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PRETREATMENT OF LIGNOCELLULOSIC BIOMASS OF *PINUS ROXBURGHII* AND *PINUS WALLICHIANA* FOR BIOFUEL PRODUCTION

Project report submitted in the partial fulfillment for

the requirement of the degree of

Bachelor of Technology

in

Biotechnology

by

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MAY 2013

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CERTIFICATE

This is to certify that the work titled "**Pretreatment of lignocellulosic biomass of *Pinus roxbhurgii* and *Pinus wallichiana* for biofuel production**" has been submitted by **Anant yadav & Abhilash Kumar Tripathi** in partial fulfillment for the award of degree of **B. Tech Biotechnology** from Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or Institute for the award of this or any other degree or diploma.

Signature of Supervisor:

Name of Supervisor: Dr. Sudhir Kumar

Date:- 25/5/13

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Date:- 25/05/2013

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ABSTRACT

The Pines belong to the category of coniferous, evergreen and resinous trees. These trees are abundant in Himachal Pradesh and in the Himalayan belts of India. However, the accumulation of Pine needles throughout the year possesses a great threat to the environment. Accumulation of Pine needles increases the risk of forest fire and may cause infertility of soil due to the release of tannins (Selvakumar et al. 2007) [1]. Two pine trees were selected for this study: *Pinus roxburghii* and *Pinus wallichiana*. This study was basically conducted with a motive to use Pine needles in an useful manner which would be environment friendly. The prime objective of the study was to find out optimal pretreatment methods that would generate sufficient quantity of reducing sugars that could be subsequently fermented to ethanol. The present study also focused on the production of charcoal briquettes from fallen dried pine needles that could be used as biofuel. Determination of cellulose, hemicellulose and lignin content in needles of both the trees were carried out in the present study. Various methods such as (water steam explosion, acidic steam explosion, ammonia steam explosion) were carried out. The sugar content in the supernatant of acid catalyzed steam explosion was found out to be the greatest in both the trees taken for study. Further comparison for reducing sugar was done using wood samples of both the trees. The reducing sugar content was found to be low when compared to the needles. The pretreated pine needles were also subjected to enzymatic hydrolysis using the enzyme cellulase from *Trichoderma reesei*.

The distinguishing part of this study was the utilization of starch in fresh pine needles for the production of reducing sugars that could also be used to produce ethanol.

1. Introduction

Himachal Pradesh is a forest rich state (forest area 37,033 km^2) and generates large biomass (annual production 163 x 10^9 tons) and over half of these is in form of lignocellulose [2]. Pine trees are abundant in the mid and high altitudes of Himachal Pradesh.

All the Himalayan states in India contain pine forest. 8950 sq km of area in Himachal Pradesh comprises of pine forest [3] .Uttrakhand has around 3.43 lakh hectares [4] and Manipur has about 2442.77 sq km of pine forest [5]. So, we get pine needles in high quantity to be used as a feedstock for bio-ethanol production.

Pine trees are lingo-cellulosic biomass and biodegradation of these lingo-cellulosic biomass to fermentable sugars is a key factor for the viability of bio-fuel industries. Pine needles can serve as an abundant and inexpensive source of cellulose and hemi-cellulose that could be hydrolyzed into sugars. Pine needles being rich in cellulose and to some extent hemicelluloses can serve as a substrate for biodegradation and for the production of economical products like bio-fuel and at the same time playing an important role in safe disposable of lingo-cellulosic biomass that will also help to solve the burning problem of global warming. Pretreatment is required to alter and remove the recalcitrant layer of lignin which in turn exposes cellulose and hemi-cellulose to enzymatic hydrolysis by cellulases and xylanases. Enzymatic hydrolysis in turn produces reducing sugars that could be fermented to ethanol. For better degradation, lignocelluloses must be deconstructed into fermentable sugars by various combinations of physical, physicochemical and biological processing steps [6]. Pine needles if not utilized can result in forest fires and may even result in premature parturition in cattles during later stages of pregnancy [7]. Thus Pine needles may result in air pollution and can have devastating effects on the wildlife as well. This study also provides us a potential lignocellulose that can be used to produce bioethanol. Over the few years India has seen an exponential increase in the cost of petrol and petroleum products. Utilization of lignocellulosic biomass such as Pine needles for bioethanol production can provide us an alternative to petrol and can help stabilize the economy of our country.

2. Review of literature

2.1 Composition and structure of lignocellulosic biomass

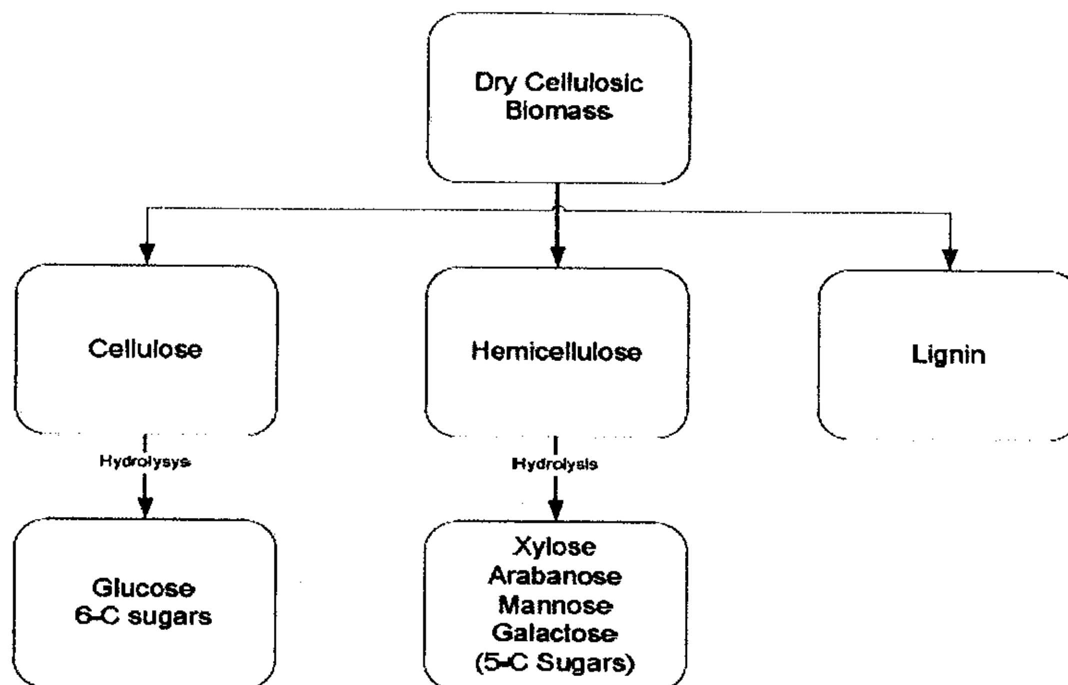
Lignocellulose is the primary building block of plant cell walls. The composition of lignocellulosic biomass is shown in Figure 1. Plant biomass is mainly composed of cellulose, hemicellulose, and lignin, along with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash.[8]

Cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure. This linear polymer consists of D-glucose subunits linked to each other by beta (1,4)-glycosidic bonds. Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains. The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which causes the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the microfibrils. Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes that breaks the beta (1, 4) - glycosidic linkages. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises a major proportion of cellulose, whereas a small percentage of unorganized cellulose chains forms amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form.[9]

The main feature that differentiates hemicellulose from cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars. These monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (e.g., 4-*o*methylglucuronic, D-glucuronic, and D-galactouronic acids). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by beta (1,4)-glycosidic bonds and occasionally beta (1,3)-glycosidic bonds[10]. Also, hemicelluloses can have some degree of acetylation, for example, in heteroxylan. In contrast to cellulose, the polymers present in hemicelluloses are easily hydrolyzable. These polymers do not aggregate, even when they co-crystallize with cellulose chains.

Lignin is a complex, large molecular structure containing cross-linked polymers of phenolic monomers. It is present in the primary cell wall, imparting structural support, impermeability, and resistance against microbial attack [11]. Three phenyl propionic alcohols exist as monomers of lignin: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (*p*-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds link these phenolic monomers together.

Fig 1. Components of lignocellulosic biomass.



2.2 Overview of the Conversion of Biomass to Fuel

Action of microorganisms and enzymes on biological sources can lead to the production of mostly ethanol and, less commonly, propanol and butanol. These agents carry out the fermentation of sugar, starch, hemicellulose, or cellulose, with cellulose fermentation being the most difficult.

A schematic flow diagram for the conversion of biomass to fuel is shown in Figure 2. The conversion includes the hydrolysis of various components in the lignocellulosic materials to

fermentable reducing sugars and the fermentation of the sugars to fuels such as ethanol and butanol. The pretreatment step is mainly required for efficient hydrolysis of cellulose to its constituent sugars. The hydrolysis is usually catalyzed by acids or cellulase enzymes, and the fermentation is carried out by yeasts or bacteria. The factors affecting the hydrolysis of cellulose include porosity (accessible surface area) of the biomass materials, cellulose fiber crystallinity, and content of both lignin and hemicellulose. The presence of lignin and hemicellulose makes the accessibility of cellulase enzymes and acids to cellulose more difficult, thus reducing the efficiency of the hydrolysis process. Pretreatment is required to alter the size and structure of the biomass, as well as its chemical composition, so that the hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved rapidly and with greater yields. The hydrolysis process can be significantly improved by removal of lignin and hemicellulose, reduction of cellulose crystallinity, an increase of porosity through pretreatment processes.[12]

In the hydrolysis process, the sugars are released by breaking down the carbohydrate chains, before they are fermented for alcohol production. The cellulose hydrolysis processes include

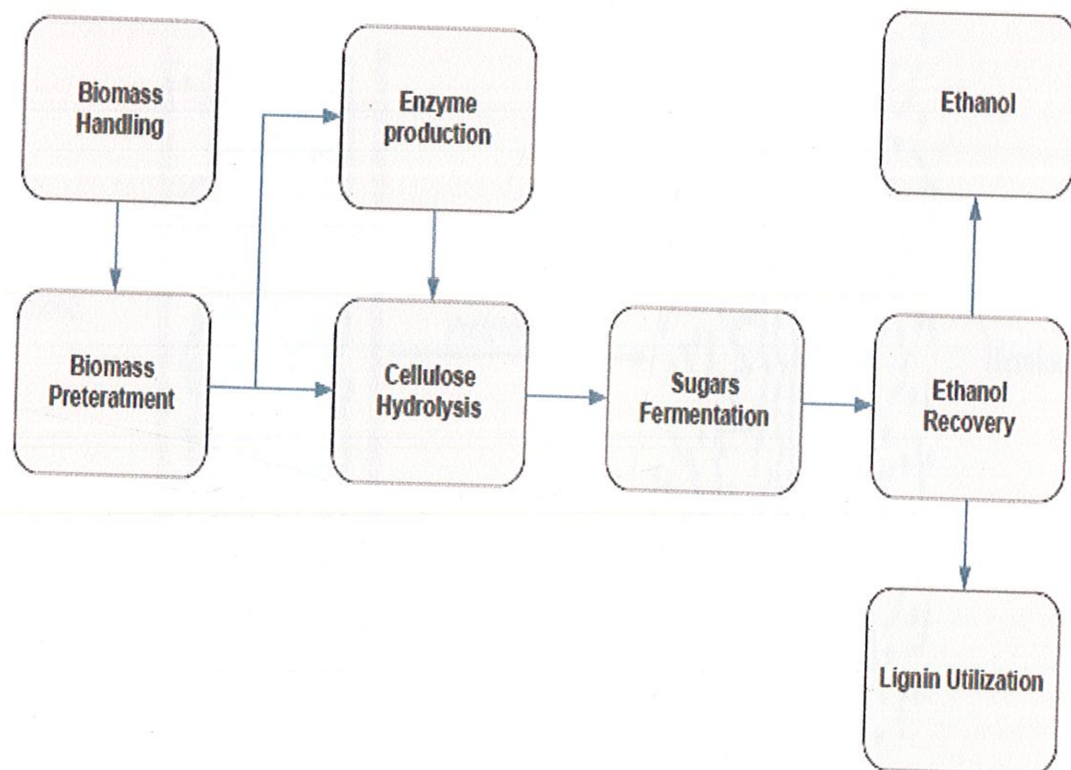
(1) Acid hydrolysis and
(2) Enzymatic hydrolysis.

The decrystallized cellulosic mixture of acid and sugars reacts in the presence of water to release individual sugar molecules. The dilute-acid process is a harsh process that leads to the formation of toxic degradation products that can interfere with fermentation. Cellulose chains can also be broken down into individual glucose sugar molecules by enzymes known as cellulase. Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the hydrolysis of cellulose. Lignocellulosic materials can similarly be enzymatically hydrolyzed under relatively mild conditions (50 °C and pH 4.8), enabling effective cellulose breakdown without the formation of byproducts that would otherwise inhibit enzyme activity.
The six-carbon sugars, or hexoses, glucose, galactose, and mannose are readily fermented to ethanol by many naturally occurring organisms[13] Baker's yeast, or *Saccharomyces cerevisiae*, has been traditionally used in the brewing industry to produce ethanol from hexoses. Because of the complex nature of the carbohydrates present in lignocellulosic biomasses, fivecarbon sugars such as xylose and arabinose, derived from the hemicellulose portion of the lignocellulose, are

also present in the hydrolysate. Microbes such as *Zymomonas mobilis* can be used to ferment pentoses to produce ethanol. The pentosan fraction of hemicelluloses cannot be fermented by hexose fermenting microbes such as *Saccharomyces cerevisae.*

The recovery of fuels from the fermentation broth is achieved by distillation or a combination of distillation and adsorption. The other components, including residual lignin, unreacted cellulose and hemicellulose, and enzymes, accumulate at the bottom of the distillation column.

Fig 2 Flow diagram of bioethanol production.



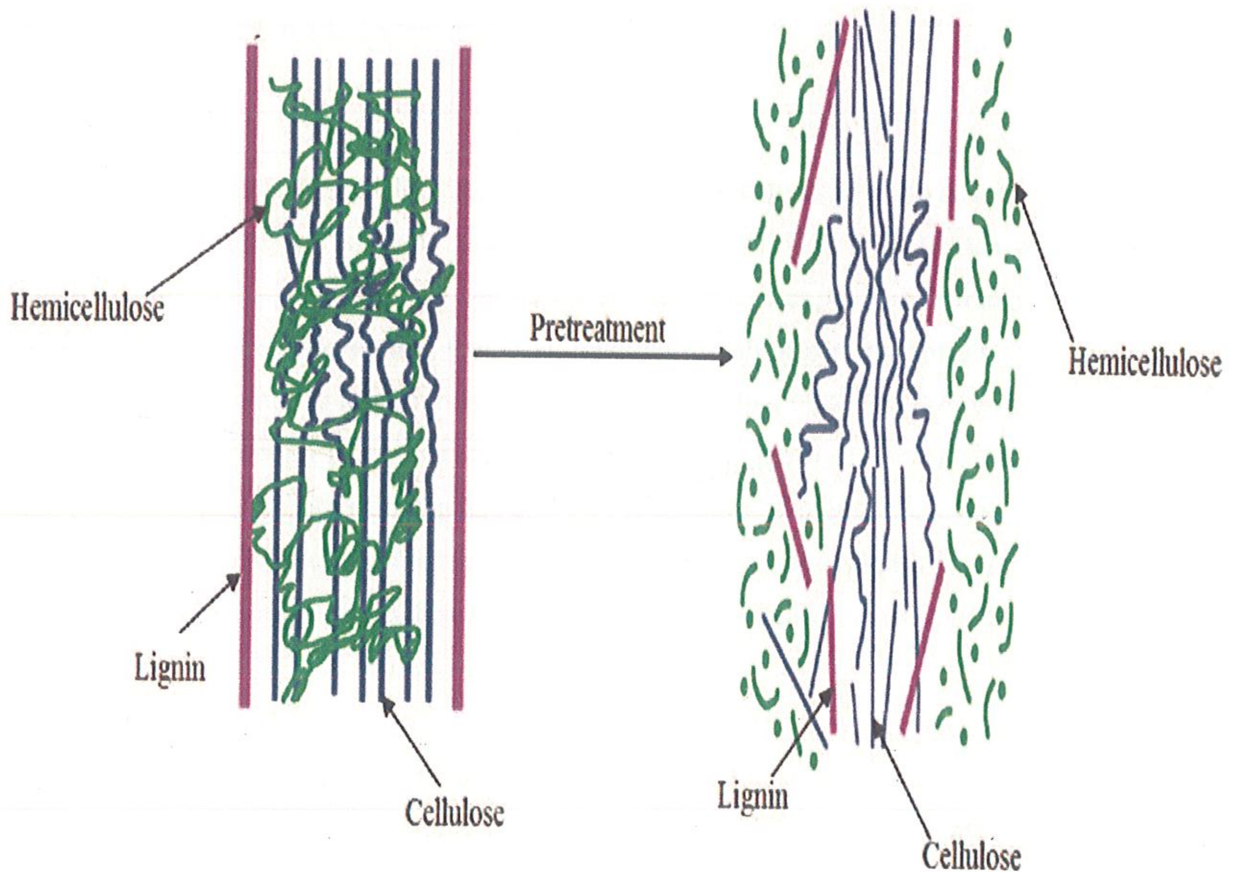
2.3 Pretreatment of lignocellulosic biomass

The goal of the pretreatment process is to remove lignin and hemicellulose, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic materials. The role of pretreatment in altering the regular structure of lignocellulose is shown in fig 3. Pretreatment must meet the following requirements: (1) improve the 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 that are inhibitory to the subsequent hydrolysis and fermentation processes, and (4) be cost-effective.

Pretreatment methods can be roughly divided into different categories: physical (milling and grinding), physicochemical (steam pretreatment/autohydrolysis, hydrothermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), biological, electrical, or a combination of these.

Fig 3. Schematic of the role of pretreatment in the conversion of biomass to biofuel.



2.3.1 Physical Pretreatment.

A) Mechanical .

Mechanical pretreatment of lignocellulosic materials through a combination of chipping, grinding, and/or milling can be applied to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding. Vibratory ball milling was found to be more effective than ordinary ball milling in reducing cellulose crystallinity of spruce and aspen chips and in improving their digestibility [14]. Irradiation of cellulose by γ-rays, which leads to cleavage of beta 1,4-glycosidic bonds and gives a larger surface area and a lower crystallinity, has also been tested[15]. This method is far too expensive, however, to be used in a full-scale process.

B)Pyrolysis

Pyrolysis has also been used for thepretreatment of lignocellulosic materials. Cellulose rapidly decomposes to gaseous products and residual char when biomass is treated at temperatures greater than 300 °C.[16],[17] At lower temperatures, the decomposition is much slower, and th products formed are less volatile. Fan et al. reported that mild acid hydrolysis (1 N H_2SO_4, 97 °C, 2.5 h) of the products from pyrolysis pretreatment resulted in 80-85% conversion of cellulose to reducing sugars with more than 50% glucose.[18] The pyrolysis process is enhanced when carried out in the presence of oxygen.[17] Zwart et al. reported production of transportation fuels from biomass via a so-called biomass-to-liquids (BtL) route, in which biomass is converted to syngas from which high quality Fischer-Tropsch (FT) fuels are synthesized.[19]

2.3.2 Physico-chemical pretreatment.

Pretreatments that combine both chemical and physical processes are referred to as physicochemical processes.

A) Steam explosion (autohydrolysis)

Among the physico-chemical processes, steaming with or without explosion (autohydrolysis) has received substantial attention in pretreatment for both ethanol and biogas production. The pretreatment removes most of the hemicellulose, thus improving the enzymatic digestion. In steam explosion, the pressure is suddenly reduced and makes the materials undergo an explosive decompression. High pressure and consequently high temperature, typically between 121 and 260 °C, for a few seconds (e.g. 30 s) to several minutes (e.g. 20 min), are used in steam explosion [20]. Its energy cost is relatively moderate, and it satisfies all the requirements of the pretreatment process. Increase in temperature up to a certain level can effectively release hemicellulosic sugars. However, the sugars loss steadily increases by further increasing the temperature, resulting in a decrease in total sugar recovery.

Ruiz *et al.* [21] studied steam explosion for pretreatment of sunflower stalks before enzymatic hydrolysis at a temperature in the range of 180–230 °C. The highest glucose yield was obtained in steam-pretreated sunflower stalks at 220 °C, while the highest hemicellulose recovery was obtained at 210 °C pre-treatment temperature.

B) Ammonia fiber explosion (AFEX)

AFEX is one of the alkaline physico-chemical pretreatment processes. Here the biomass is exposed to liquid ammonia at relatively high temperature (e.g. 90-100 °C) for a period of e.g. 30 min, followed by immediate reduction of pressure. The effective parameters in the AFEX process are ammonia loading, temperature, water loading, blowdown pressure, time, and number of treatments. The AFEX process produces only a pretreated solid material, while some other pretreatments such as steam explosion produce slurry that can be separated in a solid and liquid fractions [22]. The AFEX process can either modify or effectively reduce the lignin fraction of the lignocellulosic materials, while the hemicellulose and cellulose fractions may remain intact. At optimum conditions, AFEX can significantly improve the enzymatic hydrolysis. One of the major advantages of AFEX pretreatment is no formation of some types of inhibitory by-products, which are produced during the other pretreatment methods, such as furans in dilute-acid and steam explosion pretreatment. However, part of phenolic fragments of lignin and other cell wall extractives may remain on the cellulosic surface. Therefore, washing with water might be necessary to remove part of these inhibitory components, although increasing the amount of

wastewater from the process [23]. However, there are some disadvantages in using the AFEX process compared to some other processes. AFEX is more effective on the biomass that contains less lignin, and the AFEX pretreatment does not significantly solubilize hemicellulose compared to other pretreatment processes such as dilute-acid pretreatment. Furthermore, ammonia must be recycled after the pretreatment to reduce the cost and protect the environment [24].

2.3.3. Chemical pretreatment

A) Alkaline hydrolysis

Alkali pretreatment refers to the application of alkaline solutions such as NaOH, Ca(OH)2 (lime) or ammonia to remove lignin and a part of the hemicellulose, and efficiently increase the accessibility of enzyme to the cellulose. The alkali pretreatment can result in a sharp increase in saccharification,. Pretreatment can be performed at low temperatures but with a relatively long time and high concentration of the base. However, alkaline pretreatment was shown to be more effective on agricultural residues than on wood materials.

Zhao *et al.* [25] reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose compared with H2SO4 pretreatment. Compared with acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers. The alkaline pretreatment was also used as a pretreatment method in biogas production. A pretreatment with bases such as Ca(OH)2 could be a solution, when high loads of e.g. lipids and phenolic compounds are subjected to the digestion.

B) Acid hydrolysis

Treatment of lignocellulosic materials with acid at a high temperature can efficiently improve the enzymatic hydrolysis. Sulfuric acid is the most applied acid, while other acids such as HCl and nitric acid were also reported. The acid pretreatment can operate either under a high temperature

and low acid concentration (dilute-acid pretreatment) or under a low temperature and high acid concentration (concentrated-acid pretreatment). The lower operating temperature in concentrated-acid pretreatment (e.g. 40 °C) is a clear advantage compared to dilute-acid processes. However, high acid concentration (e.g. 30-70%) in the concentrated-acid process makes it extremely corrosive and dangerous. Therefore, this process requires either specialized non-metallic constructions or expensive alloys. On the other hand, the neutralization process produces large amounts of gypsum. Dilute-acid hydrolysis is probably the most commonly applied method among the chemical pretreatment methods. It can be used either as a pretreatment of lignocellulose for enzymatic hydrolysis, or as the actual method of hydrolyzing to fermentable sugars. At an elevated temperature (e.g. 140-190 °C) and low concentration of acid (e.g. 0.1- 1% sulfuric acid), the dilute-acid treatment can achieve high reaction rates and significantly improve cellulose hydrolysis. Almost 100% hemicellulose removal is possible by dilute-acid pretreatment. The pretreatment is not effective in dissolving lignin, but it can disrupt lignin and increases the cellulose's susceptibility to enzymatic hydrolysis.

Emmel *et al.* [26] pretreated *Eucalyptus grandis* impregnated with 0.087 and 0.175% (w/w) H2SO4 at 200–210°C for 2–5 min. The best conditions for hemicelluloses recovery were obtained at 210°C for 2 min, while a lower pretreatment temperature of 200°C was enough to obtain the highest yield of cellulose conversion (90%) by enzymatic hydrolysis. The optimum conditions for the highest hemicellulosic sugars recovery do not necessarily mean the most effective conditions for enzymatic hydrolysis.

The major drawback of some pretreatment methods, particularly at low pH is the formation of different types of inhibitors such as carboxylic acids, furans and phenolic compounds. These chemicals may not affect the enzymatic hydrolysis, but they usually inhibit the microbial growth and fermentation, which results in less yield and productivity of ethanol or biogas. Therefore, the pretreatments at low pH should be selected properly in order to avoid or at least reduce the formation of these inhibitors.

2.3.4. Biological pretreatment

Microorganisms can also be used to treat the lignocelluloses and enhance enzymatic hydrolysis. The applied microorganisms usually degrade lignin and hemicellulose but very little part of cellulose, since cellulose is more resistance than the other parts of lignocelluloses to the biological attack. Several fungi, e.g. brown-, white- and soft-rot fungi, have been used for this purpose. White-rot fungi are among the most effective microorganisms for biological pretreatment of lignocelluloses [27].

Taniguchi *et al.* [28] evaluated biological pretreatment of rice straw using four white-rot fungi (*Phanerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora,* and *Pleurotus ostreatus*) on the basis of quantitative and structural changes in the components of the pretreated rice straw as well as susceptibility to enzymatic hydrolysis. Pretreatment with *P. ostreatus* resulted in selective degradation of the lignin rather than the holocellulose component, and increased the susceptibility of rice straw to enzymatic hydrolysis. Some bacteria can also be used for biological pretreatment of lignocellulosic materials.

2.3.5. Ankom protocol fiber analysis

The Ankom Fiber Analyzer utilizes a series of extractions to determine the fiber content of a plant sample. Each of the extractions should be done in the order of NDF (Neutral Detergent Fiber), ADF (Acid Detergent Fiber), and then ADL (Acid Determined Lignin). The NDF solution is just soapy water. During NDF, the fraction that is washed off contains soluble cell contents like carbohydrates, lipids, pectin, starch, soluble proteins and non-protein nitrogen. The fraction that is left in the bag contains hemicellulose, proteins bound to the cell walls, cellulose, lignin, and recalcitrant materials. ADF solution is a 1.00 Normal sulfuric acid and detergent solution. During ADF, hemicellulose and bound proteins are washed off. The fraction left behind contains cellulose, lignin, and recalcitrant materials. The ADL solution is ultra nasty 72% sulfuric acid. During ADL, cellulose is washed off leaving only lignin and recalcitrant materials [29].

2.4 DNSA method for reducing sugar estimation

This method was first mentioned by Summer & Sisler (1944) and modified by Miller (1959), with this technique there is a possibility to dosage reducing sugar as total sugar.

The concentration of the colored complex can be determined with the spectrophotometer at Absorbance 540.The sugar concentration of unknown sample can then be read off a calibration curve(standard curve) created using known sugar concentrations. The dilutions of a solution of known concentration are used to determine the concentration of unknown. Being familiar with the background information about reducing sugars and various methods used to identify them, Biotechnology students were provided with a Fructose sample solution and were required to find its concentration [30]

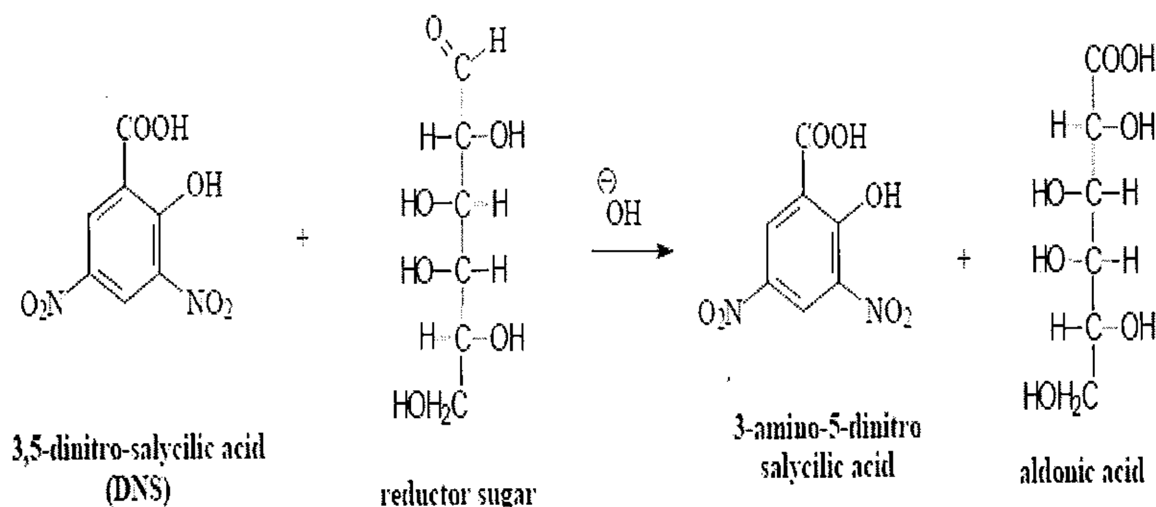
Principle

The sugar act as a chemical reductor due free aldehyde group or ketone group presence in its molecule. In an alkaline medium, the reducing sugars are able to reduce the 3-5- dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, wherever, the aldehyde group is oxidized to aldonic acid (Figure 2). The 3-amino-5-nitrosalicylic acid is a orange color product, and the intensity of the color depends on the concentration of the reducing sugar. The sodium hydroxide provides the glucose reaction with 3-5-dinitrosalicylic acid by medium alkalinization.

Besides to 3-5-dinitrosalicylic acid, it is also used in this method the Rochelles salt (Potassium sodium tartrate), phenol, sodium bisulfite, and sodium hydroxide. The phenol optimize the quantity of the colour produced and sodium bisulfite stabilize the colour in the phenol presence (Miller, 1959).

The sensitivity of the method is from 100 to 500μg.mL-1 of reducing sugar. A standard glucose or fructose solution is used to build the calibration curve and get the straight line equation to quantify samples. [31]

Fig 4 . Reaction of reducing sugar with 3,5-dinitro-salycilic acid reagent.



2.5 Pine Needle Charcoal Briquettes

More than 2 billion people use wood, charcoal, dung or agricultural residues as the primary fuel for their cooking and heating needs, leading to significant health, economic and environmental consequences. Burning wood or agricultural residues produces smoke with a variety of irritant pollutants, some of which are known carcinogens. More than 1.5 million deaths a year are caused by acute respiratory infections from breathing smoke from indoor cooking fires. Women and children are generally exposed to the greatest levels of pollutants and it is children who suffer the greatest health risk – respiratory infections are the leading cause of death of young children worldwide.

Small-scale subsistence farmers who make less than Rs 100 per day may use wood charcoal for cooking fuel. In areas where wood is scarce and farmers are not able to collect it themselves, the cost of cooking fuel is a significant drain on their limited resources. An estimated 50 billion hours are spent collecting firewood each year and families can spend over 25% of their income on fuel, leaving them with less income to cover other basic needs such as medicine, food and clean water.[32]

3. Materials and method

3.1 MATERIALS: *Pinus Roxburghii* needles were collected from the JUIT campus, Waknaghat. *Pinus Wallichiana* needles were collected from HFRI (Shimla) and Rohru. All other chemicals were procured from Fisher Scientific and Merck unless specified.

Pinus roxburghii is a large tree reaching 30–50 m (98–160 ft) with a trunk diameter of up to 2 m (6.6 ft), exceptionally 3 m (10 ft). The bark is red-brown, thick and deeply fissured at the base of the trunk, thinner and flaky in the upper crown. The leaves are needle-like, in fascicles of three, very slender, 20–35 cm (7.9–14 in) long, and distinctly yellowish green. [33]

Pinus roxburghii is the only tree with an ornamental specimen and having different medicinal values found in the Himalayan region of Bhutan, Nepal, Kashmir, Sikkim, Tibet and other parts of North India. The plant is belonging to family Pinaceae commonly known as Chir Pine. It consists of 110–120 species distributed throughout temperate regions of the Northern Hemisphere, and more than 40 taxonomic treatments have been recognized of several major divisions within the genus [34]

Pinus wallichiana is a tree to 50 m tall with straight trunk and short, downcurved branches. Branches longer in solitary trees, creating a dome-like crown. Bark on young trees smooth, becoming fissured with age. Branches in regularly spaced whorls, smooth. Young shoots glaucous, later turning pale grey-green, smooth, ribbed, darkening with age. Winter buds grey with an orange tinge, ovoid-conic, pointed. Leaves in fascicles of 5, basal sheaths deciduous, 15-20 cm long, often curved at the base, slender, flexible, abaxial side green, adaxial side with multiple bluish-white stomatal lines; usually pendant but in some trees spreading.

The male strobili are on lower branches, often in dense clusters on younger twigs. Female cones in groups of 1-6, 20-30 cm long, erect when young but later pendant, bluish-green when young, maturing to light brown with pale brown apophyses. Cone scales wedge-shaped, wide near the apex, apophysis grooved, ending in a blunt umbo; basal scales usually not, or only slightly, reflexed, very resinous.[35]

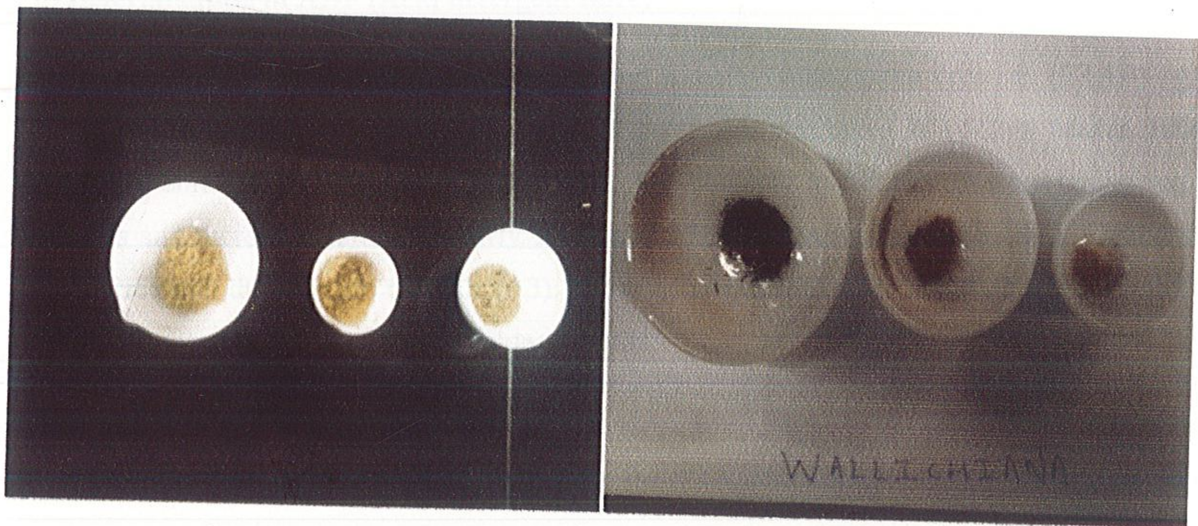
3.2 METHODS:

3.2.1) Sample collection and maintenance: Pine needles after collection were chopped into appropriate size (2 – 5cm long). Chopped pine needles were then dried overnight at 50°C. The dried pine needles were then stored in a jar for further use.

3.2.2) Chemical analysis of the substrate:

1) Determination of Cellulose content: Cellulose content of the substrate was determined by the method of Crampton and Maynard (1938). One gram of oven dried sample was taken in a 250ml beaker. Then 25ml of acetic nitrate reagent (Acetic acid = 73.86 ml; Nitric acid = 9.09ml; Distilled water = 17.04ml) was added and the contents were boiled till the evolution of brown fumes. The residue was then filtered using whatman filter papers. After the filtration, three washings each of water, alcohol and acetone were given. The residue was then transferred into a preweighed crucible and placed in an oven (150°C) overnight. After cooling in a dessicator, it was weighed (W1). Finally, the crucible was kept in a muffle furnace at 450°C for 1 hour, cooled and weighed (W2). Loss in weight (W2 – W1) was observed as the amount of cellulose present in the sample.

Fig 5. Samples after heating in muffle furnace.



2) Determination of Hemicellulose content: Hemicellulose content of the substrate was determined by estimating the percentage of NDF and ADF by the method of Georing and Vansoest (1970).

a) Determination of NDF (Neutral detergent fibre):

Reagent: NDS – Neutral detergent solution (SDS = 30g\L, EDTA = 18.61g\L, Sodium borate decahydrate = 6.81g\L, Disodium hydrogen phosphate = 4.56g\L, 2-ethoxy ethanol = 10ml).

Preparation of NDS: EDTA and sodium borate decahydrate were taken in a beaker containing 200ml of distilled water and dissolved by heating. SDS and 2-ethoxy ethanol were dissolved separately in boiling distilled water and then mixed with above solution. Disodium hydrogen phosphate was separately dissolved in boiling water and then added to above solution. The ph was adjusted to 7 to completely dissolve all the solvents and volume was made to 400ml with distilled water.

One gram (W) of dried sample was taken in a beaker. Then 100ml of NDS, 2ml decaline and 0.5g sodium sulphite were added in sequence. The contents were boiled for 5 to 10 minutes and refluxed slowly for 1 hour. The refluxed sample was filtered and transferred into the weighed crucible (A1). It was then rinsed with hot water, absolute ethanol followed by acetone. The crucible was then dried at 105°C for 12 hour and weighed (A2). The NDF (%) was calculated as:

$$NDF (\%) = (A2 - A1/W) \times 100.$$

b) Determination of ADF (Acid detergent fibre)

Reagents: ADS – Acid detergent solution (Cetyl trimethyl ammonium bromide (CTAB) 6g in 300ml of 1N sulphuric acid). One gram (W) of the sample was taken in a 500ml beaker. 100ml ADS and 2ml decaline were then added to it. The contents in the beaker were boiled for 10 minutes and thereafter refluxed slowly for 1 hour. The digested sample was then filtered and transferred into the weighed crucible (A3). The residue was then washed with hot water, ethanol and acetone respectively. The crucible was then dried at 105°C for 12 hour, cooled in a dessicator and weighed (A4). The dried weight of the residue was recorded as ADF given by:

$$ADF (\%) = (A4 - A3/W) \times 100$$

Hemicellulose content (%) in the substrate was calculated as per the formula

HEMICELLULOSE (%) = NDF (%) – ADF (%)

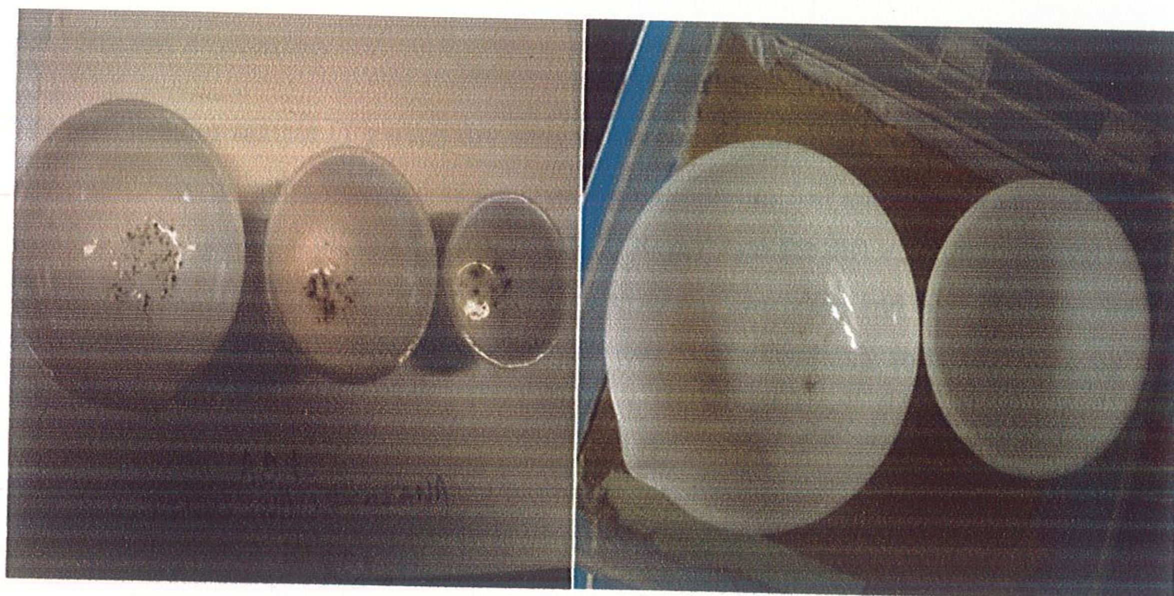
c) **Determination of ADL (acid detergent lignin) [Georing and Vansoest 1970]**

The crucible containing ADF was kept on 500ml conical flask containing water. The contents of the crucible were covered with 20ml of 72% sulphuric acid and stirred with a glass rod to a smooth paste. The crucible was then refilled with acid and kept on ice bucket. After 3 hours, excess of acid was filtered. The residue was then washed with hot water and acetone till it was acid free. The crucible was dried at 100°C till it was completely dried, cooled in a dessicator and weighed (A5). The crucible was then placed in a muffle furnace at 500°C for 3 hours and weighed (A6). The ADL was computed as

$$ADL (\%) = (A5 - A6/W) \times 100$$

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

Fig 6. Samples after heating in muffle furnace at 500 °C for 3 hours.



3.2.3) 3% ammonia pretreatment

One gram of substrate was taken and dipped in 3 % (50ml) ammonia solution. The substrate was taken as a triplicate and was given treatment for 1 hour, 2 hour and 3 hour respectively. The samples were then neutralized (ph 7) using de-ionized water and dried at 50°C for 16hours.

3.2.4) Enzymatic hydrolysis

The above dried samples were then enzymatically hydrolyzed. Enzymatic hydrolysis of pretreated samples was carried out using a 0.1 M, pH 4.8 citrate buffer at 5 different time intervals (24hr, 48hr, 72hr, 96hr, 120hr) at 50 °C and 150 rpm as described by Selig et al. Based on the literature survey and a previous study, the amount of cellulase enzyme was chosen as 15 FPU/g of dry matter [36]. All these enzymes were procured from Sigma Aldrich. The supernatant was collected at different time intervals and was analyzed for reducing sugar content using DNS assay.

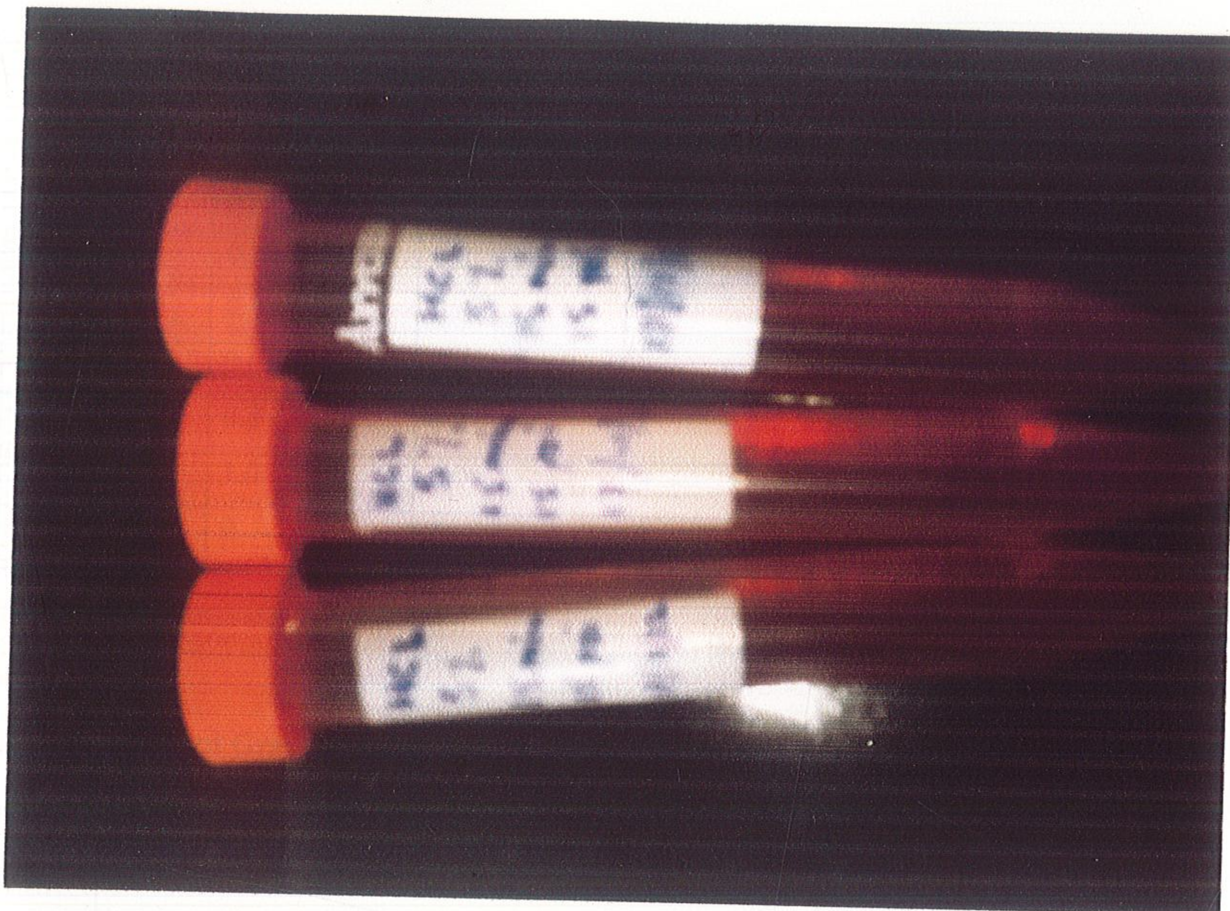
3.3) Steam explosion

The pine needles sample (1 g oven dry weight) was soaked in water (100ml) in a borosil beaker of 250ml. After this the beaker was covered with aluminum foil and kept in an autoclave. The samples were treated at different pressure (15 & 20 psi) and for different time intervals (15, 30, 45 & 60 min). After treating the sample the supernatant was taken and centrifuged at 5000 rpm for 5 min. After centrifugation the supernatant was used to estimate reducing sugar by DNSA method.

3.4) Acid catalyzed steam explosion

The pine needles sample (1 g oven dry weight) was soaked in 2% solution of 98% HCL (100ml) in borosil beaker of 250ml. After this the beaker was covered with aluminum foil and kept in an autoclave. The samples were treated at different pressure (15 & 20 psi) and for different time intervals (15, 30, 45 & 60 min). After treating the sample the supernatant was taken and centrifuged at 5000 rpm for 5 min. After centrifugation the supernatant was used to estimate reducing sugar by DNSA method.

Fig 7. Acid catalyzed steam explosion.



3.5 DNSA method

Preparation of DNS reagent

A) Sodium potassium tartarate 300 g/l

B) Sodium sulphite 0.50 g/l

C) Phenol crystals 2.00 g/l

D) Sodium hydroxide 19.8 g/l

E) Di-nitro salicylic acid 10.0 g/l

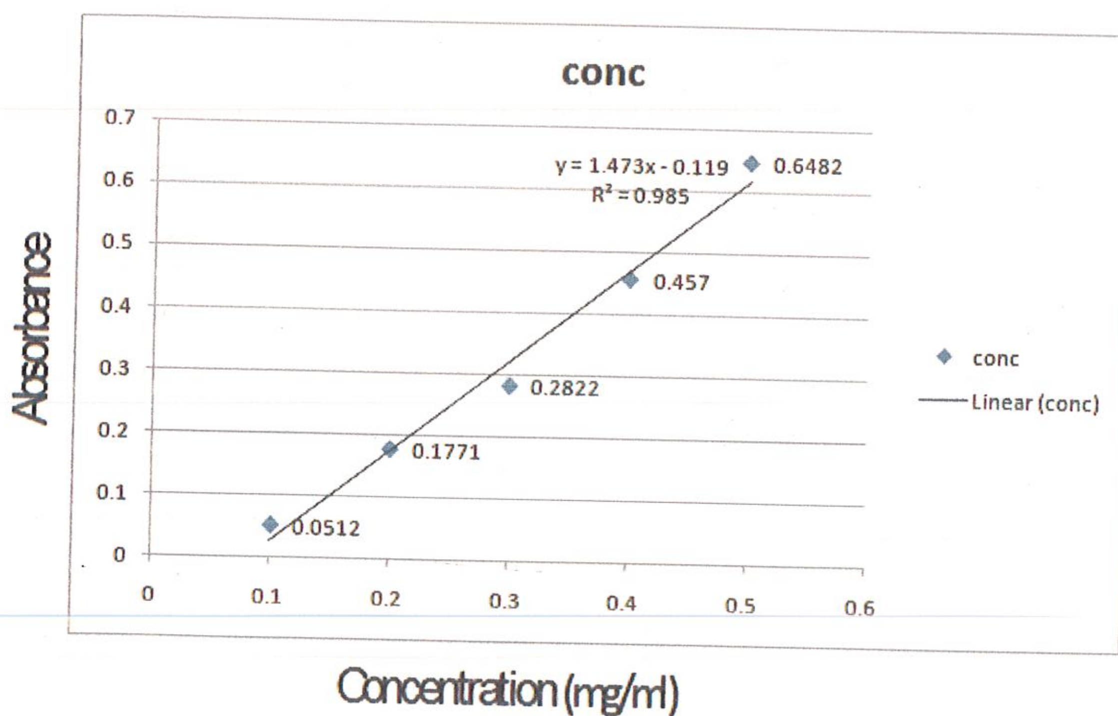
1 ml of sample was dissolved with 3 ml DNS reagent in a test tube. It was then heated in boiling water bath for 10 minutes. The test tubes were then cooled to room temperature and optical density was measured at 540 nm after proper dilution (if required). A reference was run simultaneously using 1 ml of distilled water.

Standard curve was prepared using 0.5 mg/ml of glucose as stock solution.

Table 1. Optical density by DNSA

Working solution (M)	Volume of stock solution (ml)	Volume of distilled water (ml)	Volume of DNS (ml)	Incubation in boiling water bath (minutes)	Optical density
0.1	0.2	0.8	3	10	0.0512
0.2	0.4	0.6	3	10	0.1771
0.3	0.6	0.4	3	10	0.2822
0.4	0.8	0.2	3	10	0.4570
0.5	1.0	0.0	3	10	0.6482
Blank	0	1	3	10	

Fig 8 . Standard Graph for DNSA.



Concentration (mg/ml)

3.6 Charcoal production

Sample collected were pine needles *(Pinus roxburghii)* from JUIT Solan campus , Himachal Pradesh, India. The biomass collected were air dried for ten days to reduce moisture content of the material.

3.2.7.1) Preparation of the briquette samples:

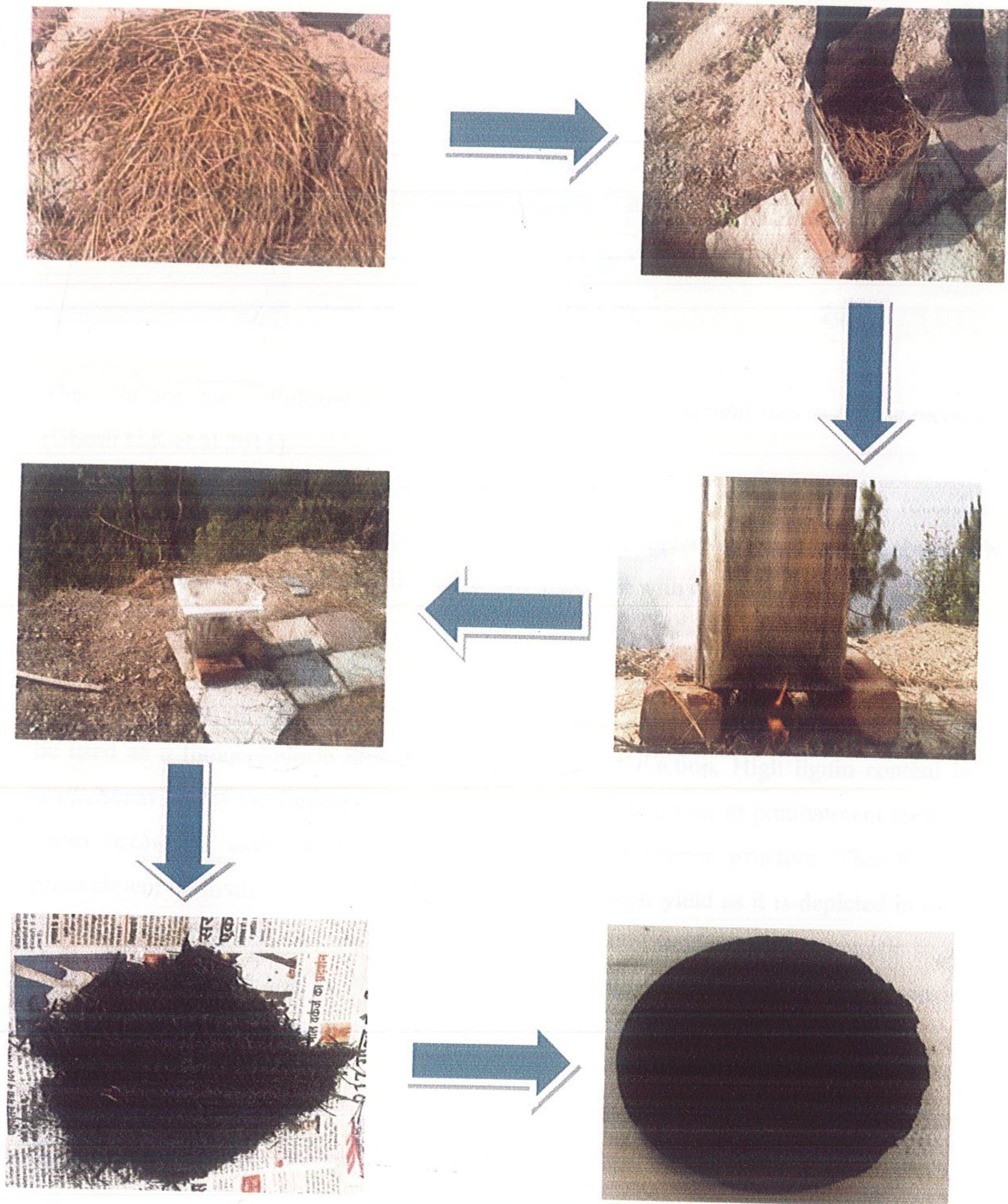
Materials required: forest wastes (pine needles), binding materials (wheat flour and rice starch), Charcoal kiln/drum and Briquetting mould/machine.

Carbonization: For carbonization, the kiln was loosely packed with dried pine needles. The kiln accommodated around ~1kg dry biomass. After loading the biomass into the kiln it was ignited from below and the top was closed with a marble slab after 10 to 15 minutes. Using little amount of biomass in the firing portion to ignite in the kiln and closing the openings tightly for 45 minutes to 1hr (depending upon the biomass) using biomass increases carbonization. In the absence of air, the burning process is slow and the fire slowly spreads to the biomass though the hole in the perforated sheets. In this method 30 % of carbonized char can be obtained.

Binder preparation and mixing: A binder is used for strengthening the briquettes. The carbonized char powder can be mixed with different binders (100 kg of char +5kg of starch) such as commercial starch, rice powder, rice starch (rice boiled water), wheat flour and other cost effective materials like brown clay soil. Binder was mixed with water and boiled for 20 minutes. After boiling the liquid solution is poured into char powder and mixed to ensure that every particle of carbonized charcoal material is coated with binders. It enhances charcoal adhesion and produce identical briquettes.

Briquettes: The charcoal mixture is made into briquettes either manually or using machines. Pour the mixture directly into the briquetting mould/ machine to form uniform sized cylindrical briquettes. Briquettes were then sun dried for 4 to 5 days.[37]

Fig 9. Flow diagram of charcoal production.



4. Results and discussions

4.1 Cellulose, hemicellulose and lignin content

Table 2. Cellulose, Hemicellulose and lignin content in pine needles

Samples	Cellulose	Hemicelluloses	Lignin
Pinus Roxburghii	**61.73 %**	**11.80 %**	**21.5%**
Pinus Wallichiana	**58.23 %**	**15.2 %**	**26.6%**

The cellulose, hemicelluloses and lignin content in *Pinus roxburghii* was as per the recorded data (Ghosh U.K et.al 2011).

The cellulose and hemicellulose content *Pinus Wallichiana* was also as per the recorded data. T.M khattakh and A. Mhamood et.al 1989 [38] reported holocellulose content of about 70% and the reported lignin content in this literature was matching with our data.

Cellulose is close to 60% in both the softwood trees: *Pinus roxburghii* and *Pinus wallichiana*. This high amount of cellulose content is sufficient for *Pinus roxburghii* and *Pinus wallichiana* to be used as a lignocellulosic biomass for bioethanol production. High lignin content in *Pinus wallichiana* makes the lignocellulosic structure more recalcitrant to pretreatment methods used. *Pinus roxburghii* with low lignin content has less recalcitrant structure. Therefore different pretreatment methods used resulted in more reducing sugar yield as it is depicted in our results. Hemicellulose fractions can also be utilized for bioethanol production. Monomeric sugars from pretreated sample are mainly obtained in the hydrolysate and so it should not be considered as waste.

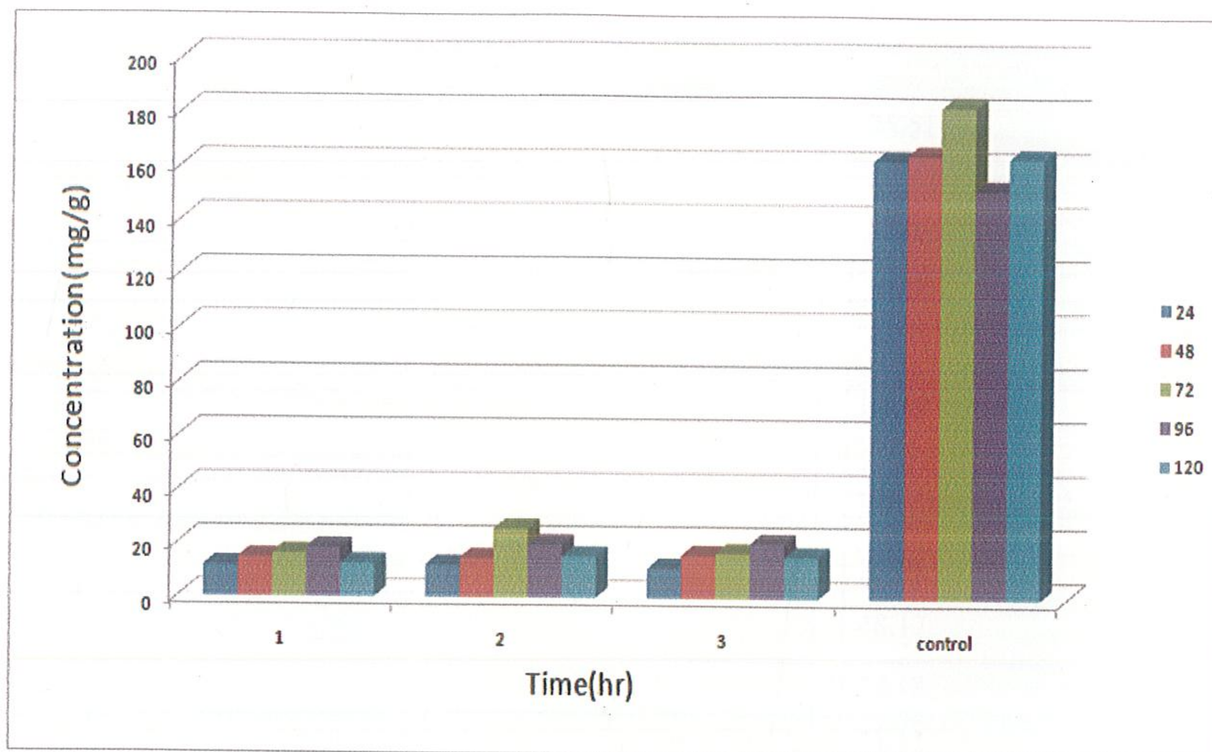
4.2 NH_3 pre-treatment and Enzymatic hydrolysis

4.2.1 Pinus roxburghii

Table 3. NH_3 pre-treatment and Enzymatic hydrolysis of *Pinus roxburghii*

NH3 pertreatment(hr)	Enzymatic treatment(hr)	Reducing sugar(mg/g)
1	24	11.7
	48	14.45
	72	16.2
	96	18.2
	120	12.57
2	24	12
	48	14.5
	72	25.5
	96	19.8
	120	15.4
3	24	11
	48	15.9
	72	16.75
	96	20.42
	120	15.53
Control	24	162.69
	48	164.86
	72	182.91
	96	152.04
	120	163.98

Fig 10. NH3 pre-treatment and Enzymatic hydrolysis of *Pinus roxburghii.*



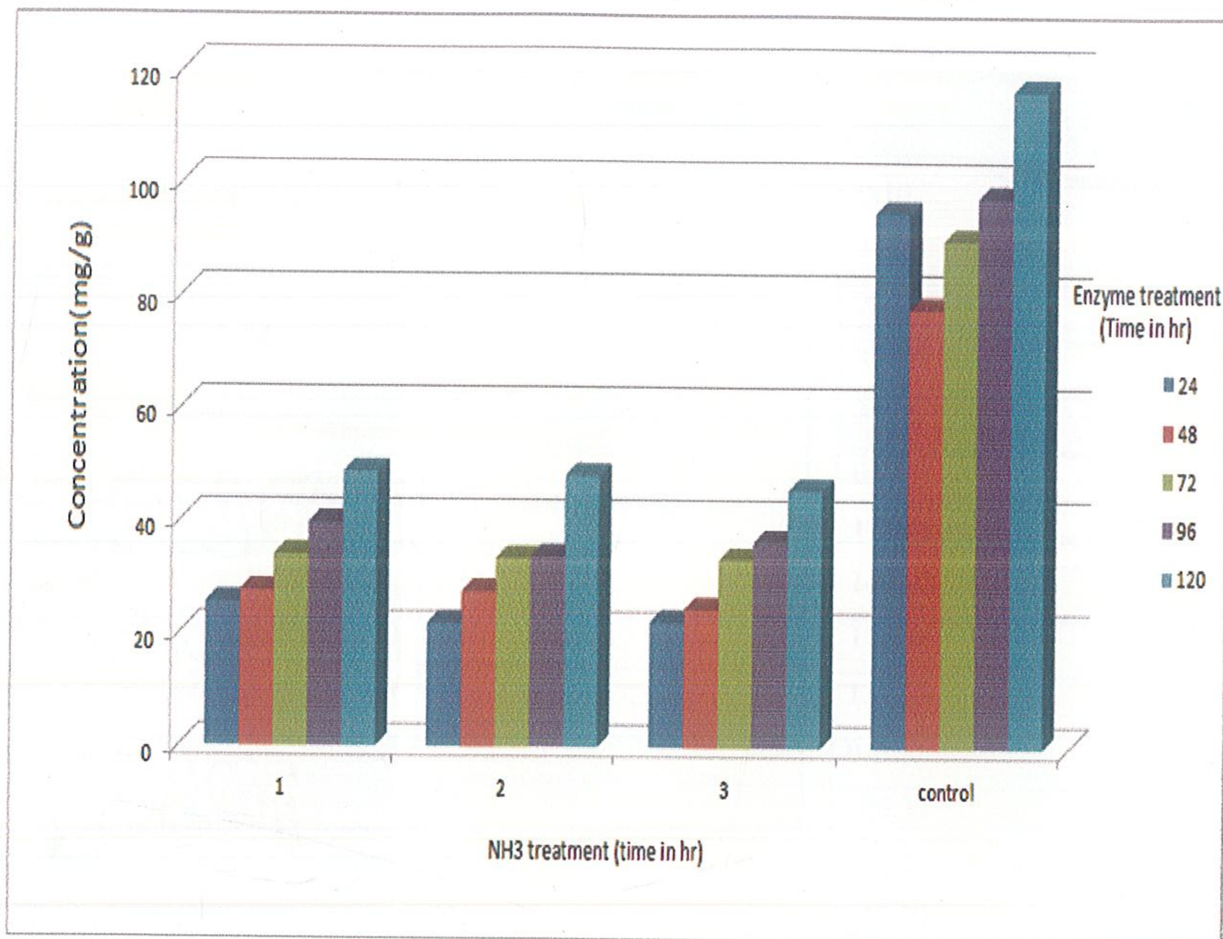
From the above graph, it is apparent that NH3 pretreatment has little effect on the yield of reducing sugars per gram of substrate. Enzymatic hydrolysis was not successful due to the generation of harmful inhibitors and ph of the pulp used. Enzyme did not worked efficiently under the prevailing ph conditions. A method that could result in the recovery of ammonia used may prove to be more efficient in enzymatic hydrolysis of the pretreated pulp. Control samples (with no NH3 pretreatment) had good reducing sugar yield. The reason is that NH3 pretreatment results in the destruction of most of the sugars present in the form of starch. The pretreatment conditions resulted in the dissolution of hemicellulose. In the control samples, increased contents of reducing sugar were obtained due to presence of soluble starch because of enzymatic hydrolysis.

4.2.2 Pinus wallichiana

Table 4. NH3 pre-treatment and enzymatic hydrolysis of *Pinus wallichiana*.

NH3 pertreatment(hr)	Enzymatic treatment(hr)	Reducing sugar(mg/g)
1	24	25.51
	48	27.54
	72	34.05
	96	39.69
	120	48.71
2	24	21.91
	48	27.47
	72	33.44
	96	33.78
	120	48.17
3	24	22.18
	48	24.42
	72	33.24
	96	36.43
	120	45.66
Control	24	94.91
	48	77.75
	72	90.03
	96	97.69
	120	116.56

Fig 11. NH3 pre-treatment and Enzymatic hydrolysis of *Pinus wallichiana*



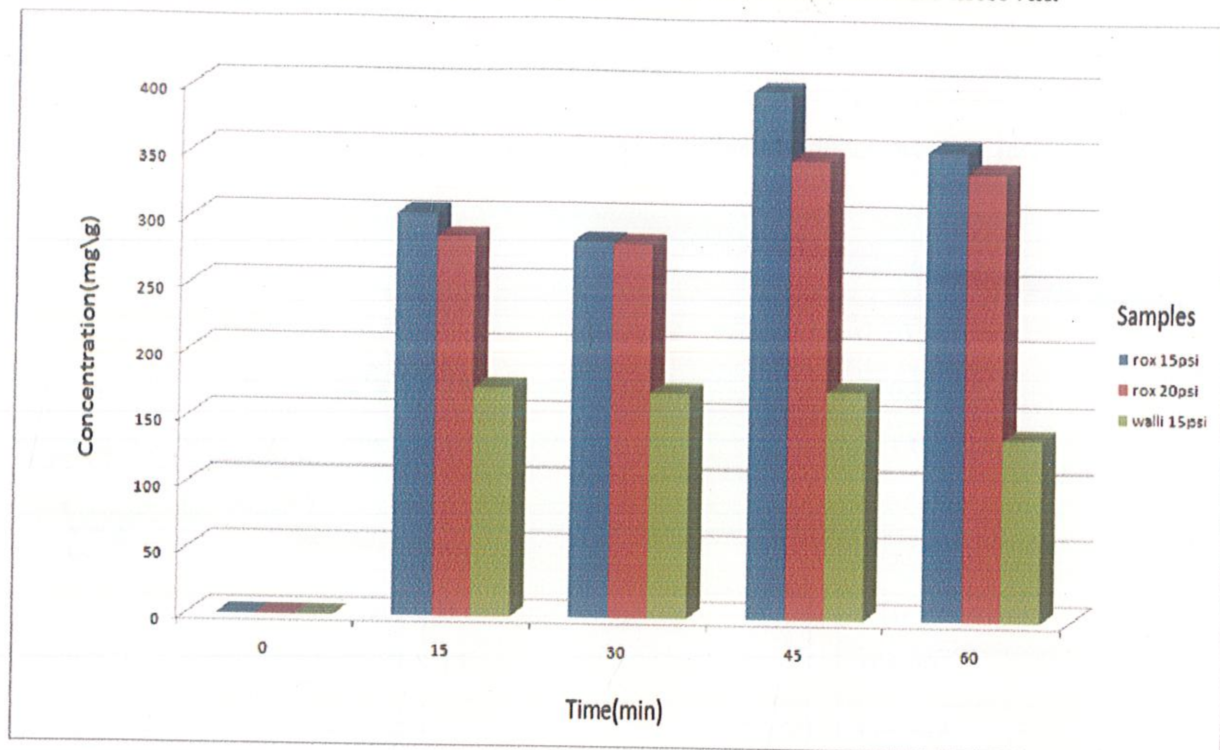
As compared to *Pinus Roxburghii*, pretreatment of *Pinus Wallichiana* with NH3 followed by enzymatic hydrolysis had slightly better reducing sugar yield. But similar to *Pinus Roxburghii*, control samples had very good reducing sugar yield due to the presence of soluble starch due to enzymatic hydrolysis. Pretreated samples had an increasing concentration of reducing sugar as enzymatic hydrolysis time was increased. This shows that more units of enzyme and more hydrolysis and pretreatment time may result in more reducing sugar yield from starch as well as from cellulose. Unlike *Pinus Roxburghii, pretreatment* of *Pinus Wallichiana* resulted in efficient breakdown of recalcitrant layer of lignin and may be to some extent hemicelluloses.

4.3 Steam explosion pretreatment of pine needles

Table 5. Steam explosion pretreatment of pine needles at different time interval

Samples	Time(min)	Reducing sugar(mg/g)
Pinus Roxburghii needles(15psi)	0	0.10
	15	304.0
	30	283.6
	45	397.8
	60	345.6
Pinus Wallichiana needles(15psi)	0	0.10
	15	172.51
	30	169.85
	45	172.15
	60	137.29
Pinus Roxburghii needles(20psi)	0	0.10
	15	286.3
	30	282.3
	45	345.99
	60	338.53

Fig 12. Steam explosion pretreatment of pine needles at different time interval.



Steam explosion of *Pinus Roxburghii at* 121°C and at two different pressures (15psi and 20psi) had good reducing sugar yield in the supernatant. Reducing sugar yield initially showed an increase in concentration till 45min and then a slight drop in concentration. Working at two different pressures had almost no effect on the yield of reducing sugars. This shows that reducing sugar content was mostly due to starch content which got dissolved in supernatant in the form of reducing sugar.

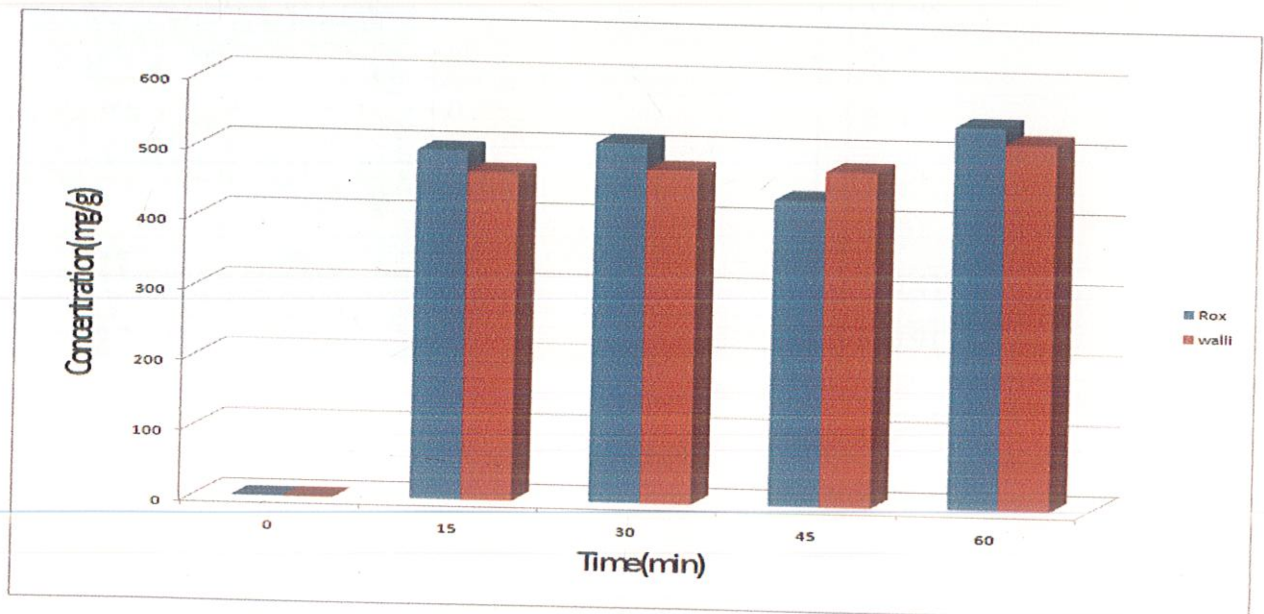
Pinus wallichiana had less and very constant reducing sugar yield as compared to *Pinus Roxburghii* . This was due to less starch content in *PinusWallichiana.* Less starch content in the needle may be related to age of tree as starch content varies with age of tree.

4.4 Acid catalyzed Steam explosion of pine needles

Table 6. Acid catalyzed Steam explosion of pine needles for different time intervals.

Samples	Time(min)	Reducing sugar(mg/g)
Pinus Roxburghii needles(15psi)	0	0.10
	15	496.40
	30	511.025
	45	435.90
	60	543.79
Pinus Wallichiana needles(15psi)	0	0.10
	15	466.20
	30	474.432
	45	476.89
	60	520.45

Fig 13. Acid catalyzed Steam explosion of pine needles for different time intervals.



Catalyzed Steam explosion of *Pinus Roxburghii at* 121ºC and at pressures 15psi had good reducing sugar yield in the supernatant. Reducing sugar yied in this case was higher than that of uncatalyzed steam explosion. This shows that reducing sugar content was mostly due to starch and hemicellulose content which got dissolved in supernatant in the form of reducing sugar.

Pinus wallichiana had almost equal reducing sugar yield as compared to *Pinus roxburghii.* This was due to less starch content in *Pinus Wallichiana.* But in case of uncatalyzed steam explosion the yield of reducing sugar from *Pinus wallichiana* was less than that of *Pinus roxburghii.*

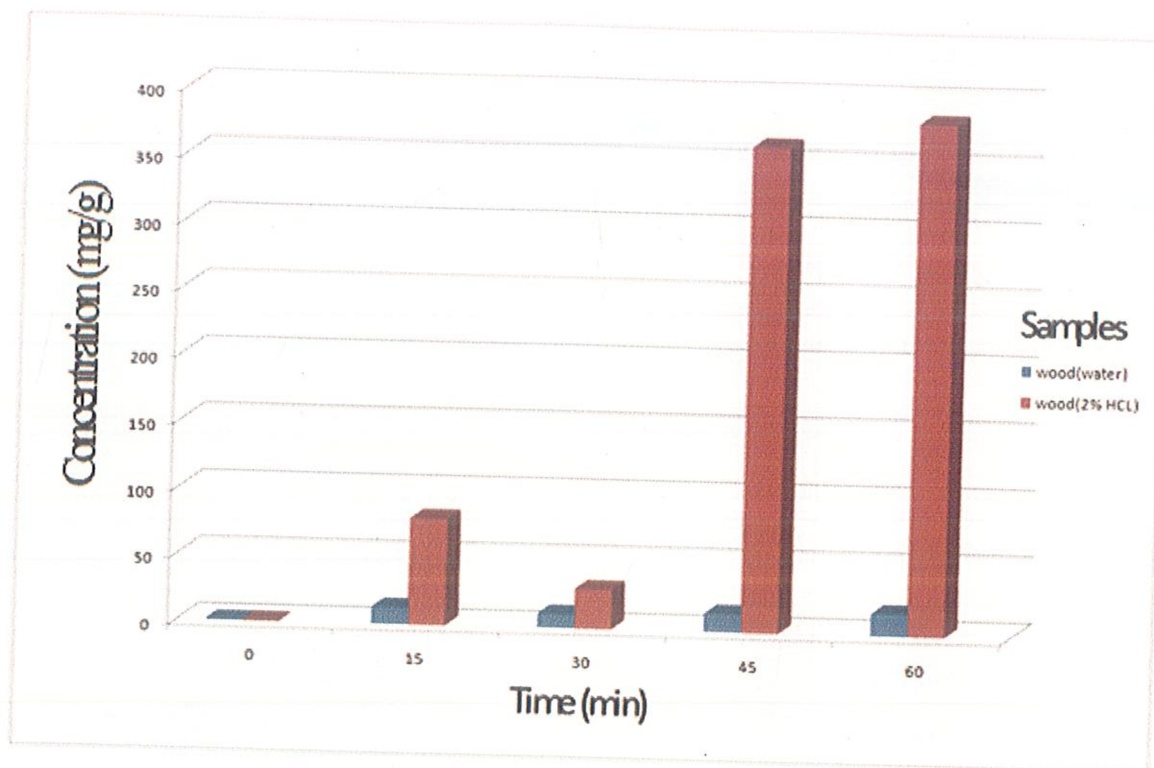
4.5 Steam explosion pretreatment of pine wood

4.5.1 *P.Roxburghii* wood

Table 7. Steam explosion pretreatment of *Pinus roxburghii* wood

Samples	Time(min)	Reducing sugar(mg/g)
Water steam explosion (15psi)	0	0.10
	15	12.38
	30	10.19
	45	13.38
	60	16.283
Acidic steam explosion (15psi)	0	0.10
	15	79.31
	30	29.31
	45	363.41
	60	383.91

Fig 14. Steam explosion pretreatment of *Pinus Roxburghii* wood.



Pinus roxburghii wood samples were also subjected to catalyzed and uncatalyzed using water and 2% HCL respectively. Water catalyzed steam explosion had very less monomeric sugar yield in the supernatant. On the other hand acid catalyzed steam explosion had good reducing sugar yield in the supernatant. As pretreatment time was increased, reducing sugar content in the pretreated samples increased in catalyzed and uncatalyzed steam explosion. The reducing sugar content was much higher in case of catalyzed steam explosion when the pretreatment time was increased 45 minutes or 1 hour.

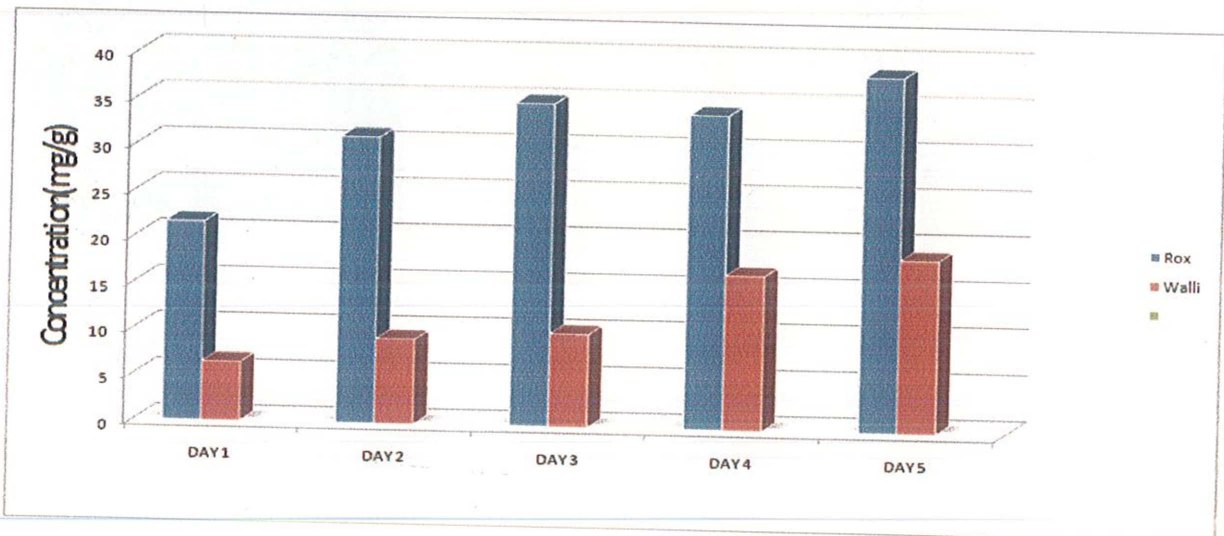
4.6 Enzymatic treatment of pretreated needles sample

4.6.1 Water steam explosion pretreated sample

Table 8. Enzymatic treatment of uncatalyzed steam exploded pulp of needles.

Samples	Tim(day)	Reducing sugar(mg/g)
P.Roxburghii needles (15 psi , 45 min)	1	21.521
	2	31.06
	3	35.009
	4	34.099
	5	38.551
P.Wallichiana needles (15 psi, 45 min)	1	6.387
	2	9.1818
	3	10.09
	4	16.756
	5	18.761

Fig 15. Enzymatic treatment of Water steam explosion pretreated needle sample



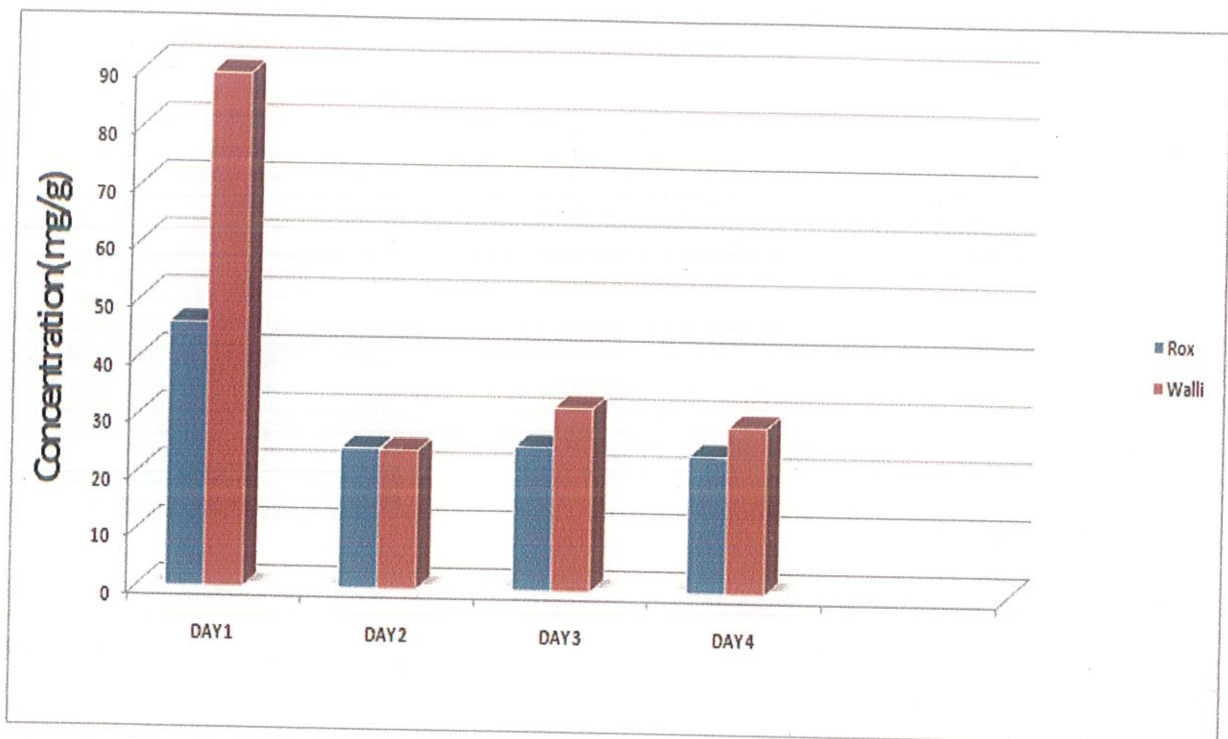
The pulp of both the samples that was obtained after uncatalyzed steam explosion were subjected to enzymatic hydrolysis using the enzyme cellulase. The concentration of reducing sugar was much higher in *Pinus roxburghii* as compared to *Pinus wallichiana* as the enzymatic hydrolysis time was increased. However, more amount of reducing sugar in *Pinus roxburghii* was due to the formation of less inhibitors and more exposed cellulose fractions as compared to *Pinus wallichiana.*

4.6.2 Acidic steam explosion pretreated sample

Table 9. Enzymatic treatment of acid catalyzed steam exploded pulp of needles.

Samples	Time(min)	Reducing sugar(mg/g)
Pinus Roxburghii needles (15psi, 45 min)	1	45.91
	2	24.42
	3	25.08
	4	23.89
Pinus Wallichiana needles (15psi, 45 min)	1	89.25
	2	24.129
	3	31.915
	4	30.956

Fig16. Enzymatic treatment of Acidic steam explosion pretreated needles sample



Enzymatic hydrolysis of pretreated samples after catalyzed steam explosion resulted in higher reducing sugar yield during first day and the reducing sugar concentration was constant during days 2, 3 and 4 in both the samples. The higher reducing sugar yield during the first day was due to efficient enzymatic hydrolysis and as the samples were neutralized, during the first day there were less inhibitors. As the enzymatic hydrolysis time was increased reducing sugar content decreased because of the formation of inhibitors. Incomplete distortion of the lignocellulosic layer during pretreatment may be one of the factors for the reduced reducing sugar yield.

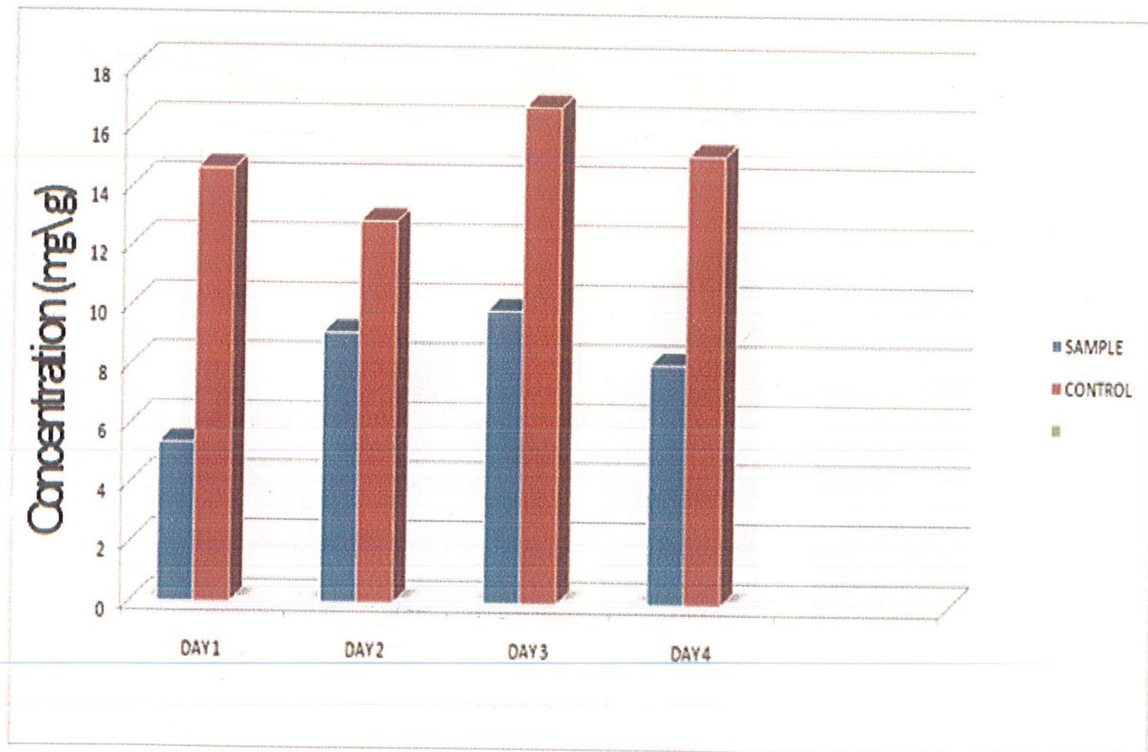
4.7 Enzymatic treatment of pretreated wood sample

4.7.1 Acidic steam explosion pretreated sample

Table 10. Enzymatic treatment of Acid catalyzed steam explosion of *Pinus roxburghii* wood pulp.

Samples	Time(min)	Reducing sugar(mg/g)	Control
Pinus Roxburghii wood (15psi,45 min)	1	5.337	14.58
	2	9.0956	12.84
	3	9.837	16.71
	4	8.06578	15.1095

Fig 17. Enzymatic treatment of Acid catalyzed steam explosion of *Pinus roxburghii* wood pulp.



The reducing sugar concentration in the wood samples of *Pinus roxburghii* were not significant after enzymatic hydrolysis. This was because the pretreatment conditions applied was not able to break the recalcitrant layer of lignin completely and so the enzymes could not work efficiently. Control samples had a slightly higher reducing sugar concentration due to the presence of starch and formation of less inhibitor.

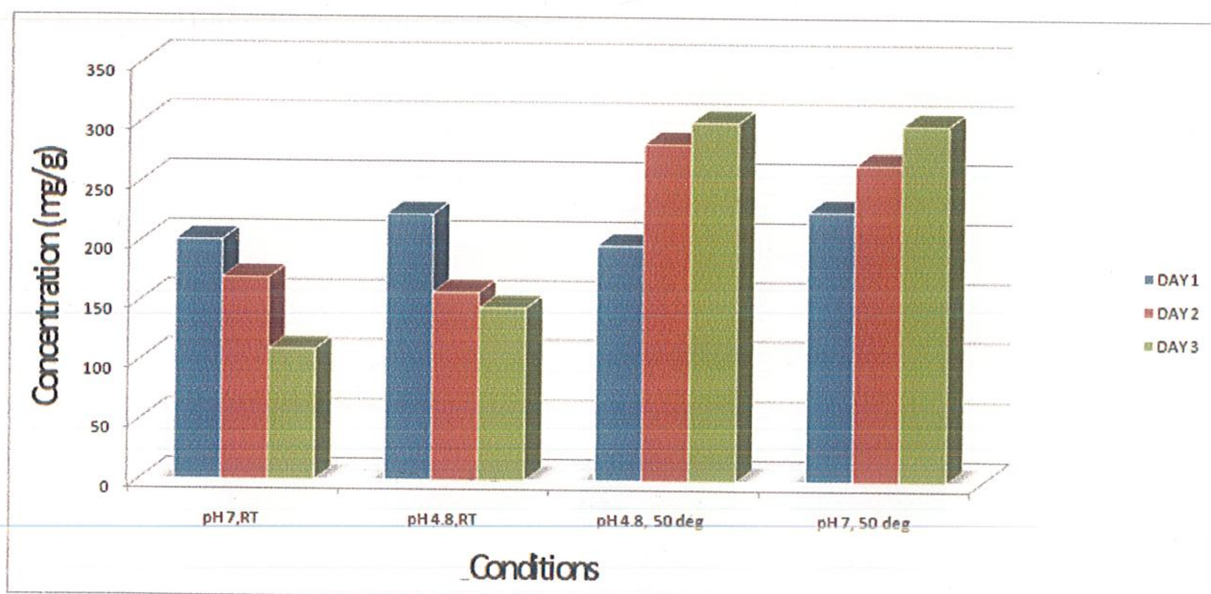
4.8 Starch analysis by changing pH and temperature

4.8.1 *Pinus roxburghii needles*

Table 11. . Starch analysis of *Pinus roxburghii* .

Condition	Day 1	Day 2	Day 3
pH7,RT(Room temperature)	201.4 mg/g	169.65 mg/g	109.605 mg/g
pH 4.8, RT	223.345 mg/g	157.81 mg/g	144.83 mg/g
pH 4.8, 50 °C(Degree Celsius)	197.59 mg/g	283.09 mg/g	301.42 mg/g
pH 7, 50 °C	226.725 mg/g	266.13 mg/g	299.48 mg/g

Fig 18. Starch analysis of *Pinus roxburghii*.



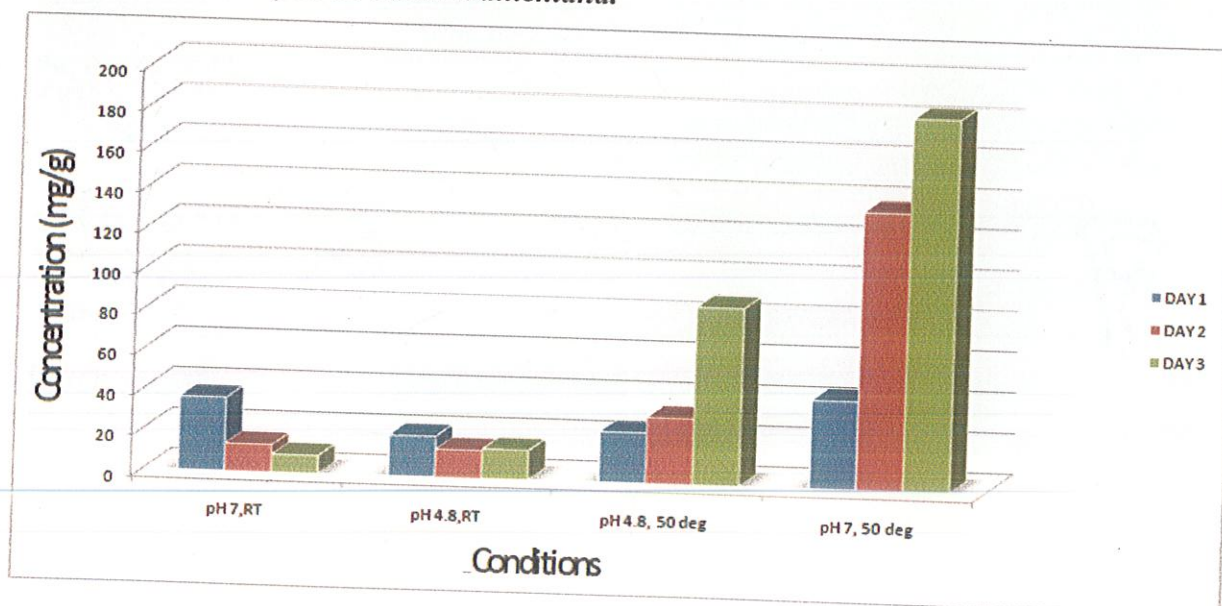
The untreated needles were kept at two different ph and temperatures to analyse the starch content in the needles. Monomeric sugar content was more when temperature was 50° C irrespective of whether the ph was 4.8 or 7. Ph of 4.8 was found to be more effective than ph of 7. The sugar content also increased exponentially from day 1 to day 3 when temperature was 50° C. This shows that temperature plays a role in efficient hydrolysis of starch and it also distorts the lignin layer thereby helping in the release of monomeric sugars from hemicellulosic fraction of biomass. At room temperature there was no release of sugars from hemicellulosic fraction and development of colour due to needles resulted in the decrease of reducing sugar content from day 1 to day 3.

4.8.2 *Pinus Wallichiana* needles.

Table 12. Starch analysis of *Pinus Wallichiana*.

Condition	Day 1	Day 2	Day
pH 7, RT(Room temperature)	36.165 mg/g	13.47 mg/g	8.88 mg/g
pH 4.8, RT	20.085 mg/g	13.759 mg/g	14.46 mg/g
pH 4.8, 50 °C (Degree Celsius)	24.765 mg/g	32.42 mg/g	87.50 mg/g
pH 7, 50 °C	43.605 mg/g	136.745 mg/g	183.475 mg/g

Fig 19. Starch analysis of *Pinus Wallichiana*.



The reducing sugar content increased from day 1 to day 3 when the temperature was 50° C irrespective of ph. The same explanation that monomeric sugars were released from hemicelluloses fraction increased the content of reducing sugars. Change of ph did not had enough change in the content of reducing sugars rather it decreased from day 1 to day 3.

4.9 Charcoal product.

The results from this study have shown that the biomass briquettes bonded with starch are very efficient biomass briquette depend on the following factor: its ability to ignite easily without any danger, generate less smoke, high calorific value, generate less ash as this will constitute nuisance during cooking and to be strong enough for safe transportation and storage. The technology has a great potential for converting waste biomass into a superior fuel for household use, in an affordable, efficient and environment friendly manner.

Fig 20. Charcoal briquettes

5. Significance of the Project

The price of petroleum products in India has increased about 3 times in the last decade. This has resulted in about 75% increase in green house gases from 2001 to 2011[39]. In addition to this, world oil use is expected to grow from about 80 million barrels/day (mbpd) in 2003 to about 118 mbpd in 2030 as per Energy Information administration(EIA), International Energy Outlook(IEO) 2006. India is expected to consume additional 2.2 mbpd by 2030 and India already imports around 80% of its crude oil. This will not only put great pressure on the budget but also on the national security of the country.

So, with a view of reducing dependence on imported gasoline, the use of lignocellulosic biomass for bioethanol production will provide great opportunity for our country to reduce its dependence on imported crude oil products. Apart from this, the use of lignocellulosic biomass as second generation feedstock will reduce dependence on the conventional use of first generation feedstock for bioethanol production. This is because first generation feedstocks are cash crops and require arable land for their cultivation. On the contrary, a use of second generation feedstock is found in abundance, at low cost and does not require arable land.

Through our project we also intend to increase the use pine needles that generally go as waste. Pine needles results in high risk of forest fires and could result in infertility of soil. Our project demonstrates two efficient use of pine needles for biofuel production. Pine needles that generally go unused can be used as lignocellulosic biomass for bioethanol production and as dried biomass for charcoal production.

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