquid ammonia (NH3) at a loading of about 1-2 kg NH3/kg dry biomass. Pressures exceeding 12 atm are required for operation at ambient temperature. The mixture is incubated from several minutes up to an hour to enable the ammonia to penetrate the lignocellulosic matrix. When the reaction is complete, a valve is opened to explosively release the pressure. AFEX pretreatment has been demonstrated to improve the saccharification rates of herbaceous crops and grasses. Materials pretreated using the AFEX process include cotton wastes, alfalfa, corn stover, rice straw, bagasse, coastal Bermuda grass, newspaper, kenaf newspaper, switchgrass , wheat straw, barley straw, and municipal solid waste.

The two mechanisms that AFEX pretreatment appears to act by are the increase in reactivity of cellulose due to exposure to liquid NH3 and the increase in accessible surface area following AFEX treatment, probably as a combined effect of hemicellulose hydrolysis and explosive decompression. AFEX pretreatment has not proven effective on hardwoods or softwoods.. The AFEX pretreatment was clearly different from the other pretreatments since it

did not significantly solubilize hemicelluloses, so the use of AFEX pretreatment was not used widely.

1.4.3 Chemical Pretreatment

1.4.3.1 Acid Treatment

Acid pretreatment can utilize either dilute or concentrated acids to improve cellulose hydrolysis. At moderate temperatures, direct saccharification suffers from low yields due to sugar decomposition. However, prehydrolysis with dilute acid at temperatures higher than 121ºC is very effective for increasing the enzymatic digestibility of cellulose. There are primarily two types of dilute acid pretreatment processes: low solids loading (5-10% [w/w]), high-temperature (T > 160ºC), continuous-flow processes and high solids loading (10-40% [w/w], lower temperature (T < 160ºC), batch processes. Dilute acid pretreatment (0.2-2.0% sulfuric acid, 121-220ºC) of lignocelluloses serves three important functions in the conversion process: 1) hydrolysis of the hemicellulose components to produce a syrup of monomeric sugars; 2) exposure of cellulose for enzymatic digestion by removal of hemicellulose and part of the lignin; and 3) solubilization of heavy metals which may be contaminating the feedstock.

In spite of these benefits, acid pretreatment presents potential problems such as the production of an acid waste stream that must be neutralized or reused, the formation of compounds such as acetic acid and furfural in the hydrolysate which are toxic to bacteria or yeasts during fermentation, and the need for corrosion-resistant equipment.

The operating costs of pretreatment are highly contingent upon the consumption of steam that is needed to heat the biomass and acid to elevated temperatures. The simplest way to reduce steam consumption is by increasing the dry weight concentration of solids in the reactor. Many

of them investigated dilute acid pretreatment of aspen wood and wheat straw at solids concentrations from 10 to 40%. A monomeric soluble sugar stream (mostly xylose) was produced with little sugar degradation and the cellulose remaining in the solids was highly digestible by enzymes thus proving that using higher solids concentrations is a feasible option for reducing the cost of steam. Pretreatment with 5% H2SO4 or HCl solubilized 85% of the hemicellulose fraction, but the enzymatic conversion increased only two times compared to untreated stover. Much better results were obtained when acid pretreatment was combined with NaOH pretreatment as described below.

1.4.3.2 Alkaline Treatment

Alkaline solutions can be used to pretreatment of lignocellulosic materials, and the effectiveness of pretreatment is dependent upon the lignin content of the material. The mechanism of alkali pretreatment is believed to be saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components such as lignin and hemicellulose. After alkali pretreatment, the porosity of the material is increased due to the extensive swelling facilitated by removal of the crosslink's. Pretreatment of cotton waste with 10% sodium hydroxide (NaOH) for 60 minutes under pressure at 121ºC in the autoclave decreased the lignin fraction by more than 95% and increased the enzymatic conversion more than four times to 79.4% as compared to untreated stover. In addition, by using dilute NaOH (0.5% w/w) and increasing the reaction time to 90 minutes, 80.1% enzymatic conversion was achieved over pretreated cotton wastes with aqueous ammonia in a flow-through column reactor, in a process termed ammonia recycled percolation (ARP). This method delignified the biomass by 70-85% with 70% of the lignin removal occurring in the first 10 minutes of treatment. Subsequent

enzymatic hydrolysis of cotton wastes treated for 90 minutes exhibited digestibility of 99% with 60 FPU/g glucan enzyme loading, and 92.5% with 10 FPU/g of glucan. Alkali soaking can also be used in conjunction with other pretreatment methods such as acetic acid, acid-catalyzed steam explosion, and hydrochloric acid and sulfuric acid. There was improved enzymatic conversion with 6% NaOH followed by acid treatment as compared to pretreatment with 9 or 15% acetic acid alone. Experiments were done when the cotton wastes were soaked in NaOH for 24 hours prior to H2SO4 pretreatment and achieved nearly theoretical maximum enzymatic conversion (95.7%). NaOH was the most effective at removing the lignin (29% removed), however the susceptibility of the treated material to enzymatic hydrolysis was lower than the untreated control and decreased with increasing lignin removal. Furthermore, ethanol production by simultaneous saccharification and fermentation was similar for the control and the NaOH-treated material and lower for the other bases.

1.4.3.3 Oxidative delignification

The rate and extent of lignocellulose digestion by microorganisms present in the stomachs of ruminants are both greatly enhanced when the lignocellulose is first treated with an alkaline (pH 11.5) solution of hydrogen peroxide (H2O2). The increase in digestibility has been attributed not only to oxidative delignification but also to a possible decrease in cellulose crystallinity. Martel and Gould (1990) concluded from their study on wheat straw and kenaf that alkaline hydrogen peroxide (AHP) treatment loosened the lignocellulosic matrix and caused a more open three-dimensional relationship between lignin, cellulose, and hemicellulose at the molecular level. They also observed that there was an increase in cellulose crystallinity after AHP treatment thus supporting the contention that the main effect of AHP treatment is that it

detaches and makes soluble the lignin, thus increasing the amount of cellulose available for hydrolysis by enzymes, while it does not decrease cellulose crystallinity as previously hypothesized.

Various experiments examined the effect of pretreatment with ammonia, sulfuric acid, and water with and without hydrogen peroxide on the enzymatic digestibility of oak. In both acid and ammonia pretreatments, the addition of hydrogen peroxide improved enzyme hydrolysis, but decomposition of soluble sugars occurred. For water pretreatment, as the concentration of hydrogen peroxide increased from 0 to 0.8, 1.6, and 3.2%, hemicelluloses recovery and delignification increased from 72 to 77, 89, and 92%, and from 24 to 37, 49, and 53%, respectively. For 1.6% H2O2, both hemicellulose recovery and enzymatic digestibility were about 90%, which was comparable to 0.2% sulfuric acid treatment, but with 23% higher delignification. It was also noted that glucose degraded significantly as hydrogen peroxide concentration increased, while hemicellulose was preserved. Optimization of the amount of hydrogen peroxide for the water-H2O2 pretreatment could provide better results than sulfuric acid pretreatment, and the neutralization step required for dilute-acid treatment could be avoided.

1.4.3.4 Ozone

Ozone has been used to degrade lignin and hemicellulose in lignocellulosic materials such as cotton stalks, corn stover, wheat straw, bagasse, and sawdust. Some of the benefits of ozone pretreatment include the fact that no toxic residues are formed since ozone can be easily decomposed to oxygen using a catalytic bed or an increase in temperature thus eliminating the need for extensive downstream processing and ozonation reactions take place at ambient temperature and pressure so energy and investment costs are minimized, pretreated cotton straw with ozone to examine the effect on the composition of the cell wall fractions and on *in vitro*

organic matter digestibility. The most notable effects of ozone treatments were demonstrated by the 50% decrease in both lignin and hemicelluloses. The pH of ozone-treated cotton stalks was considerably more acidic and it was concluded that the low pH values were probably the result of the release of a mixture of formic, acetic, glyoxylic, or other acids from the oxidized lignin. It was later confirmed this by showing the appearance of glycolic, oxalic, malonic, glyoxylic, glyceric, and malic acids in a chromatographic analysis of the aqueous extract of oxidized, extractive free corn stover, due to the generation of carboxylic acids from extensive lignin degradation. It was showed through NMR analysis that lignin degradation by ozone is the result of ring cleavage directly evidenced by the decline in aromatic C from 13.0% in untreated cotton stalks to 7.40% in ozone-treated stalks. The rate of enzymatic hydrolysis increased by a factor of 5 following removal of 60% of the lignin from wheat straw during ozone pretreatment. As the lignin content of poplar sawdust decreased from 29 to 8% after ozonolysis, enzymatic conversion increased from 0 to 57%. The optimal moisture content of 60% was found to provide the highest degree of solubilization during ozone treatment of corn Stover. Results from the same study showed that lignin was the most affected polymer, followed by hemicelluloses and then cellulose.

1.4.4 Biological Pretreatment

Biological pretreatment involves microorganisms such as brown-, white- and soft-rot fungi that are used to degrade lignin and solubilize hemicellulose. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials. Lignin degradation by white-rot fungi, specifically *Phanerochaete chrysosporium*, *Pleurotus ostreatus*,

and Trametes versicolor, is an oxidative process with lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases acting as the key enzymes.

Fungal pretreatment could potentially lower the severity requirements of chemical, temperature, and time resulting in less biomass degradation and lower inhibitory concentrations compared to conventional thermochemcial pretreatment. It was investigated effects of fungal pretreatment and steam explosion pretreatment on enzymatic conversion of cotton wastes or agricultural wastes. They found that fungal pretreatment by *P. chrysosporium* for 28 days followed by steam explosion at 215ºC for 6.5 minutes provided the maximum saccharification. Biodegradation of cotton stalks by the oyster mushroom, *Pleurotus ostreatus* was studied. The study found that during four weeks of solid-state processing, lignin content decreased significantly and *in vitro* rumen digestibility increased 2.2 times from 10% for untreated cotton stalks to 22% for fungal pretreated stalks. Preliminary results on fungal pretreatment of corn stover with *Cyathus stercoreus* showed a three to five-fold increase in enzyme digestibility from 1.2.12.1% to 8.3.35.7% of theoretical (cellulose loadings of 15, 25, 60 FPU/g cellulose). The advantages of biological pretreatment include low energy requirements and mild environmental conditions, while a disadvantage is the long time period required for lignin degradation.

1.5 Fermentation

Ethanol fermentation has become one of the most challenging biotechnological processes of our time. Research aimed at optimizing the production process has focused on four main approaches including physiological, biological, genetic, and engineering. The physiological approach recognizes that the process parameters involved in productivity are affected by

environmental factors including pH and temperature, the chemical composition of the fermentation medium, and the concentration of essential nutrients or inhibitory compounds. The biological approach replaces the more traditional alcohol producing microorganism, yeast, with more efficient and productive species.

The genetic approach aims to improve the metabolic characteristics of the microorganism by attempting to correct known weaknesses or deficiencies such as broadening the range of substrates the organism can use as carbon sources. Finally, the engineering approach aims to increase productivity by using fomenters that operate in continuous mode rather than batch mode. There are three main ethanol fermentation processes that are used: separate hydrolysis and fermentation (SHF) & simultaneous saccharification and fermentation (SSF)

OBJECTIVES

The main objectives of the proposed research work are as follows:

1. **Characterization of cotton gin waste**: Investigation of detailed chemical composition of cotton gin waste like ash content, moisture content, lignin, cellulose, and hemicelluloses.
2. **Pre-treatment of cotton gin waste:** Efforts will be given to develop an efficient and cost effective pre-treatment process for the separation of lignin from cellulose and hemicellulose in cotton gin waste.

Chapter 2

Literature Review

LITERATURE REVIEW

Different regions of the world have excess agricultural or forest waste products with high potentials for conversion into ethanol. For example, eucalyptus is abundant in Portugal pine in Chile and Brazil has surplus sugarcane. Many of these countries are looking at ways to utilize their natural resources for the production of fuel ethanol. The Brazilian government, through the implementation of the National Alcohol Program, has expended considerable amounts of effort to promote cars fuelled by ethanol produced from their sugar cane. Currently, 40% of Brazilian cars operate on 100% ethanol fuel. Even the gasoline-based cars operate on a blend of 22% ethanol with 78% gasoline.

Nikolaus A. Otto, developer of the Otto cycle, is said to have deemed alcohol as the proper fuel for his four-stroke internal combustion engine. In the United States, Henry Ford, the father of automobile, promoted the use of ethanol in the 1920's. The trend continued through the 1930's where more than 2,000 Midwestern service station carried blends of 6-12% ethanol produced from corn. However, the high costs of ethanol production soon became too restrictive and thereby resulted in the end of ethanol usage.

The general makeup of cotton gin waste consists of sticks, leaves, burs, soil particles, other plant materials, mote and cotton lint. Slight differences in the proportions of the components are usually found between varying mechanical harvest methods. The stripper harvesting method generates more waste than the spindle harvesting method.

Many avenues for the disposal or utilization of the wastes have been investigated throughout the years. The idea of recovering energy from cotton gin waste has been around for several decades. However, the initial application was to harness the energy by incineration.

Griffin (1974) determined the fuel value and ash content of cotton gin waste for the purpose of studying the feasibility of disposal by incineration. Although his primary concern was simply the disposal of the waste, he also mentions the possibility of using the heat for seed cotton drying. The study provided a method for estimating the heat value of ginning wastes.

Schacht et.al. (1978) conducted another study to further analyze the physical and chemical composition of cotton gin waste. One of the purposes was to open the possibility for ways other than combustion to utilize energy from cotton gin wastes. The possibilities mentioned are hydrogen and protein production by an enzymatic process and the production of char, condensable gases, and non-condensable gases by pyrolysis.

Parnell et. al. (1991) investigated the possibility of gasifying cotton gin waste using a fluidized bed reactor. Economic consideration of the Biomass Thermochemical Conversion System (BTCS) revealed low net revenue from the gasified products as compared to natural gas and electricity derived from traditional resources. The researchers, however, did find that the char resulting from the BTCS has a potential market as activated carbon in water and wastewater treatment facilities. At $200/ton, cotton gin waste activated carbon is ten times less costly than commercial activated carbon. The low cost of cotton gin waste activated carbon from the BTCS, coupled with the effective nature of activated carbon in meeting the increasingly stringent EPA water quality regulations showed a promising avenue for cotton gin waste utilization.

In order to develop a process for fuel ethanol production from cotton gin waste, a series of studies need to be conducted from the laboratory scale up to the industrial scale. The general issues that need to be addressed are:

1) Whether the composition of the material (i.e., cotton gin waste) is sufficiently high in fermentable sugars,

2) Accessibility of the sugars for fermentation,

3) Maximizing sugar to ethanol conversion by optimization of fermentation parameters.

The composition of the material is of importance in determining if the biomass is suitable for use as a fermentation feedstock. High fermentable sugar content of the material is of course desirable. Agricultural biomass may have higher inorganic compounds collectively termed “ash” which will lower overall yields. The content of lignin, a non carbohydrate polymer closely associated with the sugar fractions is also of concern as it may hinder access to these fermentable constituents.

Carlos A. Cardona, Oscar J.Sanchez, reviewed on current fuel ethanol research and development deals with process engineering trends for improving biotechnological production of ethanol. In their work, the key role that process design plays during the development of cost effective technologies is recognized through the analysis of major trends in process synthesis, modeling, simulation and optimization related to ethanol production. Finally some considerations on current and future research tendencies in fuel ethanol production regarding process design and integration are presented. For current technologies employed at commercial level, the main share in the cost structure corresponds to the feedstock’s (above 60%) followed by the processing expenditures. In general, the use of sucrose-containing materials as cane molasses allows producing ethanol with the lowest costs compared to the starchy materials (mostly grains). Particularly, although the ethanol yield from corn is higher than that from sugar cane, the lower annual yield of corn per cultivated hectare makes it necessary to use larger cropping areas.

Howard et.al, reviewed on finding alternatives uses for natural, renewable resources, especially organic wastes using clean technologies. Lignocellulose biotechnology offers significant opportunities to developing countries for addressing some of the issues highlighted

since most of the technology is based on the utilization of readily available residual plant biomass considered as waste to produce numerous value added products. Lignocelluloses biotechnology forms a capital cost investment perspective is an attractive technology for developing countries.

Kiran L.Kadam, James D.Mc Millan, reviewed on pretreatment technologies and give importance of pretreatment step. They conclude that pretreatment is the first step required to fractionate lignocellulosic materials into its major plant components of lignin, cellulose and hemicellulose. The mechanisms by which pretreatments improve the digestibility of lignocellulose are however not well understood. An important goal of pretreatment is to increase the surface area of lignocellulosic material, making the polysaccharides more susceptible to hydrolysis. Along with an increase in surface area, pretreatment effectiveness and hydrolysis improvement has been correlated with removal of hemicellulose and lignin and the reduction of cellulose cyrstallinity.

J.H.Reith et.al reviewed through successful technology development for coproduction of bioethanol, electricity and heat from agricultural residues and other biomass waste streams, the use of ethanol as a renewable transportation fuel can be greatly increased, leading to a substantial reduction of Co2 emissions.

Brown R. et al, reviewed on biorenewable resources are usually classified as either wastes or dedicated energy crops. Categories of waste materials that qualify as biorenewable resources include agricultural residues, yard waste, municipal solid waste, food processing waste, and manure. Agricultural residues such as corn stover, rice hulls, wheat straw, cotton stalks, and bagasse, are the portion of the crop discarded after harvest. Municipal solid waste (MSW) is waste discarded as garbage, not all of which is suitable as biomass feedstock. In

communities where yard waste is excluded, the important components of MSW are paper (50%), plastics and other fossil fuel derived materials (20%), food wastes (10%), and non-flammable materials including glass and metal (20%).

Sun.et.al reviewed on the combined bonding energies of the intermolecular and intramolecular hydrogen bonds increases the rigidity of cellulose and forms the crystalline structure that makes it highly insoluble and recalcitrant to most organic solvents. The cellulose microfibrils are imbedded in a matrix of noncellulosic polysaccharides, mainly hemicellulose and pectic substances, which complicates hydrolysis of cellulose to glucose even further. The cellulose in lignocellulosic biomass feedstocks provides the main source of glucose used during ethanol fermentation.

Liu and Wyman studied the effects of fluid velocity and contact time on corn Stover pretreatment in a flow through reactor. These studies provide useful information to understand the factors that influence pretreatment and hydrolysis, which is essential for reactor scale up and design the operating costs of pretreatment are highly contingent upon the consumption of steam that is needed to heat the biomass and acid to elevated temperatures. The simplest way to reduce steam consumption is by increasing the dry weight concentration of solids in the reactor.

Moiser.et al., Carried out H_2SO_4 pretreatments in a Batch reactor at 121°C. The treatment times were from 30 to 120 min with acid concentrations ranging from 0 to 2% (w/v) at a solid concentration of 5% (w/v). The results showed that performance of H_2SO_4 pretreatment (hemicellulose recovery and cellulose digestibility) was significantly better than that obtained with H_3PO_4.

Tengborg et al., studied on enzymatic hydrolysis and gives benefits over acid hydrolysis. Enzymatic hydrolysis of cellulose proceeds in several steps to break glycosidic bonds by the use

of cellulase enzymes. Factors effecting hydrolysis of cellulose include type of substrate, cellulase loading, and reaction conditions such as temperature and pH, and end-product inhibitors. A rapid release of glucose is usually observed in the first 24 hours of hydrolysis, with the remaining cellulose hydrolysis taking as long as 2 more days to complete. End product inhibition has been shown to play a significant role in slowing the hydrolysis rate, and glucose, cellobiose, and ethanol have demonstrated inhibitory effects on the activity of both β-glucosidase and cellulose

Chapter 3

Pretreatment methods
Cellulose, Hemicellulose and Lignin estimations

MATERIALS AND METHODS

3.1.1 **Collection, isolation, identification and culturing of white rot fungi**

The white rot fungi *Pleurotus Ostreatus* were collected from Dept. Of Microbiology, Kakatiya University, Warangal. The collected fruiting bodies are preserved by sealing in polythene bags. A small fruiting body is dipped in 0.01% mercuric chloride to remove the surface contamination and washed several times with distilled water to remove the traces of $HgCl_2$ and transferred aseptically on to 3% malt agar slants. Slants were incubated for 5-7 days and initial growth of mycelium was observed on fourth day. All the species produced white coloured mycelium and growth was spread over the medium within two days of incubation. The mycelium collected from the growing edge was transferred on to new malt agar slant and incubated further for 5-7 days. This is repeated 2-3 times to isolate pure cultures.

Table 1: Composition of Malt agar medium

	Composition % (w/v)
Malt Extract	3%
NH_4Cl	0.10%
K_2HPO_4	1.0%
Agar	2.0%
pH	5.6

Then make up the volume to 100 ml.

3.2 Screening of ligninase positive fungi:

The white-rot fungal isolates were screened qualitatively for their ability to produce ligninases. Malt agar plates supplemented with 0.02% guaiacol were used to screen the fungi. Two mm of the each fresh fungal mycelium, precultured on malt agar plates were introduced onto the center of these plates and incubated for 5 days. The fungus containing the enzyme activity developed circular zones of reddish brown colour due to the oxidation of guaiacol and noted as ligninase positive. The cultures which did not produce zones were discarded.

3.3. ESTIMATIONS

3.3.1 Determination of Moisture Content

The moisture content of the samples was determined by **oven drying method**. The sample was weighed with the glass crucible and placed in the air drying oven for 12 h at 105°C and cooled to room temperature in a desiccator and weighed. The process was repeated until a constant weight was achieved and thus making it free of moisture content. The moisture content was then calculated as follows:

$$\textbf{\% moisture content} = (W_1 - W_2 / W) * 100$$

Where:

W= Weight of the initial sample, gm.

W_1= Weight of the sample + container before drying, gm.

W_2= Weight of the sample + container after drying, gm

3.3.2Determination of Ash Content

The ash content of the samples was determined by placing the moisture free biomass in a muffle furnace at 500°C for 3 h and cooled to room temperature in a desiccator and weighed. The process was repeated until a constant weight was achieved. The ash content was then calculated as follows:

$$\% \text{ ash content} = (W_2 - W / W_1) * 100$$

Where:

W = ignited dish weight, gm.

W_1=initial moisture-free sample weight, gm.

W_2=sample weight plus dish weight after removal from furnace, thus weight of ash, gm.

3.3.3 Determination of reducing sugars

- The DNS procedure used was a modified version of LAP-006 from NREL (Adney & Baker, 1996).
- First, the DNS Reagent was prepared by mixing 400mL of distilled water with 10.0g of 3,5-Dinitrosalicylic acid and 20.75 mL of 19.3N (50% w/w) sodium hydroxide.
- Three hundred grams of Rochelle salts (sodium potassium tartrate tetrahydrate) were added and dissolved. Deionised water was added up to 1 L.
- The solution was stored in the dark by wrapping the bottle with aluminium foil.
- The Citrate Buffer was prepared by mixing 210 grams of citric monohydrate with 750 mL of Deionised water.
- The solution was diluted to 1 L and the pH was checked.
- The pH was adjusted to 4.5 by adding sodium hydroxide solution.

- The buffer was then diluted to 0.05M by adding deionised water at 1:19 buffer to water ratio. Glucose standards were prepared for each analysis.
- A stock solution was prepared by dissolving 0.4 grams of glucose to 200mL of citrate buffer and the glucose standards were prepared as follows: 0.5 mL + 0mL buffer = 1.0mg/0.5mL, 400uL + 100 uL buffer = 0.8mg/0.5mL, 300uL + 200 uL buffer = 0.6mg/0.5mL, 200uL +300uL buffer =0.4mg/0.5mL, 100 uL + 400 uL buffer = 0.2 mg/0.5mL.
- Each standard was then diluted 1:2 by adding 0.5 mL of the standard to 1.0mL buffer. Samples were prepared by adding 0.5 mL of sample to 1 mL of buffer.
- Three mL of DNS reagent was added to each sample and standard tube. All tubes were boiled for 5.0 minutes in vigorously boiling water.
- After 5.0 minutes, the tubes were transferred to an ice-cold water bath.
- After cooling, 1.5 mL from each tube was placed in a cuvette and the absorbance was read at 540 nm.

3.3.4. Estimation of Lignin:

Spectrophotometric method:

Principle

The sample is extracted in NaOH solution and the aliquot sample are adjusted to pH 7.0 and 12.3.The amount of lignin is calculated by a difference between A_{245} (pH-7.0) and A_{350} (pH-12.3)

Reagent:

1. Diethyl ether

2. 0.1M Sodium phosphate buffer, pH-7.0

3. 0.1 N and 0.5N NaOH

Method

- Moisten 100mg of oven dried material in a mortar with water.
- Grind with ether until it is free from chlorophyll pigment.
- Centrifuge at 5000 rpm for 5 min and decant the supernatant.
- Wash the sediment with water re-centrifuge and discard the supernatant .Repeat washing twice.
- Add 2ml of NaOH to the residue and extract at 70-80 ^{0}C for 12-16hr.
- Cool, add 0.45ml of 2N HCl and adjust the pH to 7 or 8 with NaOH.
- Make up the volume to 3ml with water and centrifuge at 2000 rpm for 5 ml.collect the supernatant.
- To 0.8ml of extract, add 0.8ml of 0.1M sodium phosphate buffer, pH-7.0.
- To another aliquot of 0.8ml extract, add 0.8ml of 0.1ml NaOH (pH-12.3)
- Measure the absorbance at 245and 350nm.

- Derive the lignin concentration from the difference between A_{245} and A_{350}(ΔE_{350}) on pH-7.0 and 12.3.samples diluted with buffer and NaoH,respectively express the amount of lignin as ΔE_{350}/sample.

3.3.5 .Estimation of Cellulose:

Principle

Cellulose undergoes acetolysis with acetic/nitric reagent to form acetylated cellodextrins which get dissolved and hydrolyses to form glucose units on treatment with 67% H_2SO_4 on dehydration with H_2SO_4,glucose form 5-hydroxymethyl furfural which on reaction with anthrone gives a green coloured product. the colour intensity can be measured at 630 nm.

Reagent**:**

- **A**cetic acid /nitric acid reagent: 150ml of 80% acetic acid mixed with 15ml of conc. nitric acid.
- Anthrone reagent: Dissolve 200mg of anthrone in 100ml conc. H_2SO_4
- 67% H_2SO_4.

Method:

- 0.2 gm of sample and 10ml of acetic/nitric acid was taken in test tub
- keep it in water bath at 100°Cfor 30min.
- cool it. Then centrifuge at high speed for 10-15min.
- Discard the supernatant.

- Wash the residue with distilled water
- Add 10 ml of 67r% H_2SO_4 to the residue.mix it properly.
- Allow to stand for 1 hr and measure the total volume.
- add 5 ml of anthrone reagent and mix well and keep it in the bath for 5 min
- heat the tubes in a boiling water bath for 10 min
- Cool in ice bath for 5 min.
- Measure the colour intensity at 630nm.
- Set the blank with 1ml of distilled water and anthrone reagent.

3.3.6 Estimation of Hemicellulose:

Principle:

Method is based on the formation of furfural from pentoses in acetic acid containing thiourea (an antioxidant) at 75°C and the reaction oh furfural with p-bromoaniline acetate to form a pink coloured product.

Reagent:

- P-bromoaniline reagent:
- H_2SO_4- 3% w/v
- 10N NaOH
- Standard xylose solution (0.1%):

  Dissolve 0.1gm xylose (pre-dried in oven at 105°C for 1hr) in 100ml distilled water.

Method:

- Take 2 gm of sample in conical flask and add 20ml of 3% w/v H_2SO_4 or solid to ratio of 1:10
- autoclave for 1hr (121°C, 15psi)
- cool the sample ,add 60-70ml distilled water and adjust the pH to 7.0-7.5,make the volume to 1000ml
- Take the 0.1,0.2,0.3,of sample is added with 0.9,0.8,0.7ml of distilled water in 1ml test tubes.
- Add 5ml of p-bromoaniline; incubate for 10min.then incubate at room temperature in dark 70 min.
- Measure the absorbance at 540nm.

3.4. Experimental study on delignification of Cotton gin waste

3.4.1. Experimental Design:

The following five factors were selected for optimization of the lignin degradation process:

(1) Temperature

(2) pH:

(3) rpm:

(4) Age of inoculums;

(5) Moisture content.

Each factor was assessed at four levels. The factors and their levels are shown in Table 2

Table2: Experimental design for optimizing lignin degradation

Factors	Levels

		1	2	3	4
A	Temperature (^{o}C)	30	35	40	45
B	pH	4	4.5	5	5.5
C	Rpm	0	100	200	300
D	Age of inoculums (weeks)	1	2	3	4
E	Moisture content (ml/10gm)	10	20	30	40

There are many ways to design a test, but the most frequently used approach is a full factorial experiment. For full factorial experiments, there are 2^{f} possible combinations that must be tested (f=the number of factors each at two levels). FFE use only a portion of the total possible combinations to estimate the effects of main factors and the effects of some of the interactions. Following the Taguchi method, these factors were optimized by orthogonal arrays of 16 experiments.(L16 type). The factors and their levels for each experiment are shown in Table 5.

Table 3: Experimental design for optimizing Lignin degradation: Layout of L16 orthogonal analysis

Exp.	Factors				
	A	B	C	D	E
1	1	1	1	1	1
2	1	2	2	2	2
3	1	3	3	3	3
4	1	4	4	4	4
5	2	1	2	3	4

6	2	2	1	4	3
7	2	3	4	1	2
8	2	4	3	2	1
9	3	1	3	4	2
10	3	2	4	3	1
11	3	3	1	2	4
12	3	4	2	1	3
13	4	1	4	2	3
14	4	2	3	1	4
15	4	3	2	4	1
16	4	4	1	3	2

3.4.2. Biological Pretreatment:

The 10 gm of substrate was then treated twice at 121^{o}C or 15 lbs pressure for 15 min, at room temperature 15 ml of malt agar medium along with two loops full of inoculums was added to substrate and kept the flasks at different temperatures ranging from 30-45^{o}C with a range interval of 5^{o}C. Biological pretreatment up to 40 days with an interval of 4 days were. At optimized Temperature, adjusted the pH at different levels ranges from 4-6 with a range interval of 0.5. Pretreatments protocol was repeated at regular intervals.

For the analytical estimations, 2 gm of reaction substrate was taken and ground by adding 10 ml of phosphate buffer and centrifuged at 5000 rpm for 15 min. The supernatant was used for estimation carbohydrates.

Chapter 4

Results

RESULTS

4.1. Analytical result of cotton gin waste

. Component	Percentage
Reducing Sugar	26.1%
Lignin	22.4%
Cellulose	26.8%
Hemicellulose	32.1%
Ash Content	10.2%
Moisture content	9.9 %

Table 4: Composition of cotton gin waste

4.2 Results of the effect of parameters on Biological Pretreatment

The percentages of final lignin degradation and increase in cellulose, hemicellulose & reducing sugars after 40 days of incubation using *Pleurotus ostreatus* were shown in Table 5.

Table: 5. Analytical results of % lignin removal

Exp.no.	Factors					Lignin removal by *Pleurotus ostreatus* (in %)
	A	B	C	D	E	
1	1	1	1	1	1	36.22
2	1	2	2	2	2	44.57
3	1	3	3	3	3	56.82
4	1	4	4	4	4	49.22

Fig: 5. Percentage Lignin Removal

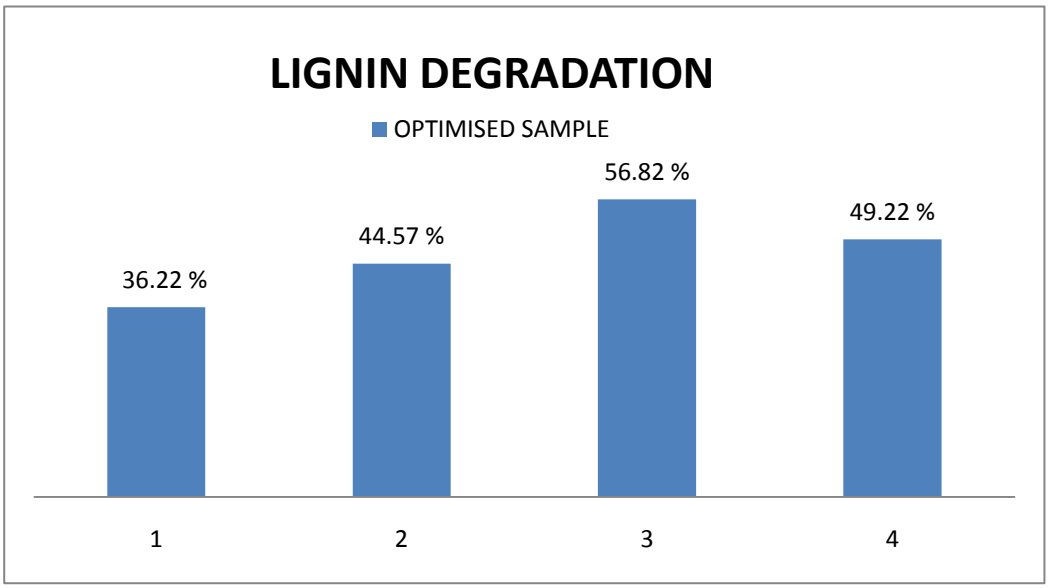


Table :6. Analytical results of % increase in cellulose

Exp.no	*Factors*					*Increase in cellulos by Pleurotus Ostreatus(in %)*
	A	*B*	*C*	*D*	*E*	
1	*1*	*1*	*1*	*1*	*1*	*26.43*
2	*1*	*2*	*2*	*2*	*2*	*32.76*
3	*1*	*3*	*3*	*3*	*3*	*37.94*
4	*1*	*4*	*4*	*4*	*4*	*35.11*

Fig: 6. Percentage Increase in Cellulose

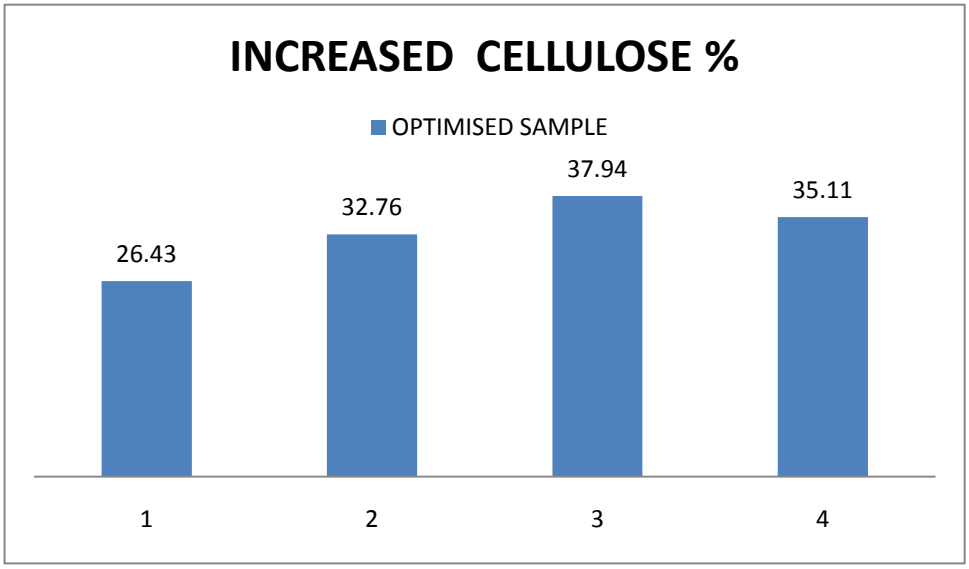


Table: 7. Analytical results on % increase in hemicelluloses

Exp.no	Factors					Increase in hemicelluloses by *Pleurotus Ostreatus*(in%)
	A	B	C	D	E	
1	1	1	1	1	1	20.94
2	1	2	2	2	2	25.76
3	1	3	3	3	3	29.96
4	1	4	4	4	4	28.22

Fig: 7. Percentage Increase in Hemicelluloses

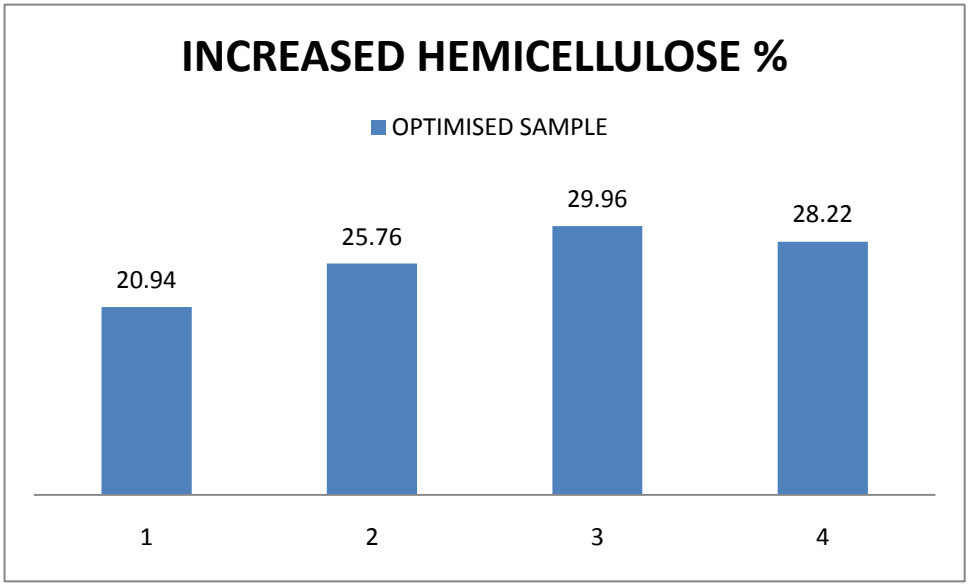
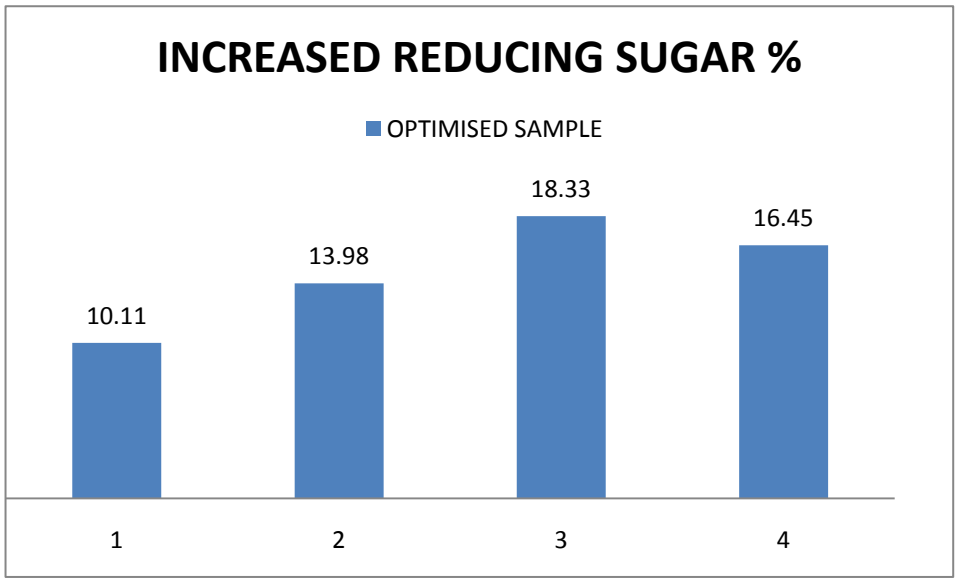


Table: 8 Analytical results on % increase in reducing sugars

Exp.no	Factors					Increase in reducing sugar by *Pleurotus Ostreatus* (in %)
	A	B	C	D	E	
1	1	1	1	1	1	10.11
2	1	2	2	2	2	13.98
3	1	3	3	3	3	18.33
4	1	4	4	4	4	16.45

Fig: 8. Percentage Increase in Reducing Sugars



Chapter 5

Discussion

DISCUSSION

Around 140 Bacediomycetous fungi were screened for their lignolytic activity among which 32 were proved to be useful for biodelignification of agro wastes. These organisms were further screened for their maximum secretion of lignolytic enzymes on production medium consisting of peptone and yeast extract. The higher zone of activity indicates the higher lignin degradation capability and vice versa. Among these organisms, *Pleurotus ostreatus* was chosen for optimizing the conditions for large scale production of the enzymes responsible for biodelignification in the pretreatment of lignocelluloses.

Characterization of substrate (cotton gin waste) has been done using standard methods. The main composition of cotton gin waste is as follows: 22.40% of acid soluble lignin, 26.80 % of cellulose, 32.10 % of Hemicellulose, 10.2 % of ash content & 9.9 % of moisture content.

Optimization experiments have been carried out using *Pleurotus ostreatus* and it is observed that *Pleurotus ostreatus* has shown maximum lignin degradation of 56.82% at optimum conditions of 30^{o}C; 5pH; 200rpm; 3weeks age of inoculum and 30ml/10gm moisture content. The maximum percentage increase in cellulose and hemicelluloses were observed as 37.94%, 29.96% and 23.37% respectively.

Chapter 6

Conclusions

CONCLUSIONS

The microorganisms like *Trametes pubescens, Pleurotus ostreatus & Gamophus clavatus* were found to secrete maximum amounts of lignolytic enzymes and hence selected as the effective organisms for lignin degradation of agrowaste.

Biodelignification experiments (pretreatment) were carried out using *Pleurotus ostreatus* for the degradation of lignin present in the cotton gin waste. The effects of the various parameters on the lignin degradation were investigated and optimum pretreatment conditions were found out. *P. ostreatus* has shown better lignin removal efficiency of 56.82% at optimum conditions of 30^{o}C; 5 pH; 200 rpm; 3 weeks age of inoculum and 30 ml/10 gm moisture content.

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Chapter 7

Rrferences

REFERENCES

1. Wyman CE, Hinman ND (1999). Ethanol. Fundamentals of production from renewable feedstocks and use as transportation fuel. *Appl Biochem. Biotechnol*. 24/25: 735-75.
2. McKendry, P., 2002. Energy production from biomass (part 1): overview of biomass. *Bioresour. Technol*. 83, 37–46.
3. Sun Y, Cheng J (2002). Hydrolysis of lignocellulosic materials for ethanol production: *a review Biores Technol* 83: 1-11.
4. Moiser N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005). Features of promising technologies for pretreatment of lignocellulosic biomass Biores. *Technol*. 96: 673-686.
5. Óscar J. Sánchez and Carlos A. Cardona(2007) Trends in biotechnological production of fuel ethanol from different feedstocks . *Bioresource Technology* 99:5270-5295
6. Lignocellulose Biotechnology: issues of bioconversion and enzyme production ,Howard R.L.etal (2003)
7. Pretreatment of lignocellulosic biomass. In: Himmel, M.E., Baker, J.O., Overend, R.P. (Eds.), Enzymatic Conversion of Biomass for Fuels Production. American Chemical Society, Washington, DC, pp. 292-324. McMillan, J.D. 1994
8. Co products of bio-ethanol,electricity and heat from biomass wastes potential and R&D issues.J.H.reith.et.al (2001)
9. Brown, R. 2003. Biorenewable Resources: Engineering New Products from Agriculture. Iowa State Press: Ames, IA.
10. Varga, E., Szengyel, Z., and Reczey, K. 2002. Chemical pretreatments of corn Stover for enhancing enzymatic digestibility. *Appl. Biochem. Biotechnology*. 98-100, 73-87.

11. Keller, F.A., Hamilton, J.E., and Nguyen, Q.A. 2003. Microbial pretreatment of biomass. *Appl. Biochem. Biotechnology*. 105-108, 27-42.
12. Tengborg, C., Galbe, M., and Zacchi, G. 2001. Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood. *Enzyme Microbial Technol*. 28, 835-844.
13. Nguyen QA, Tucker MP, Keller FA, Beaty DA, Connors KM, Eddy FP (1999) Appl Biochem Biotechnol 133:77.
14. Sehoon K, Holtzapple MT (2005) Bioresour Technol 96:1994.
15. Hsu TA (1996) In: Wyman CE (ed) Handbook on bioethanol production and utilization, Taylor & Francis, Washington, p 179.
16. Sun Y, Cheng J (2002) Bioresour Technol 83:1.
17. Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY (2005) Bioresour Technol 96:1959.
18. Mosier N, Hendrickson R, Ho N, Sedlak M, Ladisch MR (2005) Bioresour Technol 96:1986
19. Varga E, Reczey K, Zacchi G (2004) Appl Biochem Biotechnol 509:113
20. Öhgren K, Rudolf A, Galbe M, Zacchi G (2006) Biomass Bioenergy 30:863.NREL laboratory analytical procedures.
21. Albertsson A.C., Karlsson S., 1995, Degradable polymers for the future, Acta Polymer, 46, 114±123.
22. Argyropoulos D.S., Menachem S.B., 1997, Lignin. In: Eriksson, K.-E.L. (Ed.), Advances in Biochemical Engineering Biotechnology, vol. 57, Springer, Germany, pp. 127±158.
23. Ball A.S., Betts W.B., McCarthy A.J., 1989. Degradation of lignin related compounds by actinomycetes, Appl. Environ. Microbiol. 55, 1642±1644.

24. Brown A., 1985, Review of lignin in biomass, J. Appl. Biochem. 7, 371±387.

25. Buswell J.A., Odier E., 1987, Lignin biodegradation, CRC Crit. Rev. Biotechnol. 6, 1±60.

26. Moiser N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M, 2005. Features of Promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol* 96: 673-686

27. the Degree of Master of Engineering, Cork Institute of Technology, Bishops town, Cork, Ireland; The Columbia encyclopedia. 6th Ed. New York: Columbia University Press;

28. Kadam, K.L., Camobreco, V., Glazebrook, B., Forrest, L., Jacobson, W., Simerith, D., Blackburn, W., Nehoda, K., 1999. Environmental life cycle implications of fuel oxygenate production from California biomass. NREL/TP-580–25688: Golden, CO.

29. Brown, R. 2003. Biorenewable Resources: Engineering New Products from Agriculture. Iowa State Press: Ames, IA.

30. McKendry, P. 2002. Energy production from biomass (part 1): overview of biomass. Bioresource Technol. 83, 37-46.

31. Ramamurthi, R. and Bali, G. 2000. Bioenergy. In: Ramammurthi, R. Kastury, S., Smith, W. (Eds.), Bioenergy: Vision for the New Millennium: Science Publishers Inc, Enfield, NH, pp. 6.

32. Ford Motor Company. 2004. Ethanol Vehicles... Accessed 13 October, 2004.

33. Carpita, N. and McCann, M. 2000. The cell wall. In: Buchanan, B.B., Gruissem, W., and Jones, R.L. (Eds.), Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp. 61.

34. Fengel, D. and Wegener, G. 1984. *Wood: Chemistry, Ultra structure, Reactions.* Walter de Gruyter: Berli

35. Sun, Y. 2002. Enzymatic Hydrolysis of Rye Straw and Bermuda grass for Ethanol Production, Ph.D. thesis: NC State University, Raleigh, NC.

36. HUT. 2004. Module II: Basics of Wood Chemistry. Helsinki University of Technology.

37. Klass, D.L. 1998. Biomass for Renewable Energy, Fuels, and Chemicals. Academic Press: New York.

38. Brigham, J.S., Adney, W.S., and Himmel, M.E. 1996. Hemicelluloses: diversity and applications. In: Wyman, C.E (Ed). Handbook on Bioethanol: Production and Utilization. Taylor & Francis, Washington, DC, pp. 119-141.

39. Fan, L.T., Gharpuray, M.M., and Lee, Y.-H. 1987. Cellulose hydrolysis. Biotechnology Monographs, Springer-Verlag: New York.

40. Lignin Institute. 2002. Lignin and its properties. Available at: http://www.lignin.info/01augdialogue.html. Accessed 13 October, 2004.

41. McMillan, J.D. 1994. Pretreatment of lignocellulosic biomass. In: Himmel, M.E., Baker, J.O., Overend, R.P. (Eds.), Enzymatic Conversion of Biomass for Fuels Production. American Chemical Society, Washington, DC, pp. 292-324.

42. Cullis, I.F., Saddler, J.N., and Mansfield, S.D. 2004. Effect of initial moisture content and chip size on the bioconversion efficiency of softwood lignocellulosic. Biotechnology. Boeing. 85, 413-421.

43. Vlasenko, E.Yu. Ding, H., Labavitch, J.M., and Shoemaker, S.P. 1997. Enzymatic hydrolysis of pretreated rice straw. *Bioresource Technol*. 59, 109-119

44. Holtzapple, M.T., Lundeen, J.E., and Sturgis, R, 1992. Pretreatment of lignocellulosic municipal solid waste by ammonia fiber explosion (AFEX). *Appl. Biochem. Biotechnology.* 34/35, 5-21.

45. Moubsher.et.al, Analysis of plant cell wall materials 135-151.

46. Ingram, L.O. and Doran, J. 1995. Conversion of cellulosic materials to ethanol. *FEMS Microbiology Reviews* 16, 235-241.

47. Schell, D., Nguyen, Q., Tucker, M., and Boynton, B. 1998. Pretreatment of softwood by acid-catalyzed steam explosion followed by alkali extraction. *Appl. Biochem. Biotechnology.* 70-72, 17-24.

48. Malherbe, S. and Cloete, T.E. 2002. Lignocelluloses biodegradation: fundamentals and applications. *Rev. Environ. Sci. Biotechnology*. 1, 105-114.

49. Keller, F.A., Hamilton, J.E., and Nguyen, Q.A. 2003. Microbial pretreatment of biomass. *Appl. Biochem. Biotechnology*. 105-108, 27-42.

50. Sawada, T., Yoshitoshi, N. and Kobayshi, F. 1995. Effects of fungal pretreatment and steam explosion pretreatment on enzymatic saccharification of biomass. *Biotechnol. Bioeng*. 48, 719-724.

51. Hadar, Y., Kerem, Z., Gorodecki, B., and Ardon, O. 1992. Utilization of lignocellulosic waste by the edible mushroom *Pleurotus*. In: Bat-Sheva de Rothschild Symp. Use of

Natural and Genetically Engineered Microorganisms to Combat Pollution, Kiryat Anavim, Isreal.

52. Philippidis, et.al, 1996. Cellulose bioconversion technology. In: Wyman, C.E (Ed).*Handbook on Bioethanol: Production and Utilization*. Taylor & Francis, Washington, DC, pp. 253-285.

53. Xiao, Z., Zhang, X., Gregg, D.J., and Saddler, J.N. 2004. Effects of sugar inhibition on cellulases and β-glycosidase during enzymatic hydrolysis of softwood substrates. Appl. Biochem. Biotechnol. 113-116, 1115-1126.

54. Moiser N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M, 2005. Features of Promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96: 673- 686

55. Couto, S., Rodriguez, R., Gallego, P., & Sanroman, A, 2003. Biodegradation of grape cluster stems and ligninolytic enzyme production by *pleurotus ostreatus* during semi-solid-state cultivation. *Acta Biotechnology, 23*(1), 65-74.

56. Hahn-Hagerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G,2006. Bio-ethanol the fuel of tomorrow from the residues of today. Trends Biotechnology; 24:549–56.

57. Mabee WE, Saddler JN, Nielsen C, Nielsen LH, Jensen ES, 2006. Renewable-based fuels for transport. In: Renewable energy for power and transport. p. 47–50.

58. Lee YJ, 2005 Oxidation of sugarcane Bagasse using a combination of hypochlorite and peroxide. Master's Thesis, Department of Food Science, Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College.

59. Hamelinck CN, van Hooijdonk G, Faaij APC, 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass Bioenergy 28:384–410.

60. Wyman CE, 1999. Biomass ethanol: technical progress, opportunities, and commercial challenges. Annul Rev Energy Environ; 24:189–226.

61. Badger PC, 2002. Ethanol from cellulose: a general review. Trends in new crops and new uses. Alexandria, VA: ASHS Press. p. 17–21.

62. Demirbas A, 2004. Ethanol from cellulosic biomass resources. Int J Green Ener; 1:79–87.

63. Chandel AK, Es C, Rudravaram R, Narasu ML, Rao LV, Ravindra P,2007. Economics and environmental impact of bioethanol production technologies: an appraisal. Biotechnol Molec Biol Rev; 2:14–32.

64. Demirbas A, 2005. Bioethanol from cellulosic materials: a renewable motor fuel from biomass. Energy Sources; 27:327–37.

65. Jeffries TW, Jin YS, 2000. Ethanol and thermo tolerance in the bioconversion of xylose by yeasts. Adv Appl Microbial; 47:221–68.

66. Pan X, Gilkes N, Saddler JN, 2006. Effect of acetyl groups on enzymatic hydrolysis of cellulosic substrates. Holzforschung; 60:398–401.

67. Pan X, Arato C, Gilkes N, Gregg D, Mabee W, and Pye Kl, et al, 2005. Biorefining of softwoods using ethanol organosolv pulping: Preliminary evaluation of process streams for manufacture of fuel grade ethanol and co-products. Biotechnol Bioeng; 90:473–81.

68. Lu Y, Yang B, Gregg D, Mansfield S, Saddler J, 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Appl Biochem Biotechnol; 98–100:641–54.

69. Mais U, Esteghlalian AR, Saddler JN, Mansfield SD, 2002. Enhancing the enzymatic hydrolysis of cellulosic materials using simultaneous ball milling. Appl Biochem Biotechnol; 98–100:815–32.

70. Nutt A, 2006. Hydrolytic and oxidative mechanisms involved in cellulose degradation. Acta Universitatis Upsaliensis, Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 185, Upsala,