“Formation and Effects of inhibitors on fermentations of lignocellulosic hydrolysates”

Thesis on

"Formation and Effects of inhibitors on fermentations of lignocellulosic hydrolysates"

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Gitanjali Sarkar

Abstract

Lignin is a compound polymer contained in woody plant tissue. Banana pseudo-stem is an attractive alternative source of lignocellulosic biomass for biofuel production. However, the formation of inhibitor during hydrolysis and inhibitory by-products during pretreatment of lignocellulose are the main challenges for the biofuel production. Therefore, pretreatment of lignocelluloses is the key step to eliminate hemicelluloses and lignin which increases the cellulose accessibility for enzymatic hydrolysis. These issues are addressed employing alkali pretreatment. In this project work alkali pretreatment is carried out using various concentration of alkali (KOH).Maximum extent of lignin removal (78%) is done by this alkali pretreatment method.KOH having basic nature, it promotes the swelling of lignocelluloses and on the other hand presence of KOH, solubility of lignin also increases.For understanding the morphological changes of cell wall, Scanning electron microscopy (SEM) images of sample are analyzed.SEM images of untreated and pretreated banana stem are used to evaluate the changes of surface structure of cell wall and the effect on removal of ligno-cellulosic biomass.

CONTENTS

CHAPTER-I

Introduction

1. Introduction

With the growing demand of energy more and more focus is given today on alternative sources of energy to supplement petroleum and coal consumption. Renewable energy is now capturing a good share of the worldwide headlines because of concerns about declining supplies of fossil fuels, escalating population and industrialization triggering ever-increasing demand of fuels. All over the world, governments have encouraged the use of alternative sources of energy for looming energy crisis[1].The higher price of oil has attracted the greater attention to biofuels, especially bioethanol, biodiesel, biohydrogen, to list a few. Today, biomass covers about 10% of the world's primary energy demand [2]. In the past few years, a significant attention has been paid to the new sources of vegetable fibers, alternative to wood raw materials, for the pulp and paper applications and biocomposites. The process involving the conversion of biomass into a renewable source of energy is currently being studied in most of the countries in the world [3]. Industrialization of the process for conversion of biomass to an efficient fuel can provide great economic value for a country. India is the second largest producer of fruit in the world [reference]. Banana is the most important fruit crop of India having great socio-economic significance, and it contributes to 27% of world's banana production. It contributed 31% of the total food production in India. Each hectare of banana crop generates nearly 220 tons of plant residues that consist mainly of lignocelluloses material. In countries like India, where 4.796×10^5 ha of banana is cultivated, Banana (*Musa acuminata*), a monocotyledonous annual herbaceous plant, have many uses as food and as fodder[1]. From leaves to flower to the tender inner part of the stem can be used one way or another as food. But the outer layer of the stems, known as pseudo stem, is discarded as it has no significant use. So it can be a used for these types of applications. Farmers discard banana waste into rivers, lake and on roads, causing serious environmental problems [3]. An environmentally friendly solution and alternative economic use for this agricultural residue is needed. The main residuals of banana are leaves and pseudo stem, both containing high levels of lignocelluloses.

Several studies have emphasis on the use of forestry and agricultural residues for the production of lignocellulosic material. Banana stem is used as feedstock for high-value products such as

biofuel, which could replace non-renewable fuel sources, to mitigate the ever-growing co_2 emissions. Banana stem is considered as bioethanol feedstock because of its acceptable content of cellulose (42.2-63%) and low lignin content (8-12%)[2]. While the use of banana stem is particularly attractive for biofuel production, this residue is difficult to convert into bioethanol or high-value molecules, due to its chemical composition and physiochemicalproperties [3].

While the banana stem have higher cellulose content than grasses and wheat straw, representing a rich source of cellulose, the lignocellulosic nature of banana stem means that its cellulose fraction is not easily accessible to the enzyme digestion required for ethanol production [4]. The inherent resistance of lignocellulosic material to enzymatic digestion can be overcome by pretreatments. The most commonly used pretreatment to remove hemicelluloses is the exposure of biomass to alkaline (NaOH) or acid (H_2SO_4) solutions. Also some studies showed that peroxidase pretreatment of lignocellulosic materials removes lignin efficiently, producing reusable solubilized hemicelluloses.

CHAPTER-II

Literature Review

Aim and objectives

Literature Review:

Relevant literatures available on this subject are reviewed and briefly described. This chapter provides some information about the literatures reviewed to perform the research. Here an attempt has been made to identify studies involving production of ethanol from lignocellulosic biomass and various pretreatment methods using banana pseudostemand formation of inhibitors and their effects on fermentation of lignocellulosichydrolysates.A general process of the working is described in chronological manner.

2.1 Composition of biomass

Plants consisting of lignocellulose can be divided in three types according to plant taxonomy: softwood (gymnosperms), hardwood (woody angiosperms) and annual plants, e.g., crops (herbaceous angiosperms). The main component in lignocellulose is holocellulose approx. 60–70%, and is composed of the polysaccharides cellulose and hemicelluloses[5].Cellulose is a high molecular weight glucose polymer, while hemicellulose is composed of various sugars, such as xylose, arabinose, mannose, galactose, and glucose, dependent on the plant material[6].

In addition to holocellulose and lignin, plant materials are composed of extractive (soluble in water or organic solvent) and non-extractive non-cell wall materials (NCWM). The non-extractives are mainly inorganic ash components such as silica and alkalisalts, but also includes pectin, proteins, and starch. Herbaceous material, especially straw has high nonextractiveNCWM with ash contents up to 10%. Ca, Mg, and K are the major inorganic constituents in wood . In addition, Si, Cl, and Na are abundant inherbaceousmaterials[6]. Wood materials have low ash contents (<1%) and contain variable amounts of extractives or secondary metabolites such as resins, terpenes, phenols, quinones, and tannins. The extractives often have protective biological and anti-microbial activities and aid in the chemotaxonomic division of plant species by their specific biosynthetic pathways [5].

2.2 Pretreatment

Most lignocellulose-derived inhibitors form during pretreatment when hemicelluloses and/or lignin are solubilized and degraded[10].Extractives and cellulose that is unintentionally affected by the pretreatment are other sources Since the formation of inhibitory substances is much dependent on the pretreatment process, this review includes a brief discussion on the most commonly used pretreatment techniques, as summarized in. Only pretreatment methods that are relevant with respect to formation of inhibitors and that are of interest for industrial implementation are covered[7].

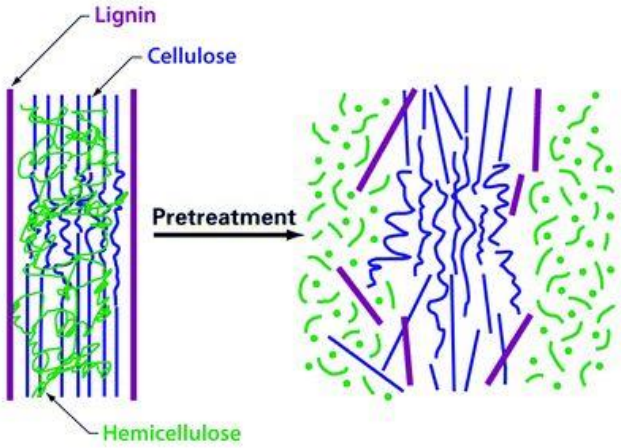

Fig:1 physicochemical deconstruction disrupts the lignocelluloses structure [46]

2.2.1 Acid-based methods

Acid hydrolysis is one of the most promising pretreatmentmethods with respect to industrial implementation. It is usuallyperformed with mineral acids. Dilute sulfuric acid pretreatment has been studiedfor a wide range of lignocellulosicbiomass[8].It results in high recovery of thehemicellulosic sugars in the pretreatment liquid, and in a solidcellulose fraction with enhanced enzymatic convertibility. Acidpretreatment has also some drawbacks, such as high cost of thematerials used for construction of the reactors, gypsum formationduring neutralization after treatment with sulfuric acid, andformation of inhibitory by-products.Steam explosion is a successful pretreatment option thatinvolves heating lignocellulose with superheated steam followedby a sudden decompression[9]. The high-pressure steam modifiesthe cell wall structure, yielding a slurry, which upon filtration rendersa filtrate with hemicellulosic sugars and a cellulose-rich filtercake containing also lignin and residual hemicellulose. Steamexplosion can be assisted by impregnation with an acid catalyst,for instance sulfuric acid or sulfur dioxide[10]. If no impregnatingagent is used, the process is catalyzed through autohydrolysis.Acetic acid and uronic acids released from hemicellulose, and formicand levulinic acids resulting from sugar degradation contribute to acidification, and can inhibit downstream biochemicalprocesses [8].

2.2.2 Hydrothermal processing

Hydrothermal processing is an approach in which water inliquid phase or in vapor phase is used to pretreat lignocellulosicsbiomass [7].It is a relatively mild pretreatmentmethod that does not require any catalysts and does notcause significant corrosion problems. Under high pressure waterpenetrates into the biomass, hydrates cellulose, and removes mostof the hemicelluloses and a minor part of lignin. The solubilizationof hemicelluloses is catalyzed by hydronium ions resulting fromwater auto-ionization. Controlling the pH around neutral valuesminimizes the formation of fermentation inhibitors [9].

2.2.3 Alkaline methods

Alkaline treatment can be used for removing lignin and therebyincreasing the digestibility of cellulose, Compared to acid andhydrothermal processes, mild alkaline pretreatments lead to lesssolubilization of hemicelluloses and less formation of inhibitorycompounds, and they can be operated at lower temperatures.Sodium hydroxide and potassium hydroxide are the most commonlyused forms of alkali, but their cost is a serious limitation.Other suitable forms of alkali are calcium hydroxide and ammonia,which can be used in processes such as lime pretreatment, ammoniarecycled percolation (ARP) and ammonia fiber expansion(AFEX)[11].

2.2.4 Oxidative methods

The use of oxidants for pretreating lignocellulosic biomassallows the reduction of cellulose crystallinity and disruption ofassociation between carbohydrates and lignin[12]. These methodsinclude alkaline peroxide pretreatment, ozonolysis, and wetoxidation. Wet oxidation is achieved by treating biomass withwater and air or oxygen at high temperatures for relatively shorttimes[13]. Hemicelluloses are extensively solubilized, and recoveredmostly as oligosaccharides. Lignin is fragmentized and oxidizedto aliphatic carboxylic acids and phenolic compounds. The combination of wet oxidationwith alkaline compounds minimizes the formation of furan andphenolic aldehydes[14].

2.2.5 Ionic liquid/alternative solvent pretreatment

The use of ionic liquids (ILs), is another alternative forpretreatment of lignocellulosics materials.ILs disrupt the non-covalent interactions between lignocelluloses components without leading to significant degradation[15]. Celluloseregenerated from IL solutions has increased enzymatic convertibility.The development of energy-efficient recycling methods, andthe implementation of effective strategies for recovery of hemicellulosesand lignin from

pretreatment liquids is required for theindustrial application of ILs. Even though the formation ofinhibitors is limited, the minor amounts of ILs remaining in thepretreated materials are potentially toxic to enzymes and fermentative microorganisms[14].

2.3 Hydrolysis & Inhibition

Various methods for the hydrolysis of lignocellulosic materials for ethanol production have recently been described such as acid hydrolysis and enzymatic hydrolysis.

2.3.1 Acid Hydrolysis

The main advantage of the acid hydrolysis is that acids can penetrate lignin without anypreliminary pretreatment of biomass, thus breaking down the cellulose and hemicelluloses polymers to form individual sugar molecules[12]. Several types of acids, concentrated ordiluted, can be used, such as sulphurous, sulphuric, hydrocloric, hydrofluoric, phosphoric, nitric and formic acid.Sulphuric and hydrochloric acids are the most commonly used catalysts for hydrolysis of lignocellulosicbiomass.The acid concentration used in the concentrated acid hydrolysisprocess is in the range of 10-30%. The process occurs at low temperatures, producing high hydrolysis yields of cellulose[16]. However, this process requires large amounts of acids causing corrosion problems to the equipments. The main advantage of the dilute hydrolysis processis the low amount of acid required (2-5%). However this process is carried out at high temperatures to achieve acceptable rates of cellulose conversion. The high temperature increases the rates of hemicellulose sugars decomposition thus causing the formation of toxic compounds such as furfural and 5-hydroxymethyl-furfural (HMF)[17]. These compounds inhibit yeast cells and the subsequent fermentation stage, causing a lower ethanol production rate. In addition, these compounds lead to reduction of fermentable sugar. In addition, high temperatures increase the equipment corrosion [16].

2.3.2 Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose is performed using cellulolytic enzymes. The cellulase enzyme system is a mixture of endo-β-1,4-glucanglucanhydrolases, exo-β-1,4-glucancellobiohydrolases (cellulases) and β-glucosidase[18]. The cellulases break down cellulose to cellobiose, which is subsequently cleaved to glucose by β-glucosidase. The cellulases and β-glucosidase are inhibited by cellobiose and glucose, respectively [19]. Product inhibition of the enzymes decreases the efficiency of hydrolysis. The rate and yield during enzymatic hydrolysis of willow have been shown to decrease because of increased concentrations of non-volatile compounds formed during steam pretreatment, which may accumulate in a process where water streams are recirculated[20]. Enzymatic hydrolysis leads to higher yields of monosaccharides than dilute-acid hydrolysis, because cellulase enzymes catalyse only hydrolysis reactions and not sugar degradation reactions. Enzymes are naturally occurring compounds which are biodegradable and therefore environmental friendly.Prior to enzymatic hydrolysis, the cellulose structure must be made available to the hydrolysing enzymes by pretreating the material. Wood can be pretreated using high-temperature steam whichsolubilises the hemicellulose. In order to improve the recovery of hemicellulose-derived sugars, the wood can be impregnated with SO_2 or H_2SO_4 prior to steam pretreatment[21]. The pretreated wood is then washed to remove the solubilised hemicellulose from the fibrous material.

Enzymatic hydrolysis is a process in which enzymes facilitate the cleavage of bonds in molecules with the addition of the elements of water. It plays an important role in the digestionof food[22]. According to the anion accumulation theory, the anionic form of the acid is captured in the cell and undissociated acid will diffuse into the cell until equilibrium is reached. Since the equilibrium concentration of the undissociated acid is a function of pH, the extent of intracellular anion accumulation will be a function of the pH gradient over the plasma membrane (Russell, 1992). At low extracellular pH, intracellular anion accumulation reaches high levels in *S. cerevisiae* as the yeast maintains a neutral intracellular pH[19]. In media with mixtures of glucose and acetic acid at low pH the accumulation ratio has been shown to increase by a factor of 10–1000 when the pH was decreased from 6.0 to 3.5. The activity of glycolytic

enzymes in the presence of acetic acid has been investigated, showing that enolase was the most sensitive enzyme, and that the inhibition was due to both internal acidification and direct interference with the acid [23].

A lot of research still needs to be carried out on the development and optimization of microbial and enzymatic detoxification of lignocellulosichydrolysate. Much of the research has been carried out on the laboratory-scale and there is little work in pilot-scale and full-scale investigations on the use of enzymes to detoxify lignocellulosichydrolysate before fermentable sugar conversion to chemicals or ethanol. Pilot-scale and full-scale research would facilitate better evaluation of the technology, itsconstraints and opportunities. The cost of the enzymes is of prime importance in order to realize the full potential of detoxification of lignocellulosichydrolysates[24]. The enzymes that are presently being investigated are still expensive because of their cost of production. However, this should not thwart the efforts to carry out more extensive research to identify the most promising enzymes and determine the optimal conditions for their application[25]. In fact, the results of such research should provide the incentive for commercial development to finally produce the enzymes economically on a large-scale. The costs are expected to decrease as technology and techniques advance and as cheaper growth substrates are explored for cultivating the parent micro-organisms. Enzymes to be used in detoxifying lignocellulosichydrolysates do not have to be highly purified fibrous material[26].

2.4 Inhibitors and their formation

An inhibitor is a molecule to be precise enzyme inhibitor molecule binds to a substrate and helps to decrease itsactivity.Not all molecules that bind to enzymes are inhibitors; enzyme activators bind to enzymes and increase their enzymatic activity, while enzyme substrates bind and are converted to products in the normal catalytic cycle of the enzyme[22]. In the actual process, entire process liquid streams must be circulated to minimize the requirement for fresh water and the production of wastewater, the consequence of recirculation is an accumulation of nonmetabolizable compounds in the hydrolysate. These components are referred to as inhibitors

since they may have an inhibitory effect on the process organism. Therefore, knowledge about inhibitors and howto minimize their effects is of the utmost importance forefficient fermentation in real process situations[21].

The inhibitors formed by pre-treatment of lignocellulose depend on both the biomass and the pretreatment conditions such as temperature, time, pressure, pH, redox conditions, and addition of catalysts. In high temperature pre-treatments, the formation of fermentable carbohydrates and degradation products is dependent on a combined severity factor, including reaction temperature, time, and pH. Sugar degradation products—i.e., furfural (from pentoses) and hydroxymethyl furfural (HMF) (from hexoses) —are formed in high concentrations during severe acidic pre-treatment conditions[23]. Acetic acid is ubiquitous in hemicellulose hydrolysates from all lignocellulosics, where the hemicellulose and to some extent lignin is acetylated. Hydroxycarboxylicacids such as glycolic acid and lactic acid are common degradation products from alkaline carbohydrate degradation. Formic acid is a product from sugar and lignindegradation, while levulinic acid is formed by 5-HMF degradation. Other carboxylic acids can also be found in hemicellulose hydrolysates, including aromatic acids[22].

2.4.1 Phenolic compounds

Phenolic compounds partition into biological membranes and cause loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices. Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosichydrolysates, the low molecular weight phenolic compounds being most toxic[25]. However, the mechanism of the inhibiting effect has not been elucidated, largely due to a lack of accurate qualitative and quantitative analyses. Model studies of the inhibitory action of phenolic compounds have been performed using far higher concentrations than are actually present in the hydrolysates[27]. When the results of those studies are interpreted, it should be borne in mind that the water solubility of phenolic compounds is limited. The solubility depends on the composition of the liquid and can be different in hydrolysate and in defined medium. When a high concentration of a certain compound has been used, it is therefore possible that the

concentration actually experienced by the microorganism has been lower[28].Inhibition of fermentation has been shown to decrease when phenolic monomers and phenolic acids were specifically removed from a willow hemicellulose hydrolysate by treatment with the lignin-oxidising enzyme laccase. 4-Hydroxybenzoic acid, vanillin, and catechol were major constituents in the untreated hydrolysate. 4-Hydroxybenzoic acid has been used as a model compound to study the influence of phenolic compounds on fermentation. The choice of 4-hydroxybenzoic acid was based on the abundance in hardwood hydrolysates and its reported inhibitory effect on fermentation with S. cerevisiae (1 g lit^{-1} has been reported to cause a 30% decrease in ethanol yield compared to a reference fermentation)[29]. However, no significant effects on either growth or volumetric ethanol productivity have been detected during fermentation with 2 g lit^{-1} 4-hydroxybenzoic acid. Vanillin constitutes a large fraction of the phenolic monomers in hydrolysates of spruce, pine and willow. Vanillin has been found to be less toxic than 4-hydroxybenzoic acid , and vanillic acid had no effect at concentrations up to 1 g lit^{-1}. S. cerevisiae assimilated vanillin, hydroxybenzaldehyde, and syringaldehyde during fermentation, and growth has been reported on catechol, recorcinol, salicylic acid, and p-hydroxybenzoic acid [30].

2.4.2 Furfural and HMF

Furfural is metabolised by S. cerevisiae under aerobic, oxygen-limited and anaerobic conditions. During fermentation furfural reduction to furfuryl alcohol occurs with high yields. Inhibition of aerobic growth of Pichiastipitis by furfuryl alcohol has beenreported , whereas only slight inhibition of anaerobic growth of S. cerevisiae has been detected[31]. Furfural oxidation to furoic acid by S. cerevisiae occurs to some extent, primarily under aerobic conditions. A metabolite identified as a reaction product between pyruvate and furfural has recently been discovered during fermentation in the presence of furfural[32]. The furfural reduction rate has been shown to increase with increasing inoculum size, and with increasing specific growth rate in chemostat and batch cultures. The reduction rate in anaerobic batch fermentation has been reported to increase with increasing furfural concentration up to approximately 84 mmol g^{-1}, and then decrease again, probably due to cell death at high furfural concentrations[33].

Furfural has been shown to reduce the specific growth rate, the cell-mass yield on ATP, the volumetric, and specific ethanol productivities . Growth is more sensitive to furfural than is ethanol production[34].

NADH-dependent yeast alcohol dehydrogenase (ADH) is believed to be responsible for furfural reduction. Under anaerobic conditions, glycerol is normally produced to regenerate excess NADH formed in biosynthesis. Glycerol production has been shown to be significantly reduced during furfural reduction, suggesting that furfural reduction regenerates NAD+. The fact that less carbon was consumed for glycerol production in the presence of furfural resulted in an increased ethanol yield in the presence of 29 mmol l−1 furfural compared with fermentation in the absence of furfural[35]. Elevated concentrations of acetaldehyde were excreted in the beginning of the fermentation, which was suggested to be due to a decreased NADH concentration in the cell during furfural reduction. Furfural inhibition of glycolytic enzymes in vitro has been reported, and direct inhibition of ADH might have contributed to acetaldehyde excretion. Intracellular acetaldehyde accumulation has been suggested to be the reason for the lag-phase in growth in the presence of furfural[36].

2.5 Effects of inhibitors

By-products of pretreatment of lignocellulose under acidic conditions can be divided into groups on basis of chemical functionality,origin, and effects on the fermenting microorganisms[37].
Carbohydrate degradation products such as the common aliphatic carboxylic acids acetic acid, formic acid, and levulinic acid, and the furan aldehydes furfural and HMF exhibit relatively low toxicity, but can be present in high concentrations depending on the pretreatmentconditions and the feedstock[38]. Due to the low acetyl content, softwood hydrolysateshave relatively low concentrations of acetic acid. Formationof formic acid and levulinic acid occurs at the expense of sugars and it is therefore desirable to use pretreatment conditionsin which the formation of these acids is minimized[37] For these reasonsthe concentrations of aliphatic carboxylic acids in pretreatedsoftwood may be low enough to stimulate rather than inhibit ethanolformation. This is the effect of increased demand for ATP and/orless efficient production of ATP due to uncoupling

of the respiratorychain and the oxidative phosphorylation of ADP, which leadsto increased ATP-generating glycolytic activity at the expense of biomass formation[39]. The use of hardwood and agricultural residueswith high acetyl content as feedstocks as well as the developmentof high-solid processes contribute to makinginhibition by aliphatic carboxylic acids more important[38]. Aromatic carboxylic acids are found within the group of phenylic compounds, which include both phenolic aromaticcarboxylic acids, such as for example ferulic acid and4-hydroxybenzoic acid, and non-phenolic aromatic carboxylic acids, such as cinnamic acid. There are good reasons togroup aromatic carboxylic acids with other phenylic compounds rather than with the aliphatic carboxylic acids[40]. As suggested by the phenylpropanoid structure of some of the aromatic acids, aswell as of the presence of S (syringyl), G (guaiacyl) and H (4-hydroxyphenyl) moieties, these compounds originate from ligninor from hydrolysis of esterified phenols[36]. Furthermore,in contrast with the carbohydrate-derived aliphatic carboxylic acids mentioned in, each of the aromatic carboxylic acidsare present in relatively low concentrations in lignocellulosic hydrolysates, and their inhibitory effect is typically stronger thanthat of the aliphatic carboxylic acids. For example, ferulic acid was inhibitory to *S.cerevisiae* at 0.20 g/L (1.0 mM). As judged by these experiments, the inhibitory effect of ferulic acid would tend to occur at concentrations that are two order of magnitudes lower than those of the common aliphatic carboxylic acids acetic acid, formic acid, and levulinic acid[37]. While pretreated corn stover contained up to 6.6 mg/L(0.033 mM) ferulic acid , up to 210 mg/L(1.1 mM) was found in sugarcane bagasse hydrolysates . Thus, although the concentrations are much lower than those of the common aliphatic carboxylic acids, the much stronger inhibitory effects make it possible that aromatic carboxylicacids contribute to inhibitory effects[38].

The catalytic action of cellulolytic enzymes can be inhibited by non-productive binding to constituents of the solid fraction, such as lignins and residual hemicelluloses[40]. The positive effect on enzymatichydrolysis of cellulose achieved by adding bovine serum albumin could be attributed to prevention of unproductive binding of cellulase onto lignin[41].

Inhibition of cellulases is also caused by soluble carbohydrates and aromatic substances in the pretreatment liquid. Product inhibition of cellulolytic enzymes by monosaccharides, such as

glucose, and disaccharides, such as cellobiose, is a well-known problem. More recently, the inhibitory effects of oligosaccharides derived from xylan andmannan have been investigated[42]. The presence of such oligosaccharides is dependent on the pretreatment method, and also on the potential inclusion of enzymes that degrade hemicellulose-derived oligosaccharides in the enzyme preparation. Solubilized aromatics, such as phenolics, may also affect enzymatic saccharification negatively[43]. Another finding that supports the significance of aromatic substances as enzyme inhibitors is that inhibition of cellulolytic enzymes can be alleviated through addition of sulfur oxyanions, such as sulfite and dithionite, which react with many aromatic compounds but not with sugars[44].

2.6 Removal of degradation products

Several methods have been devised to remove the inhibitors, by addition of activated charcoal (Roberto et.al), Extraction with organic solvents, Ion Exchange , Ion Exclusion, Stream Stripping, Molecular Sieves, Overliming[45].

Detoxification is a costly process,Overliming is the process which is widely used for detoxification. Overliming method can be performed in various ways. Calcium hydroxide (or some other hydroxide) is added to the medium until the pH reaches 10-10.5. After mixing, the resulting precipitate is removed[42]The precipitate consists mainly of calcium salts of low solubility dominated by calcium sulfate. This treatment can be combined with heat, because at elevated temperatures the solubility of calcium sulfate decreases and, in addition, volatile compounds such as furfural are stripped off[40].

Calcium sulfate precipitates acidic compounds. Sulfite is often added at some stage of the detoxification--before or after overliming. Sulfite functions as a reducing agent and it has been suggested that the redox potential of the fermentation broth is of importance for the fermentability[43].

An overview of the different inhibitors formed by pre-treatment of lignocellulosic materials and their inhibition of ethanol production in yeast andbacteria . Different high temperature physical pre-treatment methods are available to render the carbohydrates in lignocellulose accessible for ethanol fermentation[40]. The resulting hydrolyzsates contain substances inhibitory to fermentation—depending on both the raw material (biomass) and the pre-treatment applied.Apart from furans formed by sugar degradation, phenol monomers from lignin degradation are important co-factors in hydrolysate inhibition, and inhibitory effects of these aromatic compounds on different ethanol producing microorganisms can also be found out[45].

Detoxification of the hemicellulose fraction from pretreatedhigh feed stock concentrations is needed in order to achieve reasonable fermentation of the soluble sugars to ethanol. Removal of inhibitory components can be done by extraction, ion exchange, active coal, overliming or laccase and peroxidase treatment[38]. Detoxification methods result in removal of different types of fermentation inhibitory components: steam stripping or evaporation at low pH remove volatile inhibitors such acetic acid and furans. Over-liming (addition of $Ca(OH)_2$ to pH 11) removes the volatile and non-volatile inhibitors such as furans and phenols[40]. The effectiveness of different detoxification procedures has been compared in sprucehydrolysates where over-liming and enzyme treatment with laccase produced the best results[42]. The positive effect of detoxification on fermentation was primarily ascribed to lower furfural and phenol concentrations in the hydrolysates.

Findings	Title of paper	Authors	Journal name/Paper
The banana pseudostemand abundant waste product of banana production has considerable potential as a feedstock for the generation of biofuels and other high-value molecules. Acid and alkaline pretreatments in specific	Acid, alkali and peroxide pretreatments increase the cellulose accessibility and glucose yield of	Felipe Lange Shimizu et al.,	Industrial Crops & products, May 2018

conditions can modify the banana pseudostem accessibility and provide high glucose yields by enzymatic hydrolysis, for fermentation processes.	banana pseudostem.		
This review provides an overview of the formation of inhibitory by-products from lignocellulosic feedstocks as a consequence of using different pretreatment methods and feedstocks as well as an overview of different strategies used to alleviate problems with inhibitors. Novel methods for chemical detoxification without the need for separate process steps have recently been developed and are ready for industrial implementation. Selection and engineering of biocatalysts with improved resistance has progressed with regard to some well-known inhibitors,but more efforts are needed to cover all groups.	Pretreatment of lignocellulose: Formation of inhibitory by-productsand strategies for minimizing their effects	Leif J Jonsson et al	Bioresource Technology, 13 October 2015
This review focuses on inhibitors from lignocellulose feedstocks and how conditioning of slurries and hydrolysates can be used to alleviate inhibition problems. Rapid progress in several areas, such as conditioning or detoxification of slurries and hydrolysates in fermentation technology.	Bioconversion of lignocellulose: inhibitorsand detoxification	Leifn J Jonsson, Bjorn Alriksson and Nils OlofNilvebrant	Biotechnology for Biofuels 2013, 6:16
The inhibitory effect of the main inhibitors (acetic acid, furfural and 5-hydroxymethylfurfural) formedduring steam explosion of wheat straw was studied through ethanol fermentations of model substratesandhydrolysates from	Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichiastipitis*	Carolina Bellido, Silvia Bolado, Mónica Coca, Susana Lucas, Gerardo Gonzalez-Benito,	Bioresource Technology 102 (2011) 10868–10874

wheat straw by *Pichiastipitis*. Experimental results showed that an increase in acetic acid concentration led to a reduction in ethanol productivity and complete inhibition was observed at 3.5 g/L.		Maria Teresa García-Cubero	
An insight of the different inhibitors formed by pre-treatment of lignocellulosic materials and their inhibition effect on ethanol production when yeast and bacteria is given.	Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass	H. B. Klinke . A. B. Thomsen B. K. Ahring	ApplMicrobiolBiotechnol (2004) 66: 10–26
Biological detoxification, fermentation inhibitors, lignocelluloses hydrolysates, adaptation, genetic engineering, bioethanol	Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review	W. Parawira& M. Tekere	Critical Reviews in Biotechnology, 31 May 2010
Alkali treatment of banana fibres has been used for lignin removal. The effects of various experimental parameters, such as alkali concentration, time and temperature, on lignin removal of banana fibres have been ascertained by response surface methodology .	Optimisation of alkali treatment of banana fibres on lignin removal	K J Vishnu Vardhini,RMurugan,C Tamil Selvi& R Surjit	Indian Journal of Fibre& Textile Research Vol. 41, June 2016, pp. 156-160
This review discusses the generation of inhibitors during degradation of lignocellulosic materials, and the effect of these on fermentation yield and productivity. The fermentation process can be improved in several ways. Firstly, the formation of inhibitors can be	Fermentation of lignocellulosichydrolysates. II: inhibitors and mechanisms of inhibition	Eva Palmqvist, Barbel Hahn-Hagerdal	Bioresource Technology14 November 1999

minimised through optimisation of the pretreatment and hydrolysis conditions. Secondly, prediction of fermentability based on analysis of the hydrolysates will be possible, and, thirdly, specific detoxification methods can be developed for efficient removal of inhibitors prior to fermentation of strongly inhibiting hydrolysates.			
In this study, a dilute acid hydrolysate of spruce and an inhibitor cocktail consisting of six known inhibitors were used to investigate different alkali detoxification methods. The various treatments included the addition of calcium hydroxide, sodium hydroxide, potassium hydroxide, and ammonia to pH 10.0 and subsequent adjustment of the pH to 5.5 with either sulfuric or hydrochloric acid as well as treatment with the corresponding amounts of calcium, sodium, and potassium as sulfate or chloride salts at pH 5.5.	Effect of Different Forms of Alkali Treatment on Specific Fermentation Inhibitors and on the Fermentability of Lignocellulose Hydrolysates for Production of Fuel Ethanol	Per Persson, Jessica Anderson, Lo Gorton, Simona Larsson, Nils-OlofNilvebrant, LeifJ.Jonsson	*J. Agric. Food Chem.* 2002**,** *50,* 5318-5325

This aim of this study was to convert banana stem into bioethanol using SSF. Simultaneous saccharification and fermentation (SSF) of acid pretreated banana stem was carried out using different ratios of mixed cultures of *A. niger*, *T. reesei* and *Z. Mobilis* enzymes. At the culture ratios *A. Niger: T. Reesei : Z. mobilis* of 1:1:2 yielded the highest ethanol concentration. The highest ethanol was obtained at pH 5 as compared to pH 4 and pH 6. The highest ethanol (8.51 g/L) was obtained at SSF condition of pH 5	Bioethanol Production From Banana Stem By Using Simultaneous Saccharification and Fermentation (SSF)	Kusmiyati , A Mustofa and Jumarmi	IOP conference series: Materials Science and Engineering, 2018

Table 1: Summary of literature review

2.7 Aims and objectives

1. To identify the inhibitory byproducts formed during pretreatment and hydrolysis of lignocelluloses.
2. To study the dependency on process parameters during formation of the inhibitors in every step.
3. To study the effects of inhibitors on yield and productivity.
4. To establish efficient detoxification techniques to remove these inhibitors.

2.7 Challenges faced

- The banana pseudo stem lignocellulose biomass is difficult to handle because of stickiness and can cause stain.
- The banana pseudo stem is a natural product and is easily degradable. So it has to be stored properly.
- Washing of the lignocellulose biomass during the estimation of lignin and alkali pretreatment becomes hectic when required to bring the pH to neutrality.
- Due to low yield of product after pretreatment of banana pseudo stem,a huge quantity of initial raw material is needed.

2.8 Advantages

- Since India is a major banana producing country banana pseudostem is easily available and is also cheap raw material which is otherwise considered waste.
- In this process we don’t have to invest on any costly equipment and chemicals and is therefore economical.
- Temperature requirement for this process is low and thus saves lot of energy.

CHAPTER-III

Experimental works and Metodology

3. EXPERIMENTAL WORKS

3.1 Sample collection and preparation:

Banana stems were procured from nearby villages.Banana stems werewashed and cleaned from any impurities,, chopped into small pieces, and finally subjected to sun drying for 2 days followed by drying in a hot air oven for 5 to 6 hours at a temperature of 100-110℃ consecutively in order to remove extraneous moisture. After sufficient drying, the dried matter was passed through a Wiley mill for particle size reduction. The ground sample is passed through a 10 mesh screen. The undersized particles were kept in airtight containers until further use. The following flow sheet (Fig 1) describes the process pictorially.

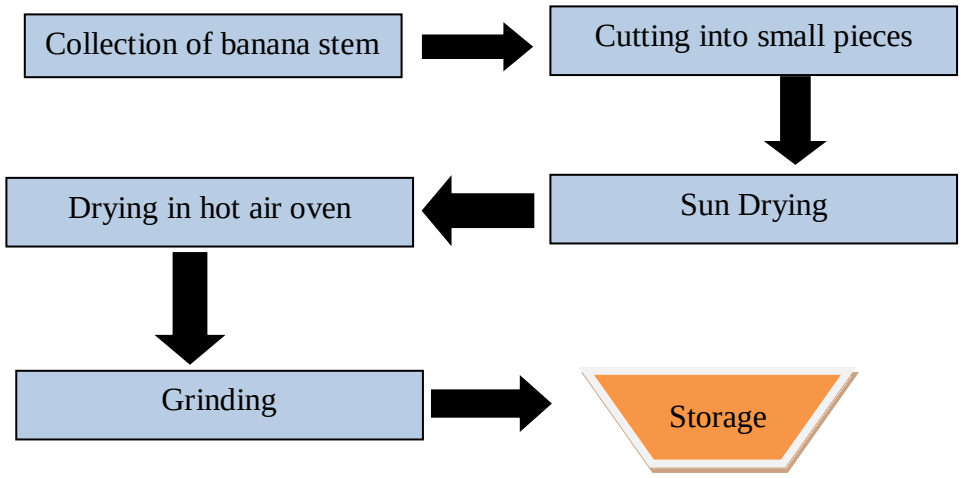

Fig- 3.1 Flow sheet of Sample preparation

3.2 Analytical methods

Finely ground banana sample have been taken and subjected to hot air oven at a temperature of 105℃ for 1 hour for moisture content determination and at 450℃ in muffle furnace for 30 minutes followed by 775℃ for 1 hour for ash content determination. Volatile matter has been estimated by subjecting sample to muffle furnace at 925^0C for 7 minutes. Fixed carbon was also estimated by deducting the sum of moisture content, ash content and volatile matter from 100. The standard protocol from Fuel Research Board and British Standard Institutions is followed to perform proximate analysis and to determine calorific value of the sample. All the analysis is done in triplicates.

Parameters	Values
Moisture content(%)	9.23
Volatile matter(%)	57.92
Ash(%)	10.22
Fixed carbon(%)	22.63
Calorific value (cal/g)	5286.81

Table- 3.1 Proximate analysis and calorific value of the banana stem

3.3 Lignin estimation (using TAPPI method)

Lignin content of the used banana stem Is measured by following TAPPI T222 om02 protocol [reference].2 grams of extracted fibre sample were placed in a flask and 20 mL of 72% sulphuric acid was added. Themixture was stirred frequently for two hours at 20℃ followed by addition of 1550 mL of distilled waterto the mixture. Then the mixture was boiled for next 4hours and cooled. After 24 h, the lignin was transferred to the crucible and washed with hot water repeatedly untill it becomes acid free. The collected lignin was dried at 110℃, cooled down at

room temperature and weighed. Thedrying and weighing were repeated till constant weight was achieved, and the lignin decomposition was calculated using the following equation:

Lignin decomposition = $(M_0-M_1) / M_0 \times 100$ ……. (1)

Where$\mathbf{M_0}$is the lignin content of the untreated banana stems and $\mathbf{M_1}$ the residual lignin content of the degummed banana fibre. All the estimations are done in triplicate.

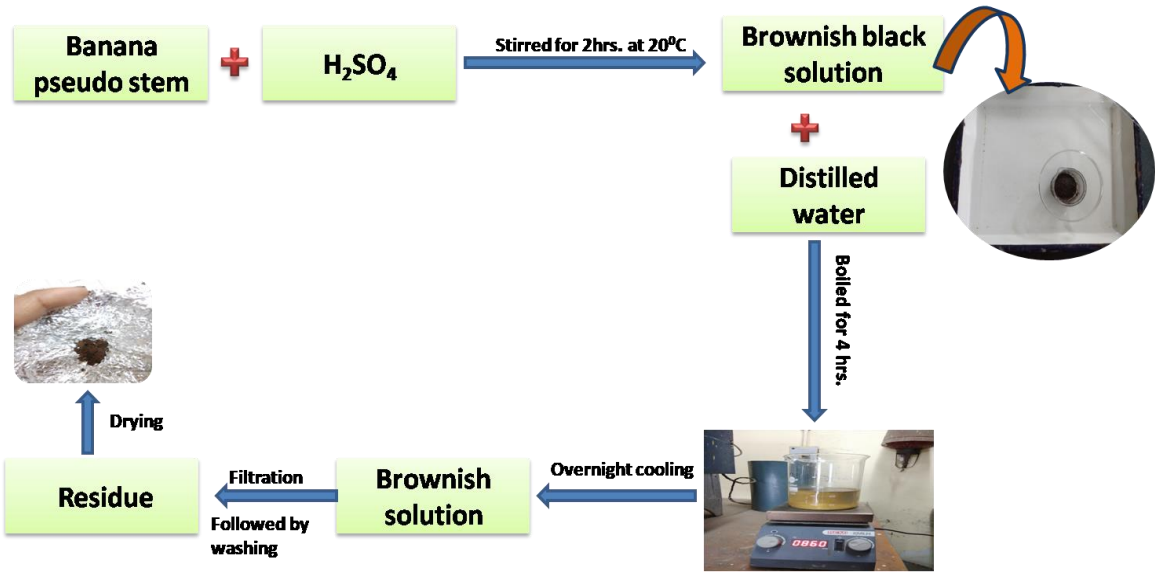

Fig- 3.2 Schematic diagram of estimation of lignin from banana pseudo stem

3.4 Pretreatment method

3.4.1 Varying the concentration of KOH

For alkaline pretreatment, 10 g of dried and milled samples were transferred to 500 mL glass bottles and mixed with solutions of 2, 5, 11, 20, 25 or 30% KOH (m/m).

After homogenization, samples were kept in hot plates for 3 hours at a temperature of 80°C and allowed to cool down at room temperature, and the soluble and solid (insoluble) fractions were separated by filtration using a paper filter. The solid fraction was washed with deionized water to reach pH= 7, dried in an oven at 45°C for 48h and stored in plastic bags until further analysis.

KOH (%)	Lignin (%)	WIS (%)
Untreated (KOH=0%)	14.5	-NA
2	12.1	43.5
5	11.3	39.5
11	8.6	36.3
20	5.4	26.5
30	3.2	23.2

Table No 3: Lignin and WIS (water insoluble solid) % after alkali pretreatment by varying concentration (2-30%) of KOH respectively

CHAPTER-IV

Result and Discussion

4. RESULTS AND DISCUSSIONS

4.1 Effect of KOH on Lignin Concentration

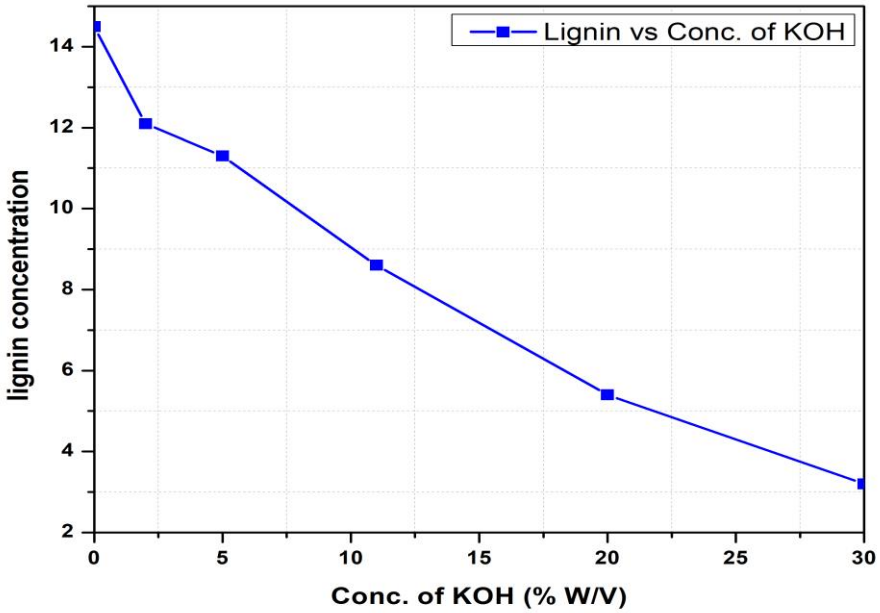

Fig- 4.1 Lignin concentration vs. Concentration of KOH (%w/v)

From the Fig-4.1 it can be seen that with increase in concentration of KOH lignin percentage decreases gradually. Initially lignin concentration was 14.5 %. After pretreatment, the concentration of lignin decreases to 3.2%. Pretreatment is carried out at different concentrations of KOH. Therefore, it can be concluded that with increase in conc. of KOH cellulose accessibility increases for enzymatic digestion and helps bioethanol production.

4.2 Effect of KOH concentration on WIS

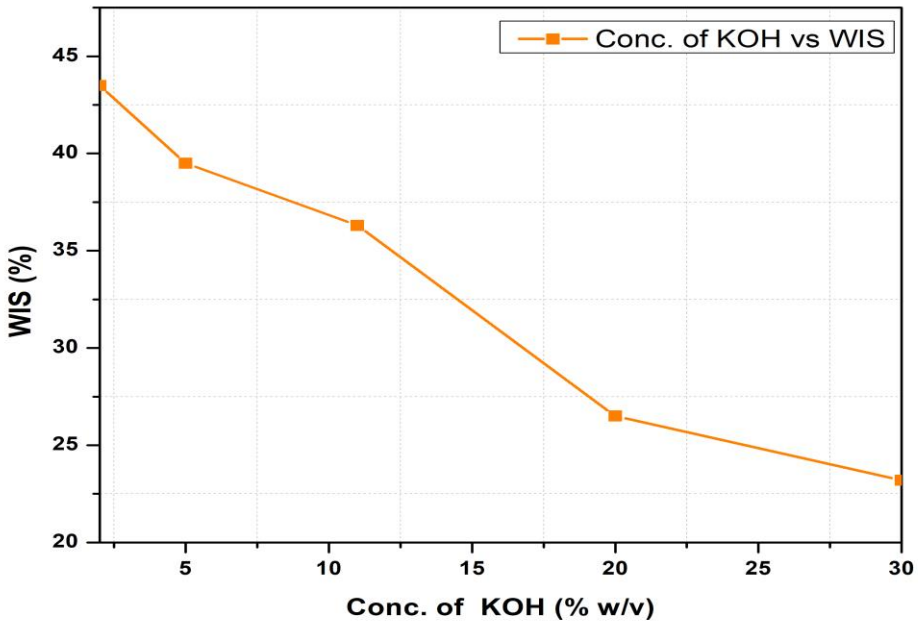

Fig- 4.2 WIS percentage vs. Conc. of KOH plot

From the Fig- 4.2 it can be seen that with increase in conc. of KOH, percentage of WIS (water insoluble solid) decreases. Initially WIS percentage was 43.5 and decreases to 23.2. This is carried out by varying KOH concentration.

Moreover, the low lignin content indicates that the banana pseudo stem is likely to be less recalcitrant than other lignocellulosic materials.

4.3 Structural Characterization: Scanning Electron Microscopy (SEM)

SEM was on done in a Hitachi S-3400 scanning electron microscope to analyze the structural characterization of different samples.

Scanning electron microscopy (SEM) analysis was used to evaluatethe changes in the cell wall surface morphology in pretreated bananapseudostem samples and untreated sample. The SEM analysis is performed with 2-5% alkaline pretreated samples to observe removal of lignin from lignocellulosic biomass of banana pseudo stem. Untreated banana pseudostem had a smooth surface with an undamaged fibrous organization (Fig. 3c), which appeared onlyslightly modified by pretreatment with a low concentration (2%) of alkali. However, when samples were pretreated with a slightly higher concentration of alkali(2-5%), a stronger morphological change was observed, compared with untreated samples(Fig 3a, 3b, 3c). The removal of biomass component such as lignin by the alkaline pretreatment resulted in morphological surface modification of banana pseudo stem samples. Damaged surfaces were no longer smooth, showing clarification and were clearly fragmented in appearance.

Three samples viz. untreated banana,2% KOH pretreated & 5% KOH pretreated pseudostem was studied by Hitachi S3400 and the results are shown below.

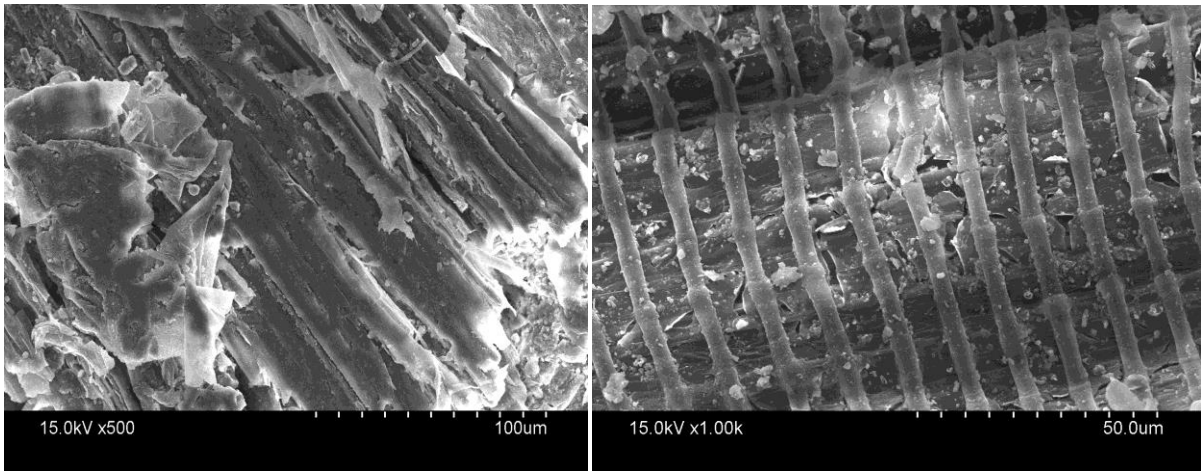

Fig- 4.3 Untreated Sample

The above image shows that the cellulose is having structured nature due to lignin and hemicelluloses bonded with the cellulose. The lining on the surface gives us an idea of the internal compactness of the fibers inside the banana pseudo stem biomass.

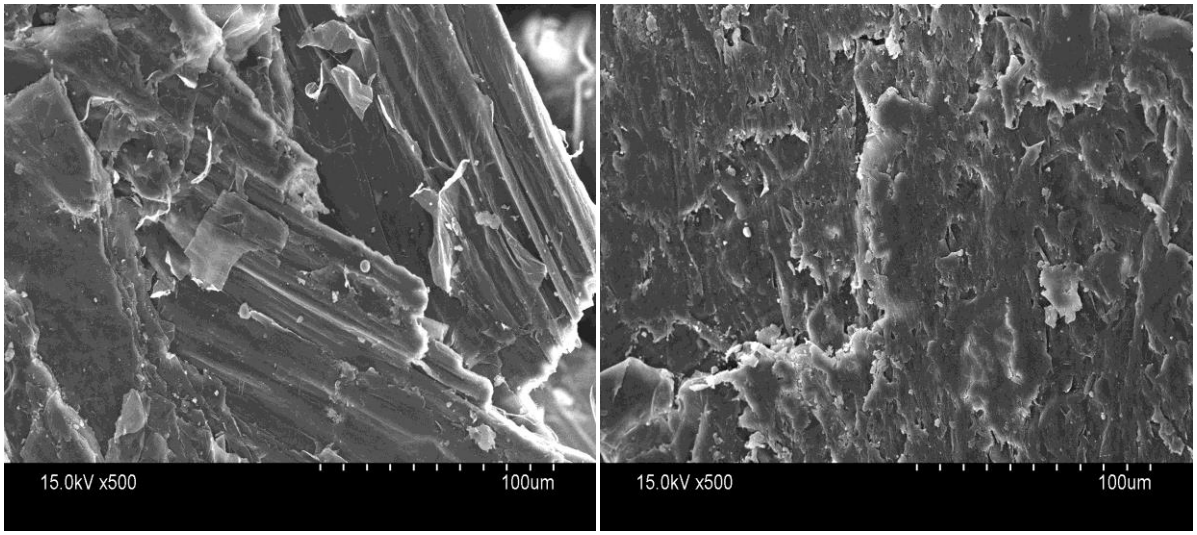

Fig- 4.4 2% KOH pretreated sample

With the pretreated sample with 2% KOH we can see a little tearing of the fibers exposing the cellulose. It can be also seen that the morphological structure of the present sample has changed than the previous untreated sample.

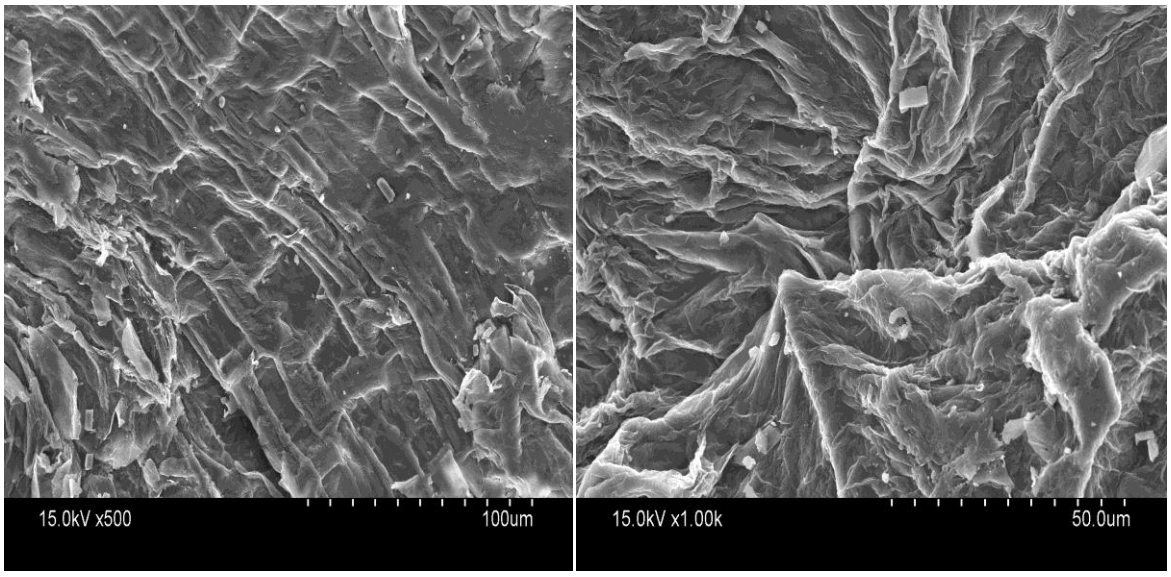

Fig- 4.5 5% KOH pretreated sample

The above SEM image shows greater changes in the structure than the untreated sample. It shows greater degradation of the cell walls and thus greater exposure of cellulose for enzyme digestion.

CHAPTER-V

Conclusion

5.1 CONCLUSION

Lignocellulosic biomass such as banana pseudo-stem contains good amount of cellulose. But this cellulose is very difficult to extract for enzymatic digestion due to the presence of lignin and hemicelluloses. To rupture the bonds between the lignin, hemicelluloses and cellulose, pretreatment is done by alkaline medium which in our case is KOH. Pretreatment is carried out by varying concentration of KOH. By alkaline pretreatment lignin percentage decreases by 78% is done. Water insoluble solid also decreases by this method of alkali pretreatment. SEM images of untreated and pretreated banana pseudo stem are also analysed. It observed that with increase in KOH concentration cell wall is ruptured and lignin concentration decreases.

5.2 Future Work

Over consumption of natural resources leading to the decrease of fossil fuels and the high demand of food, energy, chemicals will pose a serious threat to the mankind in the future. Economic feasibility of the various technologies for development of chemicals by efficient conversion of biomass as fuels and energy production is of prime importance.

The savings of energy and the emission of the green house gases are reasons for employment of bio-based polymers for socio-economic and environmental causes.

Lignocellulosic materials can be effectively be utilized to synthesize value added biochemicals and in this project pretreatment of lignocelluloses is done for mitigating hemicelluloses and lignin formation by stopping the formation of inhibitor during hydrolysis.

Further tuning and optimization by changing the operational parameters for increasing the extent of lignin removal and to enhance the efficiency of the process is crucial for industrialization of the bio-refining processes.

CHAPTER-VI

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