STUDIES ON INHIBITORS FORMED DURING THE PRODUCTION OF LIGNOCELLULOSIC ETHANOL

A thesis

submitted towards the partial fulfilment of a Master of Engineering degree in Bioprocess Engineering

by

SUBHAJIT MONDAL

Exam Roll No: M4BPE23007

Class Roll No.: 002110303006

Registration No.: 147664

Session: 2021-2023

Under the guidance of

Dr. Chanchal Mondal

&

Ms Sujata Sardar

DEPARTMENT OF CHEMICAL ENGINEERING

JADAVPUR UNIVERSITY

Kolkata - 700032

Faculty of Engineering and Technology
Jadavpur University
Kolkata-700032

CERTIFICATION FOR RECOMMENDATION

This is to certify that the thesis entitled "STUDIES ON INHIBITORS FORMED DURING THE PRODUCTION OF LIGNOCELLULOSIC ETHANOL" is a bonafide work carried out by SUBHAJIT MONDAL under my supervision and guidance for partial fulfilment of the requirement for Post Graduation Degree of Masters of Engineering in Bioprocess Engineering during the academic season 2021-2023

Dr. Chanchal Mondal
Associate Professor
Chemical Engineering Department
Jadavpur University
Kolkata-700032

Ms. Sujata Sardar
Assistant Professor
Chemical Engineering Department
Jadavpur University
Kolkata-700032

Head Of the Department (HOD)
Chemical Engineering Department
Jadavpur University
Kolkata-70003

Dean
Faculty of Engineering and Technology
Jadavpur University

CERTIFICATE OF APPROVAL

The forgoing thesis is hereby approved as a creditable study of an Engineering subject carried out and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned does not necessarily endorse or approve any statement made opinion expressed or conclusion drawn therein but approves the thesis only for the purpose for which it has been submitted.

Dr. Chanchal Mondal
Associate Professor
Chemical Engineering Department
Jadavpur University
Kolkata-700032

Signature of Examiner

Ms. Sujata Sardar
Assistant Professor
Chemical Engineering Department
Jadavpur University Kolkata-
700032

DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the Master of Engineering in Bioprocess Engineering" studies. All information in this document have been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to research work.

Name: SUBHAJIT MONDAL

Registration No.: 147664

Thesis Title: STUDIES ON INHIBITORS FORMED DURING PRODUCTION OF LIGNOCELLULOSIC ETHANOL

Signature:

Date:

ACKNOWLEDGEMENT

I would like to take this opportunity to express my deep gratitude and sincere thanks to each and Every one, for helping me in some way or the other, towards the completion of my research work and making this thesis possible. I convey my utmost respect to every person.

In the first place, I wish to express my deepest sense of gratitude, appreciation, and trustworthiness to my M.E supervisors Dr. Chanchal Mondal and Ms. Sujata Sardar for their patient guidance, monitoring, and suggestions throughout the due course of my research work. This thesis would not have been possible without their support.

I would like to thank them for encouraging, inspiring, and enriching me with the necessary research skills, which is desirable for a researcher. Their professional approach, constructive criticism, and insightful comments helped me immensely in the completion of this thesis. I am indebted to them for providing their valuable time, fruitful discussions and motivating me to explore new ideas and opportunities during my investigation. Under their supervision, I grew professionally, which I believe, will have a far-reaching impact on my career. It was a great honor and privilege to work under their esteemed guidance.

I am thankful to my present head of the Department of Chemical Engineering for their kind support. I also grate full to the faculty members of the Department of Chemical Engineering of Jadavpur University, for their development support, encouragement, and assistance

I acknowledge with thanks to the Central Instrument Facility, Department of Chemical Engineering Jadavpur University, for providing me necessary instrument facility which has been very important in this research work.

I would like to thank all my lab members like, Mr. Saswata Chakraborty, Mr. Rahul Shaikh, for their suggestions in performing experiments, cooperative assistance, and friendly support during my stay at Jadavpur University.

Further, I would like to thank all my seniors, juniors, and friends like Mr. Suvrajit Dutta Gupta, Ms. Somi Ali, Ms. Modura Roy, Mr. Sudip Haldar, Mr. Purushatam Gayen, Mr. Ayantan Biswas, Mr. Prasanta Kundu.

And biggest, I would like to thank My parents and Family members for fully supporting and encouraging me in my education carrier. If without support I am not complete my master's degree course. And special thanks to my elder sister Ms. Laxmi Mondal and elder brother mr. Bablu Mondal fully support and encourage me in my education carrier. Anther Special thanks to My Grand Father he is fully supported and encouraged me in my education carrier.

I thank the Almighty for giving me the strength and patience to work through all these years so that I can stand proudly with my head held high today.

Thank You

Subhajit Mondal

CONTENT

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. 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. 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. Industrialization of the process for conversion of biomass to an efficient fuel can provide great economic value for a country. Lignocellulosic biomass is a term used to describe a variety of plant materials that are rich in cellulose, hemicellulose, and lignin. This type of biomass is derived from non-food crops, such as trees, grasses, and agricultural residues, and can be used as a sustainable and renewable source of energy and other value-added products.

In this essay, we will explore the various aspects of lignocellulosic biomass, including its composition, properties, and potential applications.

Composition of Lignocellulosic Biomass:

Lignocellulosic biomass is composed of three main components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are polysaccharides that make up the structural framework of plant cell walls, while lignin provides strength and rigidity to the cell walls.

Cellulose is a linear polymer of glucose are connected by β-1,4 glycosidic bonds. It is the most abundant biopolymer on earth, and can be found in the cell walls of all plants. The primary function of cellulose is to provide mechanical support and protection to the plant cells. The structure of cellulose consists of long chains of glucose units that are arranged in a parallel fashion, forming microfibrils that are held together by hydrogen bonds.

Hemicellulose, on the other hand, is a more complex polysaccharide that is composed of various monosaccharides, including xylose, arabinose, mannose, galactose, and glucuronic acid. Unlike cellulose, hemicellulose is not a linear polymer and has a more branched structure [1]. Hemicellulose is also found in the cell walls of plants and serves as a glue-like substance that holds the cellulose fibers together. In addition to its structural role, hemicellulose can also be hydrolyzed into simple sugars, which can then be fermented into biofuels [5].

Lignin is a complex polymer that is composed of three main monolignols: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These monolignols are polymerized through oxidative coupling reactions, forming a complex three-dimensional network that is highly resistant to degradation [2]. Lignin is highly branched and forms a matrix around cellulose and hemicellulose in plant cell walls. The composition of lignin varies depending on the plant species and growth conditions [6].

Apart from these three major polymers, lignocellulosic materials also contain other minor components such as extractives, ash, and proteins. Extractives are non-polymeric components such as resins, waxes, and oils that can be removed by solvents. The amount and composition of extractives vary depending on the plant species and growth conditions. Ash is the inorganic residue left after combustion of the material. The ash content of lignocellulosic materials varies depending on the plant species and growth conditions. Proteins are also present in small amounts in lignocellulosic materials and can be removed by chemical or enzymatic treatments.

The chemical composition of lignocellulosic materials can be determined by various analytical methods such as proximate analysis, ultimate analysis, and spectroscopic techniques. Proximate analysis provides information on the amount of moisture, volatile matter, fixed carbon, and ash in the material. Ultimate analysis provides information on the elemental composition of the material, including carbon, hydrogen, nitrogen, sulphur, and oxygen. Spectroscopic techniques such as High-Performance Liquid Chromatography (HPLC), X-ray Diffraction (XRD), can be used to identify the functional groups and chemical bonds present in the material [7]

In addition to the major components, lignocellulosic materials also contain various types of structural features such as pits, rays, and vessels. Pits are small openings in the cell walls that allow for the exchange of water and nutrients between adjacent cells. Rays are radial structures that extend from the centre of the tree to the bark and are composed of parenchyma cells. Vessels are tubular structures that transport water and nutrients throughout the plant.

Properties of Lignocellulosic Biomass:

The properties of lignocellulosic biomass can vary depending on its source, age, and processing conditions [3]. However, some of the general properties of lignocellulosic biomass are discussed below:

❖ High carbon content: Lignocellulosic biomass contains a high amount of carbon, making it an attractive feedstock for biofuel production.

❖ Low moisture content: Lignocellulosic biomass typically has a low moisture content, which makes it easier to handle and store.

❖ High ash content: Lignocellulosic biomass contains a significant amount of inorganic materials, such as potassium, calcium, and magnesium, which can result in high ash content.

❖ High heating value: Lignocellulosic biomass has a high heating value, which makes it a valuable feedstock for bioenergy production.

❖ High lignin content: Lignin is a highly recalcitrant compound that is resistant to degradation, which can make lignocellulosic biomass more difficult to process.

2. 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. A general process of the working is described in chronological manner.

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].

Acid-based methods

Acid hydrolysis is one of the most promising pretreatment methods with respect to industrial implementation. It is usually performed with mineral acids. Dilute sulfuric acid pretreatment has been studied for a wide range of lignocellulosic biomass [8]. It results in high recovery of the hemicellulosic sugars in the pretreatment liquid, and in a solid cellulose fraction with enhanced enzymatic convertibility. Acid pretreatment has also some drawbacks, such as high cost of the materials used for construction of the reactors, gypsum formation during neutralization after treatment with sulfuric acid, and
formation of inhibitory by-products. Steam explosion is a successful pretreatment option that involves heating lignocellulose with superheated steam followed by a sudden decompression [9]. The high-pressure steam modifies the cell wall structure, yielding a slurry, which upon filtration renders a filtrate with hemicellulosic sugars and a cellulose-

rich filtrate cake containing also lignin and residual hemicellulose. Steam explosion can be assisted by impregnation with an acid catalyst, for instance sulfuric acid or sulphur dioxide [10]. If no infusing agent is used, the process is catalysed through autohydrolysis. Acetic acid and uronic acids released from hemicellulose, and formic and levulinic acids resulting from sugar degradation contribute to acidification, and can inhibit downstream biochemical processes[8].

Hydrothermal processing

Hydrothermal processing is an approach in which water in liquid phase or in vapor phase is used to pretreat lignocellulosics biomass [7]. It is a relatively mild pretreatment method that does not require any catalysts and does not cause significant corrosion problems. Under high pressure water penetrates the biomass, hydrates cellulose, and removes most of the hemicelluloses and a minor part of lignin. The solubilization of hemicelluloses is catalysed by hydronium ions resulting from water auto-ionization. Controlling the pH around neutral values
minimizes the formation of fermentation inhibitors [9].

Alkaline methods

Alkaline treatment can be used for removing lignin and thereby increasing the digestibility of cellulose, compared to acid and hydrothermal processes, mild alkaline pretreatments lead to less solubilization of hemicelluloses and less formation of inhibitory compounds,

and they can be operated at lower temperatures. Sodium hydroxide and potassium hydroxide are the most used 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, ammonia recycled percolation (ARP) and ammonia fiber expansion (AFEX)[11].

Oxidative methods

The use of oxidants for pre-treating lignocellulosic biomass allows the reduction of cellulose crystallinity and disruption of association between carbohydrates and lignin [12]. These methods include alkaline peroxide pretreatment, ozonolysis, and wet oxidation. Wet oxidation is achieved by treating biomass with water and air or oxygen at high temperatures for relatively short times [13]. Hemicelluloses are extensively solubilized, and recovered mostly as oligosaccharides. Lignin is fragmentized and oxidized to aliphatic carboxylic acids and phenolic compounds. The combination of wet oxidation with alkaline compounds minimizes the formation of furan and phenolic aldehydes [14].

Ionic liquid/alternative solvent pretreatment

The use of ionic liquids (ILs), is another alternative for pretreatment of lignocellulosic materials. ILs disrupt the non-covalent interactions between lignocelluloses components without leading to significant degradation[15].

Cellulose regenerated from IL solutions has increased enzymatic hydrolysis. The development of energy-efficient recycling methods, and the implementation of effective

strategies for recovery of hemicelluloses and lignin from pretreatment liquids is required for the industrial application of ILs. Even though the formation of inhibitors is limited, the minor amounts of ILs remaining in the pretreated materials are potentially toxic to enzymes and fermentative microorganisms [14].

Hydrolysis & Inhibition

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

Acid Hydrolysis

The main advantage of the acid hydrolysis is that acids can penetrate lignin without any preliminary pretreatment of biomass, thus breaking down the cellulose and hemicelluloses polymers to form individual sugar molecules [12]. Several types of acids, concentrated or diluted, can be used, such as sulphurous, sulphuric, hydrochloric, hydrofluoric, phosphoric, nitric, and formic acid. Sulphuric and hydrochloric acids are the most used catalysts for hydrolysis of lignocellulosic biomass. The acid concentration used in the concentrated acid hydrolysis process 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 equipment. 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].

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 environmentally friendly. Prior to enzymatic hydrolysis, the cellulose structure must be made available to the hydrolysing enzymes by pre-treating the material. Wood can be

pretreated using high-temperature steam which solubilises 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 digestion of 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. 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 lignocellulosic hydrolysate. 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 lignocellulosic hydrolysate before fermentable sugar conversion to chemicals or ethanol. Pilot-scale and full-scale

research would facilitate better evaluation of the technology, its constraint and opportunities. The cost of the enzymes is of prime importance in order to realize the full potential of detoxification of lignocellulosic hydrolysates [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 lignocellulosic hydrolysates do not have to be highly purified fibrous material [26].

Inhibitors and their formation

An inhibitor is a molecule to be precise enzyme inhibitor molecule binds to a substrate and helps to decrease its activity. 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 how to minimize their effects is of the utmost importance for efficient 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 lignocellulosic, where the hemicellulose and to some extent lignin is acetylated. Hydroxycarboxylic acids such as glycolic acid and lactic acid are common degradation products from alkaline carbohydrate degradation. Formic acid is a product from sugar and lignin degradation, while levulinic acid is formed by 5-HMF degradation. Other carboxylic acids can also be found in hemicellulose hydrolysates, including aromatic acids [22].

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 lignocellulosic hydrolysates, 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 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 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, resorcinol, salicylic acid, and p-hydroxybenzoic acid [30].

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 *Pichia stipitis* by furfuryl alcohol has been reported, 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].

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 pretreatment conditions and the feedstock [38]. Due to the low acetyl content, softwood hydrolysates have relatively low concentrations of acetic acid. Formation of formic acid and levulinic acid occurs at the expense of sugars and it is therefore desirable to use pretreatment conditions in which the formation of these acids is minimized [37] For these reasons the concentrations of aliphatic carboxylic acids in pretreated softwood may be low enough to stimulate rather than inhibit ethanol formation. This is the effect of increased demand for ATP and/or less efficient production of ATP due to uncoupling of the respiratory chain and the oxidative phosphorylation of ADP, which leads to increased ATP-

generating glycolytic activity at the expense of biomass formation [39]. The use of hardwood and agricultural residues with high acetyl content as feedstocks as well as the development of high-solid processes contribute to making inhibition by aliphatic carboxylic acids more important [38]. Aromatic carboxylic acids are found within the group of phenylic compounds, which include both phenolic aromatic carboxylic acids, such as for example ferulic acid and4-hydroxybenzoic acid, and non-phenolic aromatic carboxylic acids, such as cinnamic acid. There are good reasons to group 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, as well as of the presence of S (syringyl), G (guaiacyl) and H(4-hydroxyphenyl) moieties, these compounds originate from lignin or from hydrolysis of esterified phenols [36]. Furthermore, in contrast with the carbohydrate-derived aliphatic carboxylic acids mentioned in, each of the aromatic carboxylic acids are present in relatively low concentrations in lignocellulosic hydrolysates, and their inhibitory effect is typically stronger than that 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 [41]. 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 carboxylic acids 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 lignin and residual hemicelluloses [42]. The positive effect on enzymatic hydrolysis of cellulose achieved by adding bovine serum albumin could be attributed to prevention of unproductive binding of cellulase onto lignin[43] .

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 and mannan have been investigated [44]. The presence of such oligosaccharides is dependent on the pretreatment method, and 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 [45]. Another finding that supports the significance of aromatic substances as enzyme inhibitors is that inhibition of cellulolytic enzymes can be alleviated through addition of sulphur oxyanions, such as sulphite and dithionite, which react with many aromatic compounds but not with sugars [46].

Removal of degradation products

Several methods have been devised to remove the inhibitors, by addition of activated charcoal , Extraction with organic solvents, Ion Exchange, Ion Exclusion, Stream Stripping, Molecular Sieves, Overlining[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 [46]. 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 sulphate decreases and, in addition, volatile compounds such as furfural are stripped off[47].

Calcium sulphate precipitates acidic compounds. Sulphite is often added at some stage of the detoxification--before or after overtiming. Sulphite functions as a reducing agent and it has been suggested that the redox potential of the fermentation broth is of importance for the fermentability [46].

An overview of the different inhibitors formed by pre-treatment of lignocellulosic materials and their inhibition of ethanol production in yeast and bacteria. Different high temperature physical pre-treatment methods are available to render the carbohydrates in lignocellulose accessible for ethanol fermentation [48]. The resulting hydrolysates 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 pretreated high 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 [49]. 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 [50]. The effectiveness of different detoxification procedures has been compared in spruce hydrolysates where over-liming and enzyme treatment with laccase produced the best results [46]. The positive effect of detoxification on fermentation was primarily ascribed to lower furfural and phenol concentrations in the hydrolysates.

Mechanisms of inhibition:

Fermentation of lignocellulosic hydrolysates is an important process for the production of biofuels and other bioproducts. However, the presence of inhibitors in the hydrolysates can greatly reduce the efficiency and yield of the fermentation process. In this response, we will discuss the various inhibitors that can be present in lignocellulosic hydrolysates and their mechanisms of inhibition [8].

Furfural is produced by the dehydration of pentoses during acid hydrolysis and can also accumulate in high concentrations in the hydrolysate. Furfural inhibits fermentation by reacting with the cellular components, such as proteins and nucleic acids, and disrupting the cellular metabolism.

5 hydroxy methyl furfural (HMF) [11], is also produced during acid hydrolysis and can inhibit fermentation by acting as a non-competitive inhibitor of various enzymes involved in cellular metabolism.

The mechanisms of inhibition of these inhibitors can vary depending on the microorganism being used for fermentation [12]. For example, some microorganisms, such as Saccharomyces cerevisiae, are more tolerant to acetic acid than others, such as *Escherichia coli*. Similarly, some microorganisms, such as *Zymomonas mobilis*, are more tolerant to furfural than others, such as *S. cerevisiae*.

To overcome the inhibition caused by these inhibitors, various strategies can be employed. One strategy is to detoxify the hydrolysate by removing or reducing the concentration of the inhibitors. This can be done through physical or chemical treatments, such as activated carbon adsorption, ion exchange, or overliming.[13]

Another strategy is to adapt the microorganisms to the inhibitors. This can be done through a process called adaptive evolution, where the microorganisms are repeatedly exposed to the inhibitors and allowed to evolve to become more tolerant to them. Supporting the ADH activity in anaerobic fermentation has been reported to increase by 78% after 48 h fermentation with an initial furfural concentration of 2 g 1^-1.[14]

In conclusion, the presence of inhibitors in lignocellulosic hydrolysates can greatly reduce the efficiency and yield of the fermentation process. Understanding the mechanisms of inhibition of these inhibitors is important for developing strategies to overcome them and improve the fermentation process to produce biofuels and other bioproducts.

Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects

Lignocellulose is a complex carbohydrate material that is the most abundant organic material on earth. It is composed of cellulose, hemicellulose, and lignin, which are tightly bound and require pretreatment to break down their structure for further processing. However, during the pretreatment process, various by-products are formed, including inhibitors that can hinder the fermentation process and reduce the yield of biofuels and other value-added products.[23]

Inhibitors are formed by the breakdown of lignocellulose components during pretreatment, which results in the production of compounds such as furfural, hydroxy methyl furfural (HMF), acetic acid, formic acid, and phenolic compounds. These inhibitors can have toxic effects on microorganisms used in fermentation, and hence, reduce the yield of products such as ethanol, butanol, and organic acids.Studied inhibition of S. cerevisiae by furfural in the range .5-4g/L(540 mM)[24]

Furfural and HMF are formed through the dehydration of pentose and hexose sugars, respectively, and are highly toxic to microorganisms at concentrations as low as 1 g/L. Acetic and formic acids are formed during acid pretreatment of lignocellulose, and their accumulation can lead to the acidification of the fermentation broth, which can inhibit microbial growth.[25] Phenolic compounds, such as syringaldehyde, vanillin, and p-coumaric acid, are formed during lignin degradation and can cause oxidative stress to microorganisms.[26]

Acid based methods used chemicals such as H_2SO_4,HCL,SO_2,H_3PO_4 to help by product formation-Aliphatic carboxylic acids, phenylic compounds furans,etc.

Several strategies can be employed to minimize the effect of inhibitors on the fermentation process. One approach is to use detoxification agents that can reduce the toxicity of inhibitors. For example, the addition of activated charcoal, calcium hydroxide, and sodium bi sulfite can reduce the toxicity of furfural and HMF. The use of calcium hydroxide or magnesium hydroxide can also neutralize the acidity of the fermentation broth, thereby reducing the inhibitory effect of acetic and formic acids.[27]

Another strategy is to select robust microorganisms that are resistant to inhibitors. For instance, Saccharomyces cerevisiae is more resistant to furfural and HMF than other yeast strains. Similarly, some bacteria, such as *Clostridium thermocellum* and *Thermo anaerobacterium saccharolyticum*, are more resistant to phenolic compounds than other microorganisms.[28]

The use of co-cultures or mixed cultures can also be effective in reducing the inhibitory effect of inhibitors. In co-cultures, different microorganisms work together to metabolize the inhibitors, which can lead to higher yields of biofuels and other products. Similarly, mixed cultures can utilize different inhibitors and produce different products, thereby increasing the overall yield.[29]

In conclusion, the formation of inhibitors during the pretreatment of lignocellulose is a significant challenge for the production of biofuels and other value-added products. However, various strategies can be employed to minimize the effect of inhibitors on the fermentation process. The use of detoxification agents, selection of robust microorganisms, and the use of co-cultures or mixed cultures can all be effective in reducing the inhibitory effect of inhibitors and increasing the yield of products. Inclusion of 20mg/L of benzoquinone in fermentation experiments with S. cerevisiae was sufficient to completely inhibit growth and ethanol formation[30]

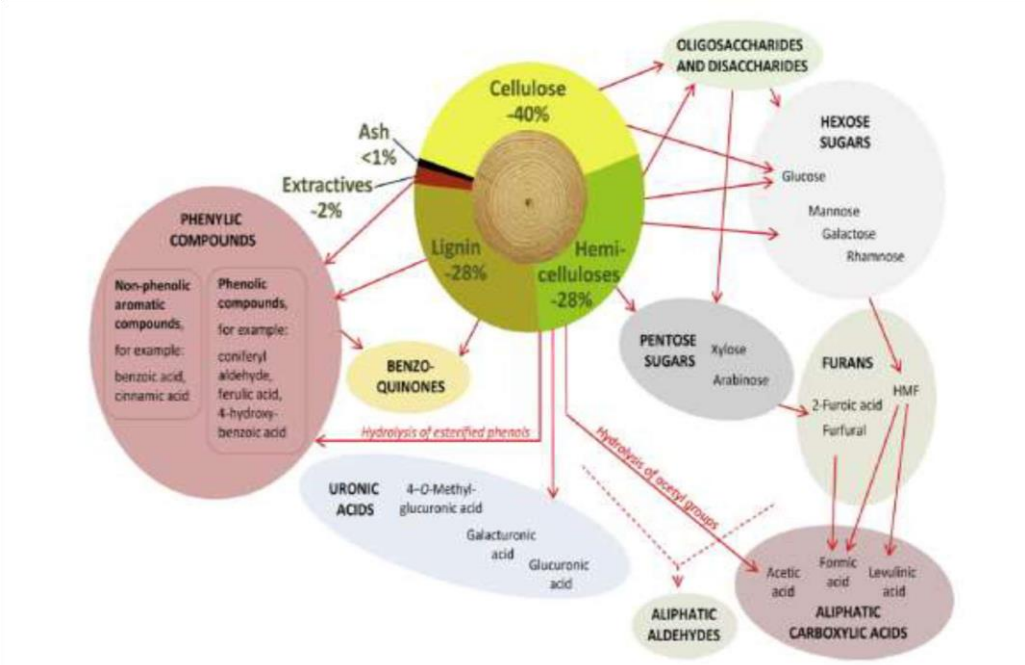


Fig. 1. Degradation products from lignocellulose as a result of pretreatment under acidic conditions. Numbers indicate fractions of constituents of wood of Norway spruce. Red arrows indicate tentative formation pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig 1: Schematic inhibitors obtained from lignocellulose (Jönsson & Martín, 2016)[31]

Bioconversion of lignocellulose: inhibitors and detoxification

Bioconversion of lignocellulose is a process that involves the conversion of lignocellulosic biomass, such as wood, agricultural waste, and other plant materials, into biofuels, chemicals, and other valuable products using microorganisms.[32]

However, lignocellulosic biomass contains a complex mixture of sugars and structural components, such as lignin, that are difficult to break down. Furthermore, the breakdown of lignocellulosic biomass is hindered by various compounds, called inhibitors, that are produced during the process. These inhibitors include furfural, 5-hydroxymethylfurfural (HMF), organic acids, phenolics, and others. Reduction of furfural has been linked to the co-factor NADH.The pKa value of formic acid(3.75) is considerably lower than those of acetic acid (4.76) and levulic acid (4.64)[33]

The presence of these inhibitors can significantly reduce the efficiency of the bioconversion process, as they can inhibit the growth and metabolic activity of the microorganisms involved. Therefore, it is necessary to detoxify the lignocellulosic biomass before bioconversion to remove or reduce the concentration of these inhibitors.[34]

Detoxification methods can be physical, chemical, or biological in nature. Physical methods include washing, soaking, and steaming, while chemical methods involve the use of acids, bases, and oxidizing agents to remove or reduce the concentration of inhibitors. Biological methods involve the use of microorganisms that can metabolize the inhibitors or convert them into less toxic compounds.[35]

Overall, the detoxification of lignocellulosic biomass is a critical step in the bioconversion process, as it can significantly improve the efficiency and yield of the process. However,

the ethanologenic microbes S. cerevisiae and *Zymomonas mobilis* can tolerate ethanol concentrations up to 18 and 12%.[36]

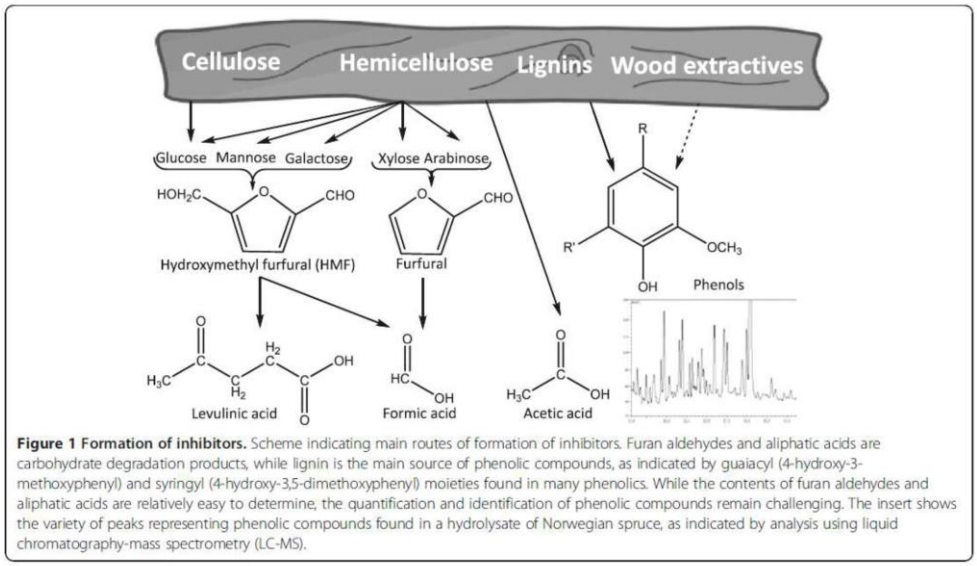


Figure 1 Formation of inhibitors. Scheme indicating main routes of formation of inhibitors. Furan aldehydes and aliphatic acids are carbohydrate degradation products, while lignin is the main source of phenolic compounds, as indicated by guaiacyl (4-hydroxy-3-methoxyphenyl) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) moieties found in many phenolics. While the contents of furan aldehydes and aliphatic acids are relatively easy to determine, the quantification and identification of phenolic compounds remain challenging. The insert shows the variety of peaks representing phenolic compounds found in a hydrolysate of Norwegian spruce, as indicated by analysis using liquid chromatography-mass spectrometry (LC-MS).

Fig 2: Type and sources of inhibitors (Jönsson et al.,2013)

3. AIMS AND OBJECTIVE

Aim: To identify, detect and characterise the inhibitors formed during the production of lignocellulosic ethanol.

Objective:

- To identify the inhibitors formed during pretreatment as well as hydrolysis and fermentation.
- To study the dependency on process parameters during formation of the inhibitors in every step.

- To study the effects of inhibitors on yield and productivity.
- To understand the mechanisms behind the formation of these inhibitors.
- To determine the method to detect the inhibitors formed.
- To evaluate of the impact of inhibitors on ethanol production
- To determine the techniques to quantify and characterise the inhibitors formed.
- To find out cost effective techniques to remove the inhibitors.

4. MATERIALS AND METHODS:

4.1 **MATERIALS:**

Dried fruit shell of *Sterculia foetida* is collected from the garden of Jadavpur University. All the reagents and chemicals such as NaOH, H_2SO_4, has been procured from Sigma Aldrich and Merck.

4.2 **EXPERIMENTAL WORK**

Sample collection and preparation:

The sample is washed in running water to remove the clay and dust products followed by drying in a hot air oven for 5 to 6 hours at a temperature of 100-110°C 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.

(a) (b) (c)

Fig 3: Raw materials, (a) *Sterculia foeitda*, (b) Willey mill, (c) *Sterculia foetida* after grinding

Analytical methods

Proximate Analysis:

Proximate analysis of the *Sterculia foetidae* carried out to find basic composition of the collected *Sterculia foetida*. For the determination of moisture content, oven dry method was used. 1 g of *Sterculia foeitda* were placed in a clean petridish. This petridish was then placed in a hot air oven for drying at 105°C for 12 hours. The dried sample was then weighed.

$$Moisture\ content\ (\%) = \frac{W_0 - W_d}{W_0} X\ 100$$

Here, W_0 is the initial weight of the *Sterculia foeitda* before drying and $W_d$is the weight of the *Sterculia foeitda* after drying.

For volatile matter calculation, the preheated *Sterculia foeitda* was placed in a crucible and was heated in a programmable muffle furnace at 900°C for 7 minutes. The weight of the heated sample was then measured.

$$Volatile\ matter\ content\ (\%) = \frac{(W_d - W_h)}{W_0} X100$$

Here, W_h is the weight of the *Sterculia foeitda* after heating in the muffle furnace at 900°C for 7 minutes.

Ash content determination was then carried out by placing 1 g of *Sterculia foeitda* sample in a crucible. The crucible was then heated in a programmable muffle furnace at 600°C for 4 hours. After the heating was completed, the weight of the remaining sample was calculated.

$$Ash\ content\ (\%) = \frac{W_f}{W_0} * 100$$

Here, W_f is the final weight of the *Sterculia foeitda* sample after heating at 600°C for 4 hours.

The fixed carbon content of the *Sterculia foeitda* determined by-

$$Fixed\ carbon\ content\ (\%) = 100 - (Moisture\ content + Volatile\ matter\ content + Ash\ content)$$

Table 1. Proximate and ultimate analysis of *Sterculia foeitda*

Content (%)	Weight (%)
Moisture content	3.6
Volatile Matter content	80.4
Ash content	5
Fixed carbon	11
Carbon	42.91
Hydrogen	6.07
Nitrogen	0.36
Oxygen	50.66

Chemical pretreatment:

Acid Pretreatment:

Sulfuric acid (3-8% v/v) was added to the biomass slurry with a variable solid to liquid ratio of 10:100 (w/v) in an autoclave at 121 °C temperature,15 psi pressure for 60 minutes. The liquid fraction was separated from the solid fraction by filtering through a double-layered muslin fabric followed by washing with distilled water until neutral pH was obtained. The acid hydrolysate was recovered and characterisation was done.

Table 2: Reaction matrix of acid and alkaline pretreatment

Reagent	NaOH			H_2SO_4		
Content (%)	1	3	6	1	3	6

Alkaline Pretreatment:

In order to improve the extraction of lignin from the lignocellulose matrix, the acid treated biomass was further treated with a weak alkaline solution in this case sodium hydroxide (NaOH). To get rid of the lignin portion from the interlaced mesh, the acid-treated lignocellulosic biomass was then treated with a weak NaOH solution. According to a combination of acid-treated rice straw was further treated with aqueous NaOH (2-6% w/v) at 121 °C and 15 psi pressure for a time of 60 min at a solid to liquid ratio of 1:50. Following the hydrolysis period, a double-layered muslin cloth was used to wash the biomass with water until a neutral pH is obtained. The amount

of holocellulose (cellulose and hemicellulose) and lignin in the sieved biomass was measured. Lignin was recovered using the black liquor that was produced as a by-product of the alkali process.

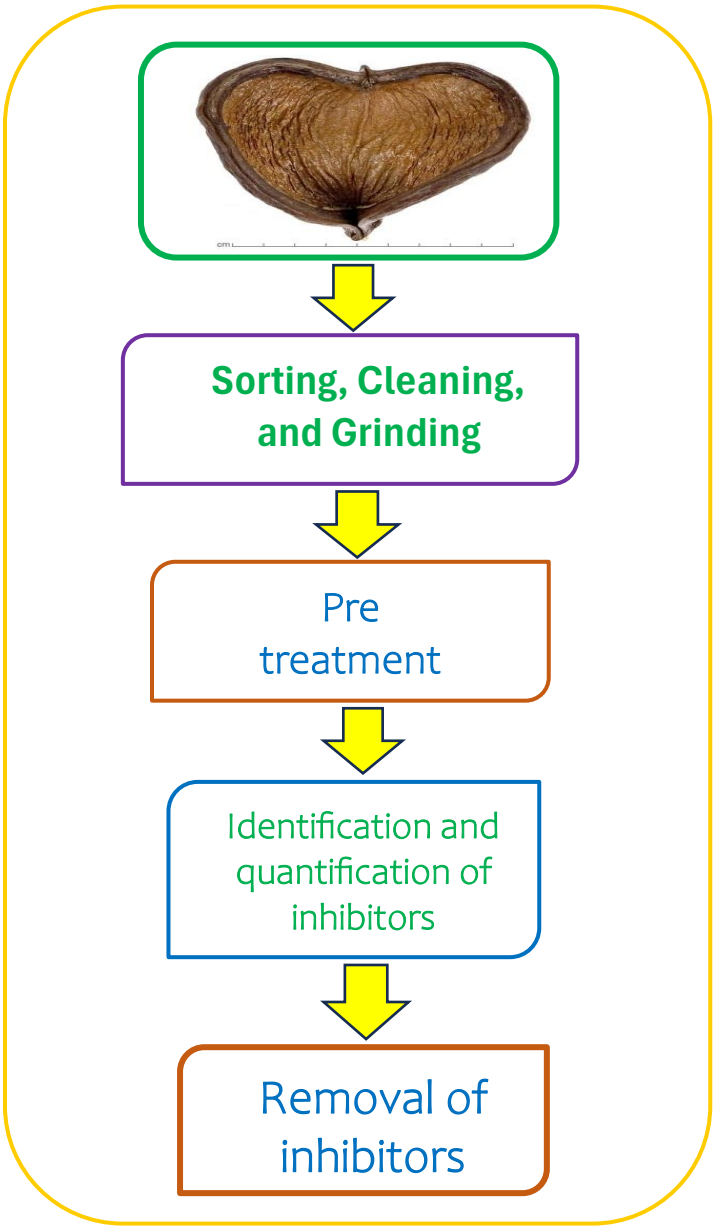


Fig 4: Schematic of the process from sample collection to removal of inhibitor

Fig 5: Experimental set up of pretreatment and the subsequent washing and separation process

Estimation of Acid insoluble lignin in wood: According to the TAPPI protocol, firstly, 72% H_2SO_4 solution is prepared. 1gm dried sample is taken in three 50ml biker and solid to liquid ratio was 1:20 (w/v). Then the solutions are cool down at 20°C for 2 hours and the temperature is restricted to 20°C by checking using a pH meter. A sample with 560ml distilled water is added to a conical flask and boiled for 4 hours and maintained the range at 575ml.After 4 hours the sample is filtered with hot water until neutral pH is obtained. Now the crucible is dried in a hot air oven at 105°C.next day weigh the insoluble lignin in the crucible and put it into a zip lock.

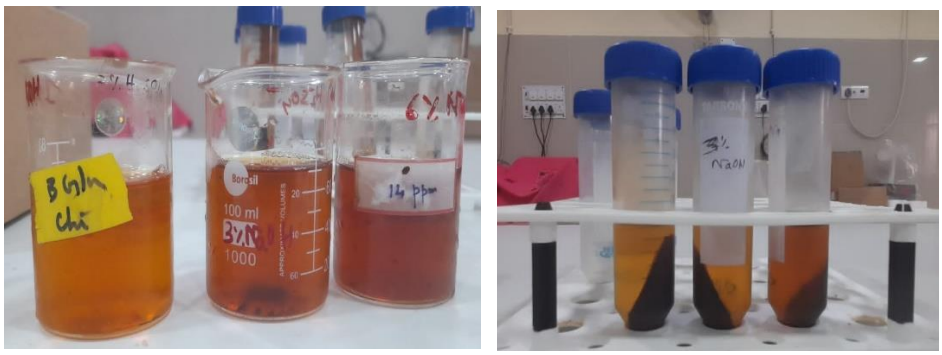


Fig 6: Visuals of sample obtained after centrifuged soluble lignin separated and insoluble lignin deposited

Fig 7: Insoluble lignin obtained in the crucible after washing with hot distilled water

5. Result and Discussion:

Effect of lignin Yield:

Lignin yield obtained with alkali and acid combination pretreatment is presented in table 3. The lignin yield lies in between 0.36gm to 1.24gm on the other hand, overall yield lies in between 1.14gm to 2.08gm. The best lignin yield of 1.24 gm was obtained for sample treated at 6%NaOH and 1% H_2SO_4 concentration. Highest overall yield of 2.08gm was obtained for sample treated at 3%NaOH and 1% H_2SO_4 concentration.

Table 3: Determination of %yield of lignin obtained from chemical pre-treatment

SAMPLE	COMPOSITION	REAGENT REACTION	TEMPERATURE	TIME	LIGNIN %	YIELD %
1	5gm sample+100ml solution	1%NaOH+3% H_2SO_4	121	15MIN	0.74gm	1.42gm
		3%NaOH+6% H_2SO_4	121	15MIN	0.78gm	1.3gm

		6%NaOH+1% H_2SO_4	121	15MIN	1.24gm	1.57gm
2	5gm sample+100ml solution	1%NAOH+6%H_2SO_4	121	15MIN	0.49gm	1.14gm
		3%NaOH+1% H_2SO_4	121	15MIN	0.73gm	2.08gm
		6%NaOH+3% H_2SO_4	121	15MIN	0.61gm	1.49gm
3	5gm sample +100ml solution	1%NaOH+1% H_2SO_4	121	15MIN	0.8gm	1.67gm
		3%NaOH+3% H_2SO_4	121	15MIN	0.36gm	1.44gm
		6%NaOH+ 6% H_2SO_4	121	15MIN	0.37gm	1.11gm

In case of AIL, highest percentage of acid insoluble lignin was obtained at the sample treated with 3% H_2SO_4 . As presence of lignin can be considered also as an inhibitor to the process it can be said that the pretreatment is effective and will aid the process. Table 4 represents the AIL percentage obtained and the pretreatment details.

Table 4: Acid Insoluble Lignin (AIL) obtained after acid pretreatment

Wt. of the sample (g)	H_2SO_4 (%)	Extracted lignin (g)	AIL (%)
1	1	0.27	27
	3	0.43	43
	6	0.29	29

XRD Analysis :

X-ray diffraction (XRD) analysis is a technique used to determine the crystal structure of a material. It is widely used in materials science, geology, chemistry, and other fields to study the arrangement of atoms in solids. In XRD analysis, a sample is exposed to a beam of X-rays, and the X-rays interact with the crystal lattice of the sample. This interaction causes the X-rays to scatter in different directions, resulting in a diffraction pattern. By analyzing this pattern, scientists can obtain valuable information about the crystal structure, including the lattice parameters, crystal symmetry, and the positions of atoms within the crystal.

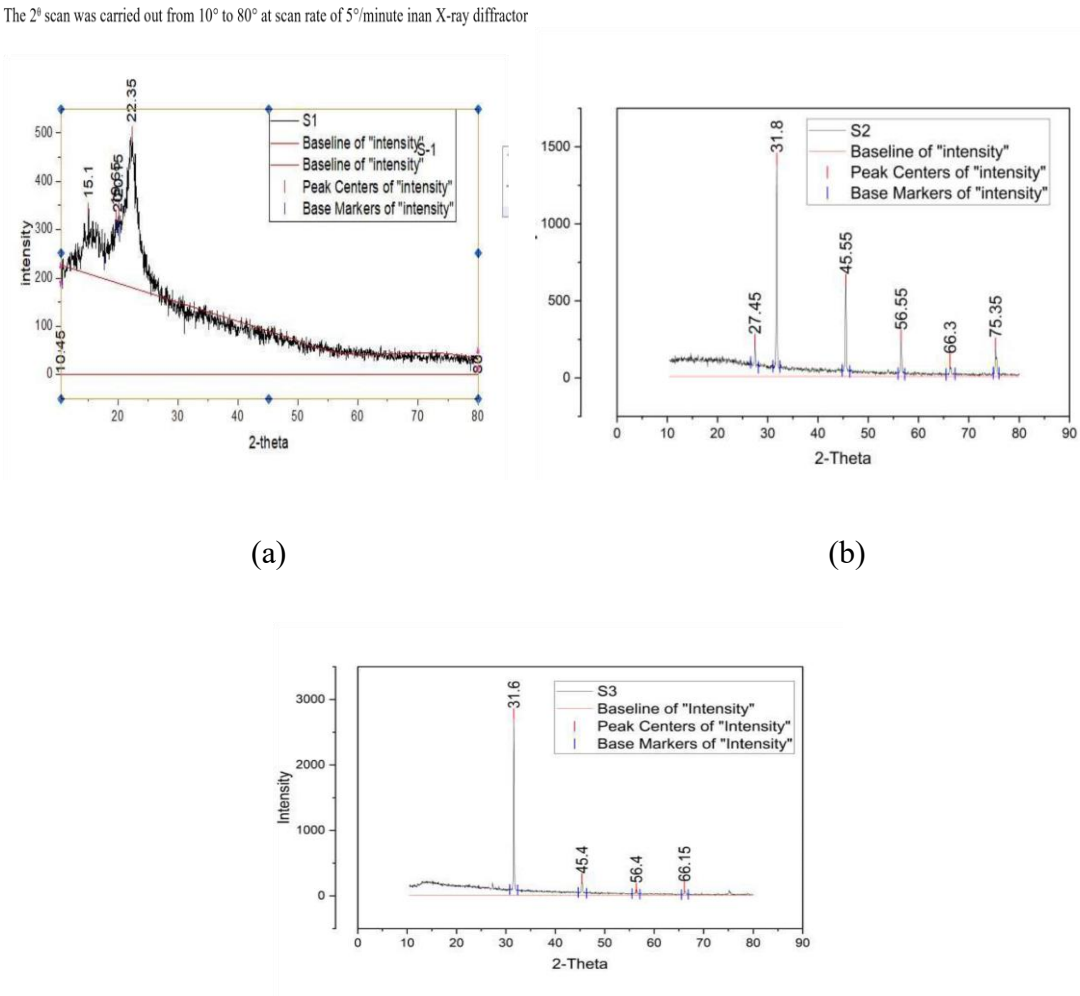
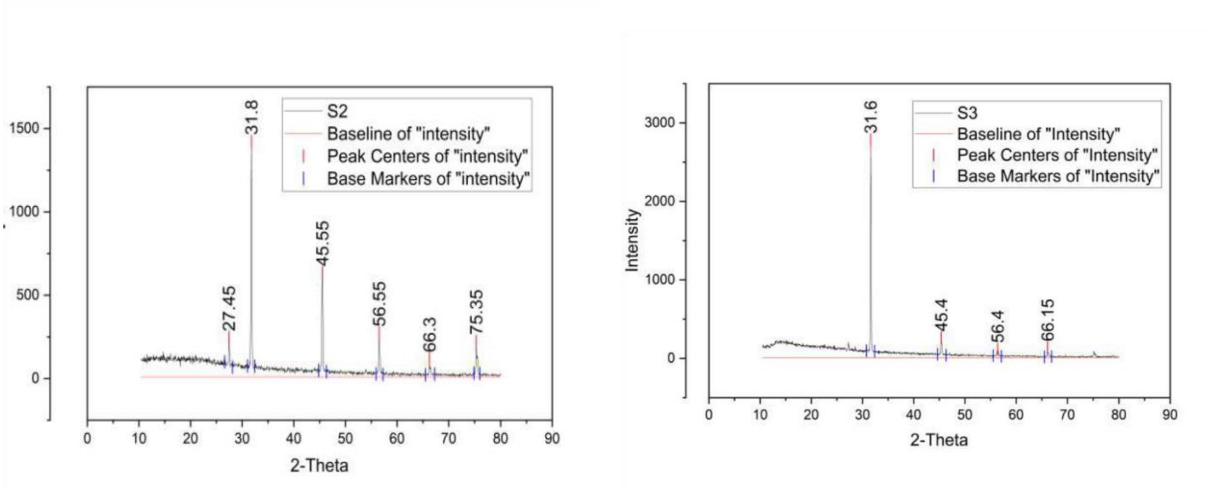


Fig 8: Images of XRD spectra for S1 (1% H_2SO_4)

Figure 8 represents the XRD spectra of 1% H_2SO_4 . and it can be seen from the spectra and from table 5 as well that the crystallinity index is 99% here. Which indicates that 1% H_2SO_4 helps in changing the lateral order structure to some extent.

Table 5: Crystallinity data of each sample calculated from XRD spectra

Area of Crystalline peak	Sum of Crystalline peak area	Total area of peak	%Crystallinity	Sample
1809.1	8202.875	8210.975	99.90135	S1 (1% H_2SO_4)
623.55				
30.825				
78.3				
5661.1				
143.05	909.025	4470.95	20.33181	S2 (3% H_2SO_4)
352.9				
204.375				
88.5				
46.025				
74.175				
551.525	779.7	5679.47	13.72839	S3 (6% H_2SO_4)
120.775				
62.575				
44.825				



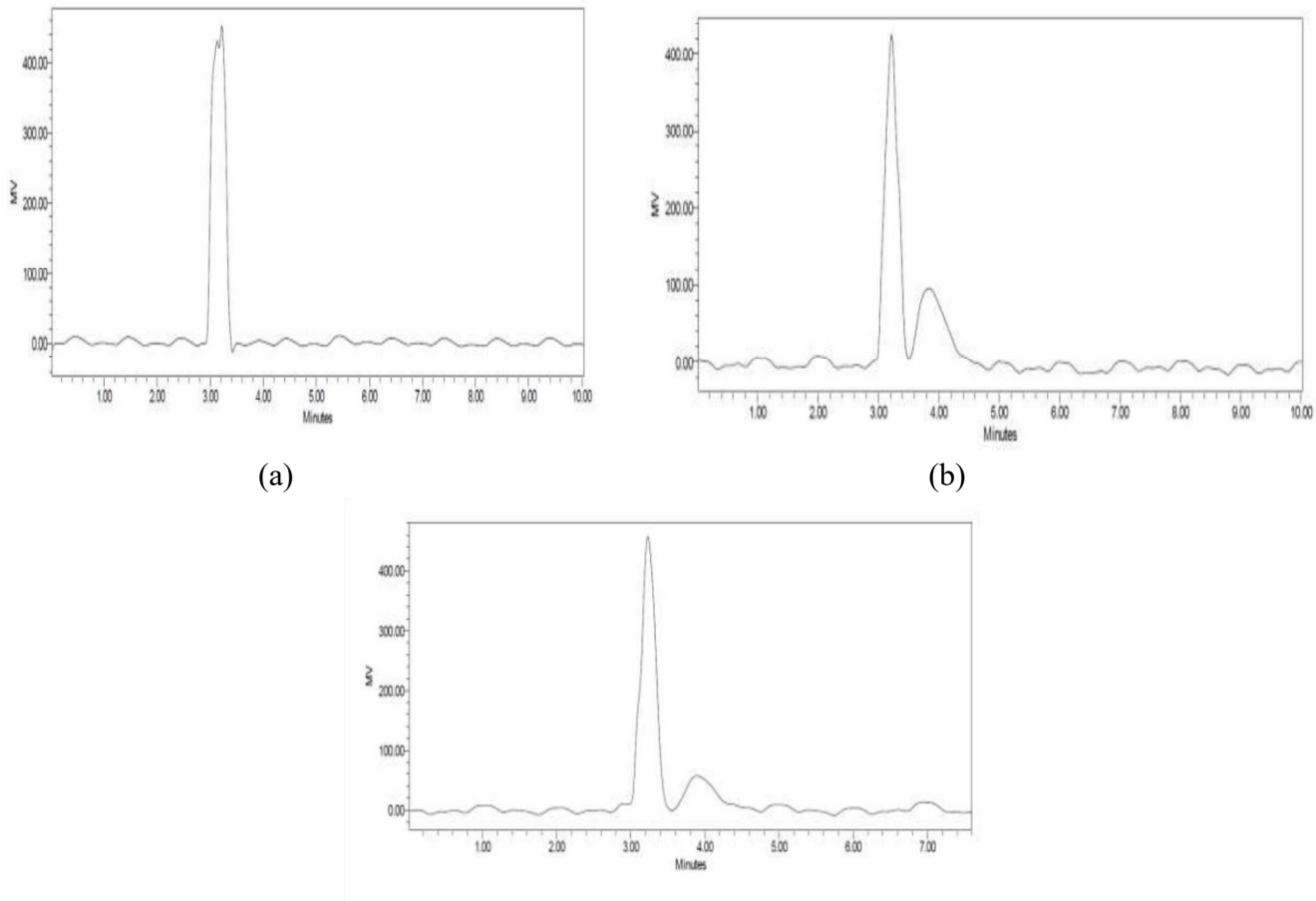
(a) (b)

Fig 9: Images of XRD spectra for (a) S2 i.e. 3% H_2SO_4 and (b) S3 i.e. 6%H_2SO_4

From table 5 and figure 9 it can be observed that for 6% H_2SO_4 the crystallinity is lower than that of 3% H_2SO_4 and for 6% H_2SO_4 crystallinity percentage was lowest at 13.72839, which indicates that as the H_2SO_4 concentration increases the crystallinity percentage decreases. It can be said that H_2SO_4 concentration plays an important role in removing the main inhibitor, lignin for an efficient pretreatment and increase in acid concentration aids in the process.

HPLC Analysis :

HPLC (High-Performance Liquid Chromatography) analysis is a widely used technique for separating, identifying, and quantifying components in complex mixtures.



(a) (b)

(c)

Fig 10: HPLC spectra of *Sterculia foetida* treated with (a) 1% NaOH (b) 3% NaOH solution (c) 6% NaOH Solution

From figure 10 the presence of sugar peaks can be observed so it can be said that some portion of the lignin may have got removed during pretreatment as sugar molecule was present in the sample and lateral order structure have to be ruptured by removing or rupturing the lignin structure in order to access the cellulose and hemicellulose.

5. CONCLUSION

To conclude it can be stated that both alkaline and acid pretreatment can be used to remove one of the inhibitor lignin. As concentration of acid increases the lignin removal percent also increases. For alkaline concentration as well the presence of sugar indicates that the lignin may have removed to some extent. Although further study is needed to completely know about the different types of inhibitors.

6. REFERENCES

1. Klemm, D., Heublein, B., Fink, H.P., et al., 2005. Cellulose: fascinating biopolymer and sustainable raw material. Angew. Chem. Int. Ed. 44, 3358 3393.

2. Nguyen, T.Y., Cai, C.M., Kumar, R., et al., 2017. Overcoming factors limiting high-solids fermentation of lignocellulosic biomass to ethanol. Proc. Natl. Acad. Sci. U. S. A. 114, 11673 11678.

3. Verbańćić, J., Lunn, J.E., Stitt, M., Persson, S., 2018. Carbon supply and the regulation of Cell Wal1 synthesis. Mol. Plant 11, 75 94.

4. Zabed, H., Sahu, J.N., Boyce, A.N., Faruq, G., 2016. Fuel ethanol production from lignocellulosic biomass: an overview on feedstocks and technological approaches. Renew. Sust. Energ. Rev. 66, 751 774. Höfte, H., Voxeur, A., 2017. Plant cell walls. Curr. Biol. 27, R853 R909.

5. Lythcke-Jøgensen, C., Haglind, F., Clausen, L.R., 2014. Exergy analysis of a combined heat and power plant with integrated lignocellulosic ethanol production. Energy Convers. Manag. 85, 817 –

827. S.C.R. Marques, J.M. Marcuzzo, M.R. Baldan, A.S. Mestre, A.P. Carvalho, Pharmaceuticals removal by activated carbons: Role of morphology on cyclic thermal regeneration, Chem. Eng. J. 321 (2017) 233 244.

8. Tengborg, C., Stenberg, K., Galbe, M., Zacchi, G., Larsson, S., Palmqvist, E., Hahn-H• agerdal, B., 1998. Comparison of SO2 and H2SO4 impregnation of softwood prior to steam pretreatment on ethanol production. Appl. Biochem. Biotechnol. 70/72, 3±15.

9. Grethlein, H.E., Converse, A.O., 1991. Common aspects of acid prehydrolysis and steam explosion for pretreating wood. Bioresour. Technol. 36, 77±82.

10. Boyer, L.J., Vega, K., Klasson, K.T., Clausen, E.C., Gaddy, J.L.,1992. The e€ects of furfural on ethanol production by Saccharomycescerevisiae. Biomass Bioeng. 3 (1), 41±48.

11. Taherzadeh, M.J., Gusta son, L., Niklasson, C., Liden, G., 1999. Physiological e ects of 5hydroxymethyl-furfural (HMF) on Saccharomyces cerevisiae, submitted.

12. Pampuhla, M.E., Loureiro-Dias, M.C., 1990. Activity of glycolytic enzymes of Saccharomyces cerevisiae in the presence of acetic acid. Appl. Microbiol. Biotechnol. 34, 375±380.

13. Verduyn, C., Postma, E., Sche€ers, W.A., van Dijken, J.P., 1990. Energetics of Saccharomyces cerevisiae in anaerobic glucose-limited chemostat cultures. J. Gen. Microbiol. 136, 405±412.

14. Banerjee, N., Bhatnagar, R., Viswanathan, L., 1981b. Inhibition of glycolysis by furfural in Saccharomyces cerevisiae. Eur. J. Appl. Microbiol. Biotechnol. 11, 226±228.

15. Scheller, H.V., Ulvskov, P., 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61, 263 2892010

16. Hilliard, M., Damiani, A., He, Q.P., Jeffries, T., Wang, J., 2018. Elucidating redox balance shift in Scheffersomycesstipitis' fermentative metabolism using a modified genomescalemetabolic model. Microb. Cell Factories 17, 140.

17. Loow, Y.L., Wu, T.Y., Jahim, J.M., et al., 2016. Typical conversion of lignocellulosic biomass into reducing sugars using dilute acid hydrolysis and alkaline pretreatment.Cellulose 23, 1491 1520

18. Kelsall, D.R., Lyons, T.P., 2003b. Grain dry milling and cooking procedures: extracting sugars in preparation for fermentation. In: Jacques, K.A., Lyons, T.P., Kelsall, D.R. (Eds.), The Alcohol Textbook, 4th ed. Nottingham University Press, Nottingham, UK, pp. 9 22.

19. Zabed, H., Sahu, J.N., Boyce, A.N., Faruq, G., 2016. Fuel ethanol production from lignocellulosic biomass: an overview on feedstocks and technological approaches. Renew. Sust. Energ. Rev. 66, 751 774

20. Bonawitz, N.D., Chapple, C., 2010. The genetics of lignin biosynthesis: connecting genotype to phenotype. Annu. Rev. Genet. 44, 337 363.

21. Duque, S.H., Cardona, C.A., Moncada, J., 2015. Techno-economic and environmental analysis of ethanol production from 10 agroindustrial residues in Colombia. Energy Fuel 29, 775 783

22. Cavka, A., Martín, C., Alriksson, B., Mörtsell, M., Jönsson, L.J., 2015. Techno-economic evaluation of conditioning with sodium sulfite for bioethanol production from softwood. Bioresour. Technol. 196, 129 135.

23. Fengel, D., Wegener, G., 1989. Wood Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin.

24. Banerjee, N., Bhatnagar, R., Viswanathan, L., 1981. Inhibition of glycolysis by furfural in Saccharomyces cerevisiae. Eur. J. Appl. Microbiol. Biotechnol. 11, 226 228.

25. Yang, B., Wyman, C.E., 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuel, Bioprod. Biorefin. 2, 26 40.

26. Cavka, A., Alriksson, B., Ahnlund, M., Jönsson, L.J., 2011. Effect of sulfur oxyanions on lignocellulose-derived fermentation inhibitors. Biotechnol. Bioeng. 108, 2592 2599.

27. Cavka, A., Alriksson, B., Ahnlund, M., Jönsson, L.J., 2011. Effect of sulfur oxyanions on lignocellulose-derived fermentation inhibitors. Biotechnol. Bioeng. 108, 2592 2599.

28. Hu, F., Ragauskas, A., 2012. Pretreatment and lignocellulosic chemistry. Bioenerg. Res. 5, 1043–1066.

29. Pan, X., Xie, D., Yu, R.W., Saddler, J.N., 2008. The bioconversion of mountain pinebeetle-killed lodgepole pine to fuel ethanol using the organosolv process.Biotechnol. Bioeng. 101, 39 48.

30. Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosichydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour. Technol.74, 25 33.

31. Larsson, S., Quintana-Sáinz, A., Reimann, A., Nilvebrant, N.-O., Jönsson, L.J., 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae. Appl. Biochem. Biotechnol. 84, 617 632.

32. Rowell RM: Handbook of wood chemistry and wood composites. 2nd edition.Boca Raton FL: CRC Press; 2012.

33. Ulbricht RJ, Sharon J, Thomas J: A review of 5-hydroxymethylfurfural HMF in parental solutions. Fund Appl Toxicol 1984, 4:843 853.

34. Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant N-O: The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb Tech 1999, 24:151 159.

35. Clark TA, Mackie KL: Fermentation inhibitors in wood hydrolysates derived from the softwood Pinus radiata. J Chem Tech Biotechnol 1984, 34:101 110

36. Martinez A, Rodriguez ME, York SW, Preston JF, Ingram LO: Effects of Ca(OH)2 treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. Biotech Bioeng 2000, 69:526 536.

37. Lin Y, Tanaka S: Ethanol fermentation from biomass resources: current state and prospects. Appl MicrobiolBiotechnol 2006, 69:627 642..

38. Selig, M., Weiss, N., Ji, Y., 2008. Laboratory Analytical Procedure: Enzymatic Saccharification of Lignocellulosic Biomass. National Renewable Energy Laboratory, Golden, CO.

39. Zheng, Y., Pan, Z., Zhang, R., 2009. Overview of biomass pretreatment for cellulosic ethanol production. Int. J. Agric. Biol. Eng. 2, 51 68.

40. Kim, K.H., Tucker, M., Nguyen, Q., 2005. Conversion of bark-rich biomass mixture into fermentable sugar by two-stage dilute acid-catalyzed hydrolysis.Bioresour. Technol. 96, 1249 1255.

41. 40.Kootstra, A.M.J., Beeftink, H.H., Scott, E.L., Sanders, J.P.M., 2009. Comparison of dilute mineral and organic acid pretreatment for enzymatic hydrolysis of wheat straw. Biochem. Eng. J. 46, 126 131

42. SaravananN, NagarajanG, PuhanS. Experimental investigation on a DI diesel engine fuelled with Madhuca Indica ester and diesel blend. Biomass Bioenergy2010;34:838 43.

43. Raju S S, Parappurathu S, Chand R, Joshi P K, Kumar P, Msangi S. Biofuels in India: potential, policy and emerging paradigms. Policy paper 27.NCAP:Chandu Press;2012.

44. Marcotullio G, Krisanti E. Selective production of hemicellulose-derived carbohydrates from wheat straw using dilute HCl or FeCl3 solutions under mild conditions. X-ray and thermo-gravimetric analysis of the solid residues.Bioresour Technol 2011;102(10):5917 23.

45. SassnerP,MartenssonC,GalbeM,ZacchiG.SteampretreatmentofH2SO4impregnatedSalixforthepr oductionofbioethanol.BioresourTechnol 2008;99:137 45.

46. RagauskasAJ,WilliamsCK,DavisonBH,BritovsekG,CairneyJ,EckertCA,etal.Thepathforwardfor biofuelsan dbiomaterials.Science2006;311(5760):484 9.

47. R. Singh et al. / Renewable and Sustainable Energy Reviews 32 (2014) 713 72

48. Blasi DA, Titgemeyer Evan C, Drouillard J. Soybean hull composition and feeding value for beef and dairy cattle. Publication MF2438. Kansas State University; January, 2000.

49. Dale BE, Weaver J, Byers FM. Extrusion processing for ammonia fiber explosion (AFEX). Appl BiochemBiotechnol 1999;77e79:35e45.

50. Chen Y, Sharma-Shivappa RR, Keshwani D, Chen C. Potential of agricultural residue and hay for bio-ethanol production. Appl BiochemBiotechnol2007;42:276e90.

51. Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 2009; 48:3713e29

52. Xie X, Cui SW, Li W, Tsao R. Isolation and characterization of wheat bran starch. Food Res Int 2008;41:882e7.

53. Green M, Kimchie S, Malester AI, Rugg B, Shelef G. Utilization of municipal solid wastes (MSW) for alcohol production. Biol Wastes 1988;26(4):285e95.

54. Tokuyasu K, Tabuse M, Miyamoto M, Matsuki J, Yoza K. Pretreatment of microcrystalline cellulose flakes with CaCl2 increases the surface area and thus improves enzymatic saccharification. Carbohydr Res 2008;343:1232e6.