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Insights on Lignocellulosic Pretreatments for Biofuel Production- SEM and Reduction of Lignin Analysis

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Abstract: Lignocellulosic biomass is a less frequently tapped source for biofuel production. Lignocelloses are composed of carbohydrate polymers (cellulose, hemicellulose) and an aromatic polymer (lignin); this intricate plant cell wall structure makes it a tedious process for bioconversion. Hence to convert the lignocellulosic biomass into a thoroughly usable form, pretreatment is done through physical, chemical and biological methods. Pretreatment of refractory lignocelluloses goes hand in hand with prolonged membrane retention throughput during downstream processing and increased yield of biofuel. Lignocellulosic biomass together with rest of the agro-waste could satiate the thirst of liquid fuel burnt each year across the globe by 100 billion gallons. Appropriate pretreatment of lignocelluloses can be awarded for serving umpteen purposes like effective disposal of agro-waste. The paper focuses on the effect of treatment time, temperature, and concentration of treating agents to separate lignin; to identify pretreatment methods that could afford the highest cellulose to glucose conversion for various lignocelluloses and the structural architecture of different lignocelluloses like rice straw, sugarcane, sawdust and softwood which are responsible for easy lignin reduction. The Latter most was analyzed by Scanning Electron Microscopy (SEM) in order to obtain an unambiguous picture of the structure that contributes to efficient lignin removal.
Keywords: lignocellulosic biomass, membrane retention, carbohydrate polymers, aromatic polymers, agro-waste, SEM.

Introduction

The voyage of exploring a best substitute for fossil fuels lead to the dependence on biochemical fermentation of agricultural feed stocks for biofuel production has increased considerably. But this is not up to the level expected since it involves utilization of feed stocks like corn kernel where the cost of the raw material exceeds the cost of the fuel itself. Hence the focus now has shifted to lignocellulosic agricultural wastes. Though it possesses drawbacks such as complex structure it does proves to overcome the drawback of utilization of feed stocks. Therefore our next step in exploration in order to find a best raw material for biofuel is to find a best way to treat the recalcitrant lignocelluloses. The lignocellulosic biomass thus can act as an efficacious raw material for production of biofuel and bioenergy such as methanol, ethanol, butanol, biohydrogen and biogas [1,2]. The three dimensional structural profile of lignocellulosic cell wall includes cross linking of celluloses and hemicelluloses which is made rigid by lignin that acts as a cement[3] making it inert to biological and chemical activity.

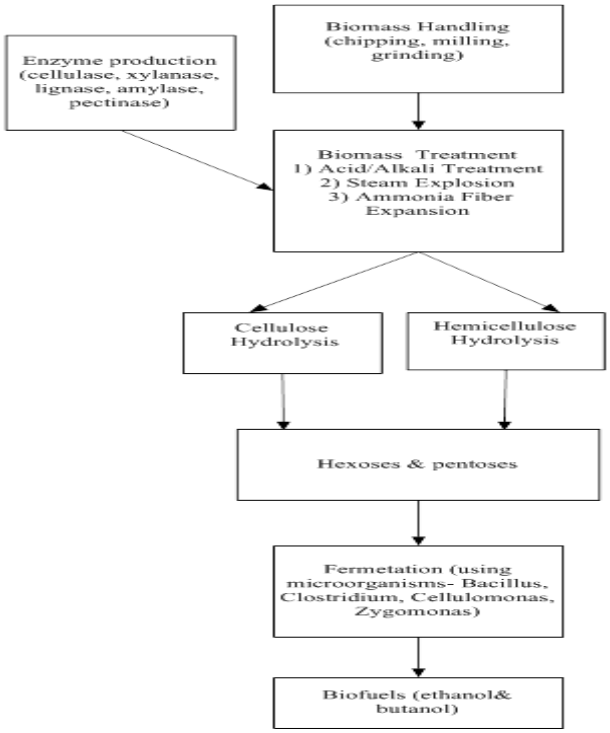
Structural profile of lignocelluloses

Cellulose is the primary component of lignocelluloses which is a linear polymer of disaccharide cellobiose. Strong linkage of cellobiose via b-1, 4 glycosidic bond forms a framework of linear cellulose. The interlinkage of cellulose is formed by hydrogen bonds and Vander Waals forces between the hydroxylic groups present in the same or vicinal cellulose chain. This leads to the formation of micro fibrils possessing high tensile strength[4]. The micro fibrils are further attached to hemicelluloses. Cellulose consists of amorphous and crystalline portions that give different orientation to its crystalline structure. The crystalline index makes the biodegradation complicated when it is high,whereas hemicelluloses are more amorphous, random and branched heterogenic polysaccharides. This characteristic of hemicelluloses makes it highly susceptible to biological, thermal and chemical hydrolysis of their monomer compounds where moisture, pH and temperature are the significant parameters of thermo chemical hydrolysis[5,6]. Lignin forms the secondary component of lignocelluloses which is a complex aromatic and hydrophobic amorphous heteropolymer having hydroxyl, methoxyl, and carbonyl functional groups[7]. Lignin acts as a barrier for the utilization of lignocellulosic biomass for bioconversion due to its water insolubility, whereas it is made soluble at higher temperatures (180 ^{0} C), neutral pH or acid/alkaline conditions based on its precursors[8]. On the analysis of lignin composition of softwood and hardwood, softwood seems to have more lignin make it more recalcitrant[1].

Pretreatment for degradation of lignocelluloses

The bioconversion of lignocellulosic agricultural waste involves three important stages that ultimately lead to production of biofuel. The stages include pretreatment, hydrolysis of cellulose to fermentable sugars and fermentation of sugars to yield the respective biofuel. Hence in order to convert it to fermentable sugar the raw material should undergo pretreatment[2]. Major types of pretreatment include physical, chemical, physicochemical and enzymatic processes and after pretreatment, the cellulose is converted to glucose by acid or enzyme catalyzed hydrolysis; where the converted glucose undergoes fermentation to yield biofuels with the aid of microorganisms. The efficiency of hydrolysis is greatly reduced when the presence of lignin and hemicellulose is between 20-25% each. Thus to improve the porosity and accessible surface area of lignocellulosic raw material and in order to reduce the cellulose crystallinity effective pretreatment is procured[9]. Therefore the objective of this paper is to bring the prospects of pretreatment such as improved formation of sugar or the ability to subsequently form sugar from hydrolysis, avoiding the loss of carbohydrates and byproduct formation, overcoming the membrane fouling during downstream processing andcost effective treatment. The conversion of lignocelluloses into biofuel by pretreatment as a precursor step is elaborated in (Figure- 1).

Figure 1: Flow Diagram for pretreatment process of lignocellulosic material



Physical pretreatment

Mechanical Comminution

Comminution of lignocellulosic materials by combination of chipping, grinding and/or milling were applied to reduce cellulose crystallinity. The size after chipping, grinding and milling was reduced to 0.2-2mm which was 10-30mm before [10]. The particle size and biomass characteristics determine the power requirement for mechanical comminution of agricultural materials [11]. It was said that, if the final particle size is with a range of 3-6 mm, the energy input for comminution is low which arround 30 kWh per ton of biomass. Irradiation ofcellulose by γ-rays leads to cleavage of1,4-glycosidicbonds that gives a larger surface area and a lower crystallinity[12].

Pyrolysis

Cellulose decomposes rapidly to gaseous products and it residual characteristics increases when biomassis treated at temperatures greater than 300 °C[13,14]. At lowertemperatures, much slower decomposition is observed, and less volatile products are formed. Scientists reported thatmildacid hydrolysis with 1 N H2SO4 at 97 °C for 2.5 h of the products frompyrolysis pretreatment resulted in 80-85% conversion ofcellulose to reducing sugars with more than 50% glucose[14] and when carried out in the presenceof oxygen the pyrolysis process is enhanced [15]. Production of transportationfuels from biomass via a so-called biomass-to-liquids (BtL)route; in which biomass is converted to syngas from which highqualityFischer-Tropsch (FT) fuels are produced[14].

Physico-chemical pretreatment

Steam explosion continuation

The chip property, size of feed stock and moisture content (12% and 30%) has been proved to have a great impact on the overall bioconversion process and also on the recalcitrant lignin removal during steam explosion. Scientists worked on the effects of chip property and found that both increased chip size and increased moisture content resulted in improved solids recovery and increased hemicelluloses derived sugar recovery and also minimized condensation of lignin. Later a post steam explosion refining step increased hemicelluloses derived sugar recovery and was delignified efficiently to 6.5%. Further the refined substrate could be enzymatically hydrolyzed to yield 98% at quite faster rate i.e. (1.23 g/L/h)[16].When small amount of mineral acids like sulfuric acid were added at reduced temperatures (acid catalysis) hydrolysis is improved due to complete hemicelluloses removal which will improve the enzymatic action on cellulose. SO_2 addition will even improve the sugar yield and enzymatic hydrolysis[17].General advantages of steam explosion processes compared to other pre-treatment technologies for chemical utilization of lignocellulose are : (1) No chemical are used except water,(2) Good yield of hemicelluloses with low degraded byproducts, (3) Equipment corrosion is minimum due to a mild pH of reaction media when compared to acidhydrolysis processes, (4) Stages of acid handling and acid recycling are avoided, (5) Disruption of the solid residues from bundles to individual fibers occur due to explosion effect[18].

Ammonia Fiber Explosion (AFEX)

AFEXis a pretreatment process where the lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. In a typical AFEX process, dosage of liquid ammonia is 1-2 kg of ammonia/kg of dry biomass, the temperature is maintained at 90 °C, and the residence time is set for 30 min. AFEX pretreatment is said to increase the fermentation process significantly for various substrates like wheat chaff, wheat straw, alfalfa, grass and crops[19]. There are two mechanisms by which AFEX pretreatment appear, (1) increase in reactivity of cellulose due to exposure to liquid NH3, (2) inrease in accessible surface area following AFEX treatment, probably as a combined effect of hemicellulose hydrolysis and explosive decompression[9]. During the pretreatment process only a small amount of the solid material is solubilized that is, almost there is no traces of hemicelluloses or lignin removed. The hemicelluloses are degraded to oligomeric sugars and aredeacetylated, which is the reason for hemicellulose to be insoluble. And the structure of the material is changed, resulting in increased water holding capacity and higher digestibility[20,21]. But it was observed that the AFEX process was not very effective for biomass with higher lignin content of 25% lignin. Therefore AFEX technology was proven to less effective for

lignocellulosic materials with high lignin content such as wood. Scientists are researching on the remedies to overcome this issue.

Chemical Pretreatment

Acid Pretreatment

Acid pretreatment is done to improve the cellulose hydrolysis by the utilization of either dilute or concentrated acids. At moderate temperatures due to sugar decomposition, direct saccharification suffers low yields. However, pre-hydrolysis increases the enzymatic digestibility of cellulose when treated with dilute acids at temperature higher than 121^{o}C[22]. 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 highsolids loading (10-40% [w/w], lower temperature (T < 160ºC), batch processes [23]. Dilute acid pretreatment with 0.2-2.0% sulfuric acid at 121-220^{o} C of lignocelluloses serves 3 functions for conversion process: (1) hydrolysis of thehemicellulose components to produce a syrup of monomeric sugars,(2) exposure of cellulosefor enzymatic digestion by removal of hemicellulose and part of the lignin, (3)solubilization of heavy metals which may be contaminating the feedstock[24]. Although the above mentioned serves as benefits, acid pretreatment posses few potential problems such as, theproduction of an acid waste stream that must be neutralized or reused, the formation ofcompounds such as acetic acid and furfural in the hydrolysate which are toxic to bacteria oryeasts during fermentation[24], and the need for corrosion-resistant equipment[25]. Scientists investigated dilute acid pretreatment of aspen, wood andwheat straw at solids concentrations from 10 to 40%[22]. A monomeric soluble sugar stream(mostly xylose) was produced with little sugar degradation and the cellulose remaining in thesolids was highly digestible by enzymes thus proving that using higher solids concentrationsis a feasible option for reducing the cost of steam.

Alkali Pretreatment

Mechanism of alkali pretreatment is the saponification ofintermolecular ester bonds crosslinking xylan hemicelluloses and other components such as lignin and hemicellulose. Alkali pretreatment aims to increase the porosity of the material due to the extensive swelling facilitated by removal of the crosslinks[26]. Pretreatment of lignocelluloses with 10% NaOH for 60 mins under pressure at 121^{o} C and 15 psi pressure in an autoclave decreased the lignin fraction more than 95% and increased enzymatic conversion by 79.4% which is 4 times more compared to untreated stover[27]. In addition, lignocelluloses was pretreated with aqueous ammonia in flow through column reactor by a process called ammonia recycle percolation which delignified the biomass by 70-85% where 70% of lignin was removed effectively.

Ozonolysis

Ozone has been used to degrade lignin and hemicellulose in lignocellulosic materials such as cotton stalks[28-30], corn stover, wheat straw[31], bagasse, and poplar sawdust. The major advantages of this process are, no toxic residues are formed as ozone can be easily decomposed to oxygen using acatalytic bed or an increase in temperature thus eradicating the need for extensive downstream processing and ozonation reactions take place at surrounding temperature and pressure so energy and investment costs are minimized. And the most notable effects of ozone treatments were 50% decrease in both lignin and hemicelluloses[28].

Biological Pretreatment

Biological pretreatment engrosses microorganisms such as brown-, white- and soft-rot fungi that are used actively to degrade lignin and solubilize hemicellulose. White-rot fungi are the most efficient basidiomycetes for biological pretreatment of lignocellulosic materials[32]. Lignin degradation by white-rot fungi, specifically *Phanerochaetechrysosporium*,*Pleurotusostreatus, and Trametesversicolor,* is an oxidative process with lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases acts as the key enzymes in the degradation[33]. Fungal pretreatment will potentially lower the severity requirements of chemical,temperature, and time resulting in less biomass degradation and lower inhibitory concentrations compared to conventional thermochemcial pretreatment processes[34]. Scientists found that fungal pretreatment by *P.chrysosporium*for 28 days followed by steam explosion at 215^{o} C for 6.5 minutes provided the maximum conversion of glucose in soft wood[35].

Enzymatic Hydrolysis

Enzymatic hydrolysis focuses on the conversion of cellulose to glucose at high yields without sugar product degradation. Enzymatic hydrolysis of cellulose involves several steps in breaking glycosidic bond by

the use of cellulase enzymes. Factors effecting hydrolysis of cellulose includes type of substrate, cellulase loading, reaction conditions such as temperature and pH, and end-product inhibitors. The significant enzyme that hydrolyses cellulose effectively is cellulases. The organisms that are involved in the synthesis of cellulases are fungi, bacteria, and plants both aerobically and anaerobically. The aerobic mesophilic fungus, *Trichodermareesei*and its mutants have been the most intensely studied and used sources of cellulases[36]. Generally cellulose is not a single enzyme, but it is made up of a family of at least three groups of enzymes: 1,4- β-D-glucanglucanohydrolases(endoglucanases), 1,4- β-D-glucancellobiohydrolases and 1,4- β-D-glucanglucohydrolases (exoglucanases), and β-D-glucosideglucohydrolases (β- glucosidases)[37,38]. This combination is required for the breakdown of the celluloses. Advantages of having all three groups of enzymesare : the enzymes are more resistant to chemical inhibitors, and exhibiting better stability at 50ºC than other fungal cellulases[38]. Enzymatic hydrolysis involves three steps: adsorption of endoglucanases and exoglucanases onto the surface of cellulose, biodegradation of cellulose to glucose, and desorption of cellulases. Native cellulose is hydrolyzed by the cellobiohydrolases to yield cellodextrins and cellobiose. Cellodextrins are further hydrolyzed to cellobiose (disaccharide), by endoglucanases, and then β-glucosidase hydrolyzes cellobiose to glucose[25]. Native cellulose is hydrolyzed by the cellobiohydrolases to yield cellodextrins and cellobiose. The cellodextrins are further hydrolyzed to cellobiose, a disaccharide of glucose, by endoglucanases, and then β-glucosidase hydrolyzes cellobiose to glucose[25].

Materials and Methodology

Requirement

The requirements such as chemicals (acids and alkalis) were purchased from S.B. Fine Chemicals, Mumbai; the enzymes (cellulases) were procured from Hi Medias Pvt. Ltd; the organisms were purchased from MTCC, Chandigarh.

Stalk Preparation

The lignocellulosic materials (saw dust, soft wood, sugarcane bagasse, rice straw) are initially chipped, ground and milled. Prior to pretreatment, they were finely powdered using a 3 mm on Laboratory Ball Mill. Once ground, the biomass was stored in a sealed plastic bag at room temperature until pretreatment. Composition of stalk (saw dust, soft wood, sugarcane bagasse, rice straw) before pretreatment is given in Table 1 and the condition requirements for the pretreatment is given in Table 2.

Table 1: Composition of lignocellulosic material (% w/w)

Lignocellusic material	Cellulose	Hemicellulose	Lignin	Ash
Rice straw	39.2	23.5	36.1	12.4
Sugarcane baggase	30.2	56.7	13.4	1.9
Soft wood	43	20	27	2
Saw dust	45.1	28.1	24.2	1.2

Table 2: Conditions, Advantages and Disadvantages of Pretreatment Processs

Pretreatment	Requirement	Conditions	Advantages &Disadvatages
Concentrated acid	H_2SO_4,HCL	Concentrated acid Low temperature	High yields Hydrolysis is mainly included Corrosion problems Material loss due to degradation High demand for chemicals Environmental issues

Diluted acid	H_2SO4	W = 0, 5-2% T > 160 °C	Corrosion problems Low yields Material loss due to degradation
ARP Ammonia recycled percoration	Ammonia	W = 15% T = ~ 170 °C	Media Recoverable Environmental issue due to ammonia
Fungi	White-rot Fungi, Brown-rot Fungi, Soft-rot Fungi		Slow conversion Low energy requirement No chemicals Mild environmental conditions
Bacteria	Spingomonas paucimoblis, Bacillus circulans		Slow conversion Low energy requirement No chemicals Mild environmental conditions
Steam Explosion		P = 2,5-7 MPa T = 180-280 .C	High Yields No corrosion Undesired side products possible High energy demand
AFEX Ammonia Fiber Explosion		Liquid Ammonia T = 90-100 °C	Low inhibitor formation Media recoverable Environmental issues due to ammonia

Steam explosion

Generally steam explosion is a process through which biomass is treated with hot steam (180 to 240 °C) under pressure (1 to 3.5 MPa) which is followed by an explosive decompression of the biomass that results in the rupture of biomass fiber's rigid structure. The sudden pressure released defibrillates the cellulose bundles and this result in a better accessibility of the cellulose for enzymatic hydrolysis and fermentation. Depending on residence time and temperature, steam explosion can result in anything from small cracks in the stalks, to total defibrillation of the stalks[39]. Acetic acid is released from the stalks, and this result in partial hydrolysis of the cell wall components[40]. It has been shown that the use of diluted acids (i.e. sulfuric or nitric acid) can accelerates the process i.e. result in higher hydrolysis rates of the hemicelluloses[41-43].

Here,the stalks are fed from a bin through a screw loading valve in a masonite gun. The stalks are then steam heated at a temperature of about 285°C and a pressure of 3.5 MPa for about 2 min. The pressure is increased rapidly to about 7 MPa (70 bar) for about 5 s, and the stalks are then discharged through restricted orifices (slotted port) and exploded at atmospheric pressure into a pulp.

Ammonia Fiber Explosion (AFEX)

Ammonia explosion or ammonia fiber explosion (AFEX) is a process in whichground, 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 mixtureis incubated from several minutes up to an hour to enable the ammonia to penetrate thelignocellulosic matrix. When the reaction is complete, a valve is opened to explosivelyrelease the pressure.

Sulfuric Acid Pretreatment

Sulfuric acid (H2SO4) at concentrations of 0.5, 1, 1.5 and 2% (w/v) was used to pretreat 10 g ground stalks samples at a solid loading of 10% (w/v). The optimal temperature for the treatment was set to be 90° C and the autoclave temperature was set at 121°C with 15 psi pressure for residence times of 30, 60, and 90 minutes. The collected solids were washed with 750 mL of hot deionized water. Using the parts of the solid residues the total residual weight and lignin, carbohydrate, and moisture content analyses was determined prior to storing at 4°C for enzymatichydrolysis. The filtrates from the lignin content analyses were collected and an HPLC carbohydrate analysis similar to that for the initial composition analysis was performed. The reduction in lignin following pretreatment was calculated based on theinitial dry-weight of lignin in the untreated sample (LU) and the dry-weight of lignin in theremaining solids after pretreatment (LP). In addition, the percentage of solids recovered wascalculated on an oven-dry basis as follows:

$$\% \text{ of solid recovered} = \left(\frac{W2}{W1}\right) * 100$$

Where, W1= dry sample weight of whole biomass before pretreatment (g)
W2= dry sample weight after pretreatment (g)
LU and LP were calculated as follows:

$$LU = \frac{\%LU}{100} * 100$$

Where, %LU = percent acid-insoluble lignin in untreated sample
W = dry sample weight (g)
The percentage of lignin reduction was calculated with the following equation:

$$\% \text{ lignin reduction} = \left(\frac{LU - LP}{LU}\right) * 100$$

WhereLP= dry-weight lignin in pretreated sample
LU = dry-weight lignin in untreated whole biomass sample

And the solubilization of xylan and glucan from the cotton stalks during pretreatment was calculated in the same manner by substituting the appropriate percentages for xylan and glucan.

Sodium Hydroxide Pretreatment

Sodium hydroxide (NaOH) at concentrations of 0.5, 1, 1.5 and 2% (w/v) was used to pretreat 10 g ground stalks samples at a solid loading of 10% (w/v). Optimal pretreatment temperature were set to be90° C and the autoclave temperature was set at 121ºC with 15 psi pressure for residence times of 30, 60, and 90 minutes which were the same as those used for sulfuric acid pretreatment. Theanalyses performed were also similar to those for sulfuric acid.

Enzymatic hydrolysis

Cellulase from *Trichodermareesei*with an activity of 96.3 FPU/mL, supplemented with cellobiase(-glucosidase) from *Aspergillusniger*at a ratio of 1:2 was used for hydrolysis experiments. Enzymatic treatments were performed at a cellulaseactivity of 39-40 FPU/g cellulose. The Filter Paper Unit (FPU) is used to define enzyme activity. The quantity 0.1875 FPU, as defined in LAP-006, is the enzyme activity that will produce reducing sugar equivalent to 1.9-2.0 mg of glucose. Now for hydrolysis stalks are pretreated in the autoclave at 121ºC with15 psis for 60 min with 2% (w/v) sulfuric acid, sodium hydroxide, or hydrogen peroxide were subjected to enzyme hydrolysis. Pretreated samples at 4-5% solids concentration (grams dry weight per 100 mL) in 50 mM acetate buffer with a pH 4.8 containing 40 µg/mL tetracycline (antibiotic against microbial contamination) were pre-incubated in flasks in a water bath shaker at 45-50°C at 150 rpm for 10 minutes. 2.20 and 3.80 mL of cellulase and cellobiase were added to start hydrolysis after temperature acclimation. Aliquots of 2.0 mL were taken after 72 hours where immediately chilled on ice, and centrifuged at 5000 rpmfor 10 min. The supernatant was stored at -20ºC until HPLC sugar analyses for glucose and xylose were performed. The sugar analysis results were used to determine the percent celluloseconversion based on the percent of glucose in the supernatant. The conversion of xylan to xylose was also determined. The percent cellulose conversion was calculated as follows:

$$\% \text{ cellulose conversion} = \frac{\%GH}{\%GP} * 100$$

Where %GH = dry-weight percentage of glucose in enzyme hydrolysis supernatant
%GP = dry-weight percentage glucose in pretreated sample.

Results and Discussion

Cellulose possesses unique structure that varies depending on its sources[44]. Narrow down of vivid lignocellulosic biomass into its utilizable cellulose structure after pretreatment has been studied under SEM. SEM is usually chosen for examining the microstructure of cellulose due to its quick examination with less cumbersome sample preparation but it lacks the required resolution for in depth information[44]. The recovery of cellulose from rice straw, sawdust, soft wood and sugarcane after pretreatment shows an overall increase and there also seemed to be a marginal increase in reduction percentage of lignin after pretreatment. This relative increase in the concentration of cellulose and lignin after pretreatment could be due to removal of hemicelluloses[45].

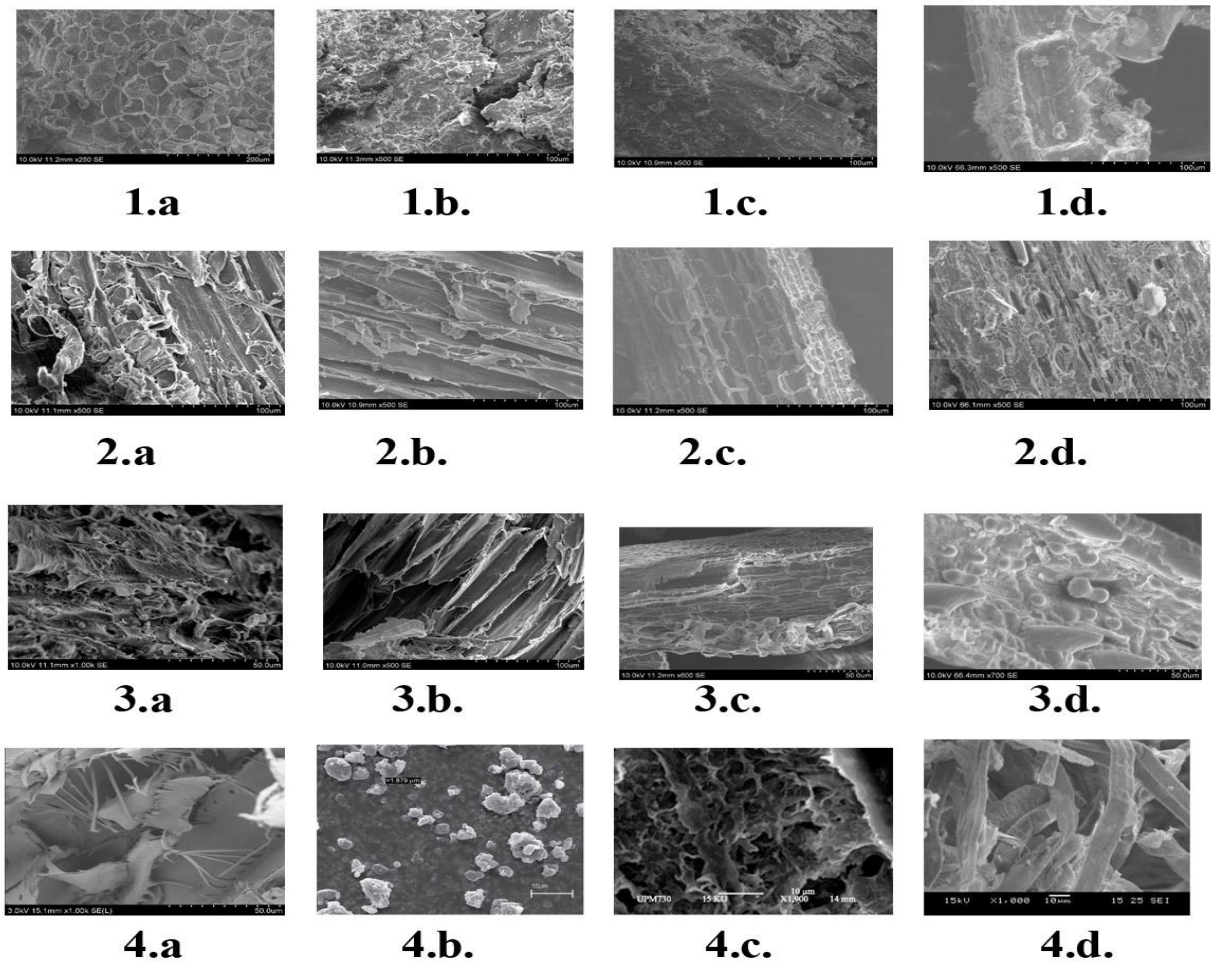
The cellulose percentage from rice straw after enzymatic hydrolysis in case of steam explosion was found to be 41.5g/100g and xylose content was 21.3g/100g; incase of lignin reduction it was 28%. The lignin reduction percentage after acid treatment at 30 mins/90^{o}C was 32%, at 60mins/90^{o}C was 28% and at 90mins/90^{o}C was 16 % for 0.5% H_2SO_4. Similarly for 1% H_2SO_4 at 30 mins/90^{o}C it was 44 %, at 60mins/90^{o}C it was 49% and at 90mins/90^{o}C it was 43%. For 1.5% H_2SO_4 at 30 mins/90^{o}C the value was 55%, at 60mins/90^{o}C it was 61% and at 90mins/90^{o}C it was 51%. In case of 2% H_2SO_4 at 30 mins/90^{o}C was 52%, at 60mins/90^{o}C was 53% and at 90mins/90^{o}C was 50%. During autoclaving temperature (121^{o}C with 15psi) the lignin reduction percentage were found to be 20%, 26% and 38% respectively at 30, 60 and 90mins for 0.5 H_2SO_4; the same for 1% H_2SO_4 were found to be 46%, 50%, 53.5% respectively for 30, 60 and 90mins; in case of 1.5% H_2SO_4 49%,52%,55% were recorded for 30,60 and 90min respectively and at 2% H_2SO_4 55%,56%,57% respectively for the same condition. The amount of lignin from NaOH pretreatment ranged from 12% (30 min, 90°C) to 14% (30 min, 121°C/15psi); 8% (60 min, 90°C) to 11% (60 min, 121°C/15psi); 19% (90 min, 90°C) to 23% (90 min, 121°C/15psi) for 0.5% NaOH. Even in the case of 1% NaOH changes in time and temperature caused no significant changes in the lignin reduction. Whereas for 1.5 and 2% of NaOH the lignin reduction saw a considerable increase with respect to time and temperature i.e from 15% (30 min, 90°C) to 17% (30 min, 121°C/15psi); 16% (60 min, 90°C) to 25% (60 min, 121°C/15psi); 21% (90 min, 90°C) to 24% (90 min, 121°C/15psi). After which the cellulose content as a result of enzymatic hydrolysis were estimated for acid and alkali pretreatment and it was observed that sodium hydroxide treatment at 2%, 60mins, 120^{o}C/ 15 psi had maximum of 48% compared to rest whereas in the case of acid hydrolysis at the same condition 46.5% was obtained. The values of lignin reduction percentage and cellulose recovery was found to be in the range similar to rice straw for sugarcane and the maximum cellulose percentage was 42% at 90 mins 120^{o}C/ 15psi in case of alkali treatment after enzyme hydrolysis. The maximum lignin reduction was seen in alkali treatment at 90^{0}C, 30 mins, 2% NaOH and it was found to be 43% for sugar cane.

Similarly pretreatment of softwood and saw dust obtained the following results; the cellulose percentage from soft wood after enzymatic hydrolysis in case of steam explosion was found to be 57g/100g and xylose content was found to be 23g/100g; lignin reduction was found to be 38%. The lignin reduction percentage after alkali treatment at 30 mins/90^{o}C was 35%, at 60mins/90^{o}C was 26% and at 90mins/90^{o}C was 22 % for 0.5% NaOH. Similarly for 1% NaOH, the range fell as same as 0.5% with very minimal variation. For 1.5% of NaOHat 30 mins/90^{o}C lignin reduction was 55%, at 60mins/90^{o}C was 61% and at 90mins/90^{o}C was 72%. For 2% of NaOHat 30 mins/90^{o}C was 52%, at 60mins/90^{o}C was 59% and at 90mins/90^{o}C was 73%. During autoclaving temperature the lignin reduction percentage were found to be 20%, 26% and 38% respectively for 30, 60 and 90mins for 0.5NaOH; the same for 1%NaOH were found to be 46%, 50%, 59% respectively for 30, 60 and 90mins; in case of 1.5% NaOH 49%, 52%, 55% were recorded for 30, 60 and 90mins respectively and in case of 2% NaOH 56%, 52%, 61% for the same condition. The amount of lignin from acid pretreatment(H_2SO_4) ranged from 15% (30 min, 90°C) to 19% (30 min, 121°C/15psi), 17% (60min,90^{o}C) to 19.5% (60 min, 121°C/15psi), 19% (90min, 90^{o}C) to 22%(90 min,121°C/15psi) for 0.5% H_2SO_4 and in case of 1% H_2SO_4 the values were similar to the former concentration. For 1.5 and 2% H_2SO_4 21% (30 min, 90°C) to 25% (30 min, 121°C/15psi), 22% (60min,90^{o}C) to 30% (60 min, 121°C/15psi), 27% (90min, 90^{o}C) to 32%(90 min,121°C/15psi), with changes in concentration, temperature and time causing the most significant changes in the lignin contents. The cellulose content after enzymatic hydrolysis were estimated for acid and alkali pretreatment and it was observed that sulfuric acid at 2%, 60mins, 15psi had maximum of 43% compared to rest and in the case of sodium hydroxide the same condition gave maximum cellulose i.e. 47% . The values of lignin reduction percentage and cellulose recovery for saw dust was found to be in the range similar to softwood and the maximum cellulose percentage ranges from 36 to 51% in case of acid and

alkali treatment respectively after enzyme hydrolysis. The maximum lignin reduction was seen in alkali treatment at 90^{o}C, 30 mins and it was found to be 42% for saw dust.

The Scanning electron microscope (SEM) studies revealed the details on tissue's ultra structural changes in the respective lignocellulosic biomass as a result of pretreatment using different methods .The SEM images of untreated and treated lignocelluloses are depicted in Figure 2. The morphological changes that take place during pretreatment were studied and it is found that the inhibitory hydrocarbons were removed; crack development on the lignocellulosic fibre and increase in porosity could be seen resulting in enhanced exposure of cellulosic material for effective bioconversion. According to the structural architecture of stalks' SEM analysis enzymatic pretreatment proves to be effective which beholds the reason behind increased sugar production.

Figure 2: SEM images of untreated and treated lignocelluloses; 1(a-d) untreated sawdust, softwood, rice straw and sugarcane; 2(a-d) acid pretreated sawdust, softwood, rice straw and sugarcane; 3(a-d) alkali pretreated sawdust, softwood, rice straw and sugarcane; 4(a-d) enzymatically pretreated sawdust, softwood, rice straw and sugarcane;



Conclusion

Exploring the feedstock availability for the production of biofuels, lignocelulosic biomass was found to be the more proficient source. However its complex structural profile (cellulose, hemicelluloses and lignin) makes the production process more complicated. Hence the technology of pretreatment was inculcated to make the lignocelluloses receptive for the production. Different pretreatment methods to make the lignocelluloses accessible to enzymes have been described and widely studied for improving the biofuel production in this paper. The main goal set by these pretreatment techniques was to make the surface area of the cellulose accessible, improve the crystallinity, degree of polymerization, porosity and the disposal of hemicellulose and lignin which proves that cellulose is the vital component that has high carbohydrate content which is obtained after the enzyme action later to pretreatment. Here, the analysis done using rice straw, sugarcane, softwood and

saw dust, ricestraw showed maximum lignin reduction of 57% at autoclaving condition(90mins) for 2% H_2SO_4 treatment, whereas the cellulose content from enzymatic hydrolysis was greater for NaOH treatment giving an output of 48% at 2%, 60mins, 120^oC/ 15 psi. For sugarcane the maximum cellulose percentage and lignin reduction was 42% at 90 mins, 120^oC/ 15psi and 43% at 90^0C, 30 mins respectively from NaOH treatment (2%). In case of sawdust there was 42% of lignin reduction and 51% of cellulose recovery at 2% NaOH 90^oC, 30 mins and for softwood the maximum output were obtained from sodium hydroxide treatment at 2% of NaOH, at 90mins/90^oC; 73% and 43% respectively for lignin and cellulose. On the whole alkaline treatment at higher temperature gives good amount of lignin and cellulose and softwood proves to be the best source of lignocelluloses compared to the rest of the stalks. Also the Scanning Electron Microscopy(SEM) was analyzed in order to obtain clear-cut picture of the lignocelluloses structure that contributed to efficient lignin removal.

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