PRODUCTION OF BIO-ETHANOL FROM LIGNOCELLULOSIC MATERIAL

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BIOPROCESS ENGINEERING

By

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CANDIDATE'S DECLARATION

I, hereby, declare that the work which is being presented in this dissertation project report entitled "**PRODUCTION OF BIOETHANOL FROM LIGNOCELLULOSIC MATERIAL**" in partial fulfilment of the requirements for the award of the degree of **MASTERS OF TECHNOLOGY** in **BIOPROCESS ENGINEERING**, submitted to the DEPARTMENT OF BIOTECHNOLOGY, INDIAN INSTITUTE OF TECHNOLOGY ROORKEE is an authentic record of my own work under the supervision of **DR. CHANDRAJIT BALOMAJUMDER** (Professor), DEPARTMENT OF CHEMICAL ENGINEERING, INDIAN INSTITUTE OF TECHNOLOGY ROORKEE.
I have not submitted the matter, embodied in this report for the award of any other degree.

Date- 9th May 2018
Place- IIT Roorkee

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CERTIFICATE

This is to certify that the above statement made by the candidate is correct to the best of my knowledge and belief.

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Date- 9th May 2018 TANMOY DE

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ABSTRACT

Lignocellulose biomass are the sole components in different waste streams obtained from various sources such as agriculture, industries, forestry and municipalities. They represent an undisturbed source of fermentable sugars for significant use in the industry for it's conversion to biofuel. It is not possible to define the best pretreatment method as it depends on many factors such as type of lignocellulosic biomass, process parameters, environmental impact, economical feasibility, etc. Lignocellulose biomasses like wood dust and banana peel were selected for the bioethanol production. Many physico-chemical, structural and compositional factors hinder the hydrolysis of constituents like cellulose and hemicellulose present in the biomass to sugars and other organic compounds that can later be converted into biofuels. Pretreatment plays a key role in breaking the complex structure of biomass. Various pretreatment processes involving acid, alkali, solvents and biological entities were used for the process. Among them, it was found that the process involving the use of H_2SO_4 and $K_2Cr_2O_7$ showed greater efficiency in breaking the biomass structure than the dilute acid pretreatment techniques. Moreover, the use of *Phanerochyta chrysosporium* resulted in the production of all three delignifying enzymes like *Lignin Peroxidase, Laccase and Manganese Peroxidase* which when used on the biomass gave good results. The biological method is a little time taking but is economical in nature.

2. OBJECTIVES

2.1 Aim

Production of bioethanol from lignocellulosic material.

2.2 Objectives

1. Approximate analysis of sugarcane leaves, sugarcane bagasse, banana peel and wood dust.

2. Determine the effect of different pretreatment methods on lignocellulose biomass.

3. Determine the effect of biological pretreatment in the lignocellulose biomass using *Phanerochaeta chrysosporium.*

4. Determination of glucose from cellulose in enzymatic hydrolysis.

5. Determination of ethanol produced with *Saccharomyces cerevisae.*

3. INTRODUCTION

3.1 Bioethanol production

Production of bioethanol from lignocellulosic material attracts the attention of various countries as an alternative fuel source in the recent past. The research on bioenergy, such as bioethanol and biodiesel, are not recent area of interest for different countries. In order to survive in the predicted depletion of fossil fuel in the near future, a number of countries have taken steps to reduce their dependence on gas and oil imports by developing and industrialising new energy forms. Potential energy forms include nuclear energy and some renewable and clean forms such as solar, hydro, biomass and wind. Amongst these, ethanol production from biomass is of particular interest due to the following advantages-

1. Low emission levels of carbon dioxide and therefore contributing less to the greenhouse effects.
2. They can be blended to gasoline and can be used in transportation industry without much modification to the vehicles.
3. Mature production techniques.

Ethanol has been produced from fruits and starch for alcoholic beverages for thousands of years. Considering these advantages, the potential of fuel ethanol has been thoroughly explored for further development and industrialisation. The research and application of bioethanol for energy purposes were pioneered by the United States and Brazil. In the United States, bioethanol is produced mainly from the traditional starch crops such as corn; whereas in Brazil, bioethanol production is derived from and is closely related to the sugarcane industry, where both sugarcane and waste material from the sugar manufacture process serve as substrate for the ethanol production. Both countries have brought out a series of strategies to promote and popularise the utilisation of ethanol for fuel purposes, e.g. the price reduction of ethanol blended gasoline. Other strategies adopted in United States include the enticement for growing plants for ethanol production and the additional duty levied for imported ethanol to encourage the domestic ethanol production industry. As a result, corn plantations have been greatly extended in the United States, replacing other crops such as soy for greater profit, e.g. 15.5% increase in average in 2007. Even the harvested corn in the market has been increasingly diverted to the ethanol production instead of using it as an agricultural commodity. The biofuel industry consumed 25% of the overall crops produced in 2007 in the United States and is projected to consume more than 30% in 2008. Due to all these measures, the bioethanol industry has rapidly developed and prospered. The production of renewable fuels in the United States is projected to achieve 36 billion gallons by 2022, which will mostly include corn and cellulose. Cars and other vehicles driven by ethanol or it's blends are commonly used in Brazil. In Australia, a program entitled "Ethanol Production Grants" was set up as early as in 2002 to

encourage the use of biofuels for vehicles. At the beginning of 2008, a global food crisis was observed worldwide. As pointed out by numerous reports, bioethanol production was considered one of the major factors responsible for this crisis. Corn and other starch crops are the basic food; the mass production of ethanol from these substrates in the United States substantially decreases the production of products used for food consumption, reduces the plantation area of other edible crops and indirectly causes the shortage of both primary product and livestocks. Bioethanol, however, remains as a promising alternative to the fossil fuels; it's rapid development and spread in Brazil confirms this viewpoint. The utilisation of ethanol is one of the safest methods to counteract the depletion of non-renewable sources. In order to retain bioethanol for fuel purposes as well as to avoid the unnecessary pressure on the food supply, new substrates for ethanol production need to be set up, ensuring that all the starch crops are returned back to the agricultural commodity market. The sugarcane program in Brazil is considered acceptable because unlike starch, the sucrose is not a necessary food material. Nevertheless, for long term consideration, material which cannot be consumed by humans should be the ultimate choice for bioethanol production, e.g. lignocellulose.

Lignocellulose is mainly the woody part of the plants, constituting the most abundant raw material in plants. Each year approximately 200 billion tons of lignocellulosic waste is produced from agriculture and industry worldwide. These mainly include various agricultural residues, deciduous and coniferous woods, municipal solid wastes, pulp and paper industry wastes and herbaceous energy crops. Ethanol production from this kind of substrate can serve as a waste treatment and convert this apparently useless material into value added products. Because the carbohydrate in the lignocellulose is originally produced by photosynthesis, the oxidation of carbon atoms does not increase the level of carbon dioxide in the atmosphere, only simply recycles them. Consequently, lignocellulose is an ideal substrate for ethanol production, on account of it's cheap cost and clean energy yield.

3.2 Lignocellulosic fuel

The concept of conversion of lignocellulose to ethanol dates back as early as 1917. However, currently ethanol production from lignocellulose substrate remains at a laboratory stage, while ethanol production from sugarcane and corn has already been commercialised. The major drawback in the utilisation of lignocellulose is it's natural structure. Briefly, the intimate association of several macromolecules in the lignocellulose makes it recalcitrant to breakdown to single fermentable sugars. Even after the breakdown, the fact that it comprises a variety of mono sugars makes the ethanol production process considerably difficult. The conversion efficiency of lignocellulose to ethanol is thus greatly limited. As a potential fuel, the final price of ethanol products needs to be moderate and acceptable compared to the market price of commercial gasoline. On the other hand, it should bring into profit to the organisation. Both factors have an important role in the feasibility of producing ethanol from lignocellulose. A standard proposed in earlier research requires an ethanol producing capability of 50-60 g/l within 36 hours, with a yield no less than 0.4 g/g.

Research on the lignocellulosic fuel production has continuously worked on the improvement of the efficiency of both the hydrolysis process to depolymerise the macromolecular complex to fermentable sugars and the microbial fermentation process to convert the sugar mixture to ethanol. The current research was therefore with the aim of assisting the bioenergy industry.

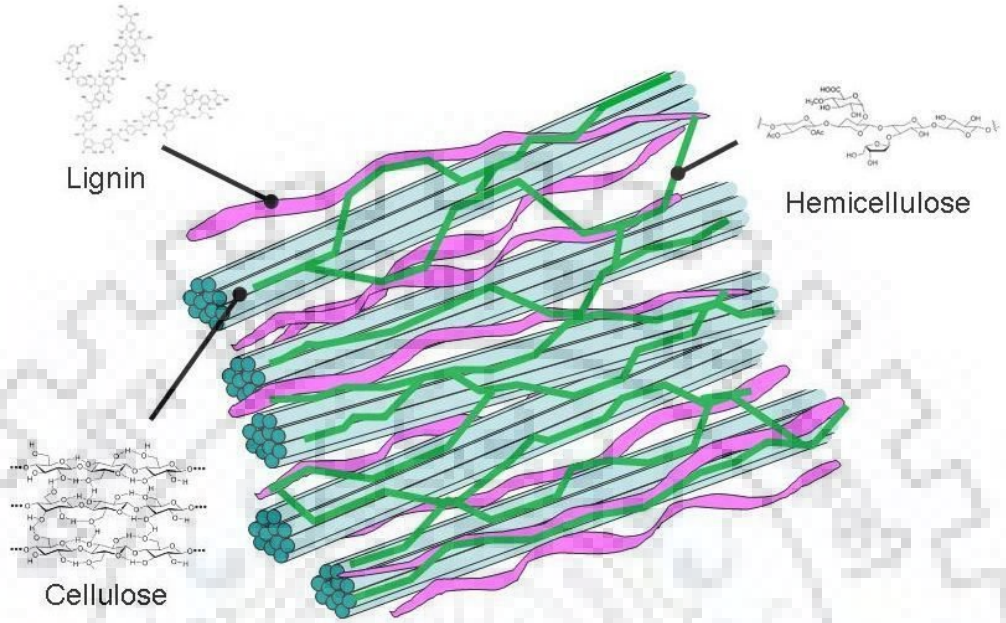

Fig1.1: Structure of lignocellulosic material

3.3 Understanding Lignocellulosic Biomass

Understanding the lignocellulosic structure of the biomass, mainly it's chemical composition, is a prerequisite for developing effective pretreatment techniques to disrupt its rigid and firm structure, designing of the enzymes to liberate sugars, particularly cellulase to release glucose, as well as engineering microorganisms to convert sugars into bioethanol.

Lignocellulosic biomass is mainly composed of plant cell walls and the plant cell walls are composed of three main components which are cellulose, hemicellulose and lignin. The cellulose and hemicellulose are the structural homogeneous carbohydrates and the lignin is the heterogenous phenolic polymer. Fig. shows the structure. However, their contents varies substantially, depending on the species, climate, fertilization practice, etc. [1].

3.3.1 Cellulose

Cellulose is a polysaccharide which comprises linear glucan chains which are linked together by beta-1,4-glycosidic bonds with cellobiose residues as the repeating unit at different degrees of

polymerization, and packed into microfibrils which are held together by intramolecular hydrogen bonds as well as intermolecular vander Waals forces [2]. Although, cellulose has polymorphy, native cellulose occurs as cellulose I, which is a mixture of two polymorphs Ia and Ib [3,4]. Cellulose Ia is formed simultaneously with the extension of the microfibril network, and thus is dominant in lower plants to form the primary wall, and also in some bacteria. But, cellulose Ib is deposited within the secondary wall of higher plants for strength. The decipherment of crystalline structure indicates that cellulose Ia is characterized by the triclinic unit containing one chain, while there are two chains in the monoclinic unit of cellulose Ib providing more intramolecular hydrogen bonds, making it more stable [5].

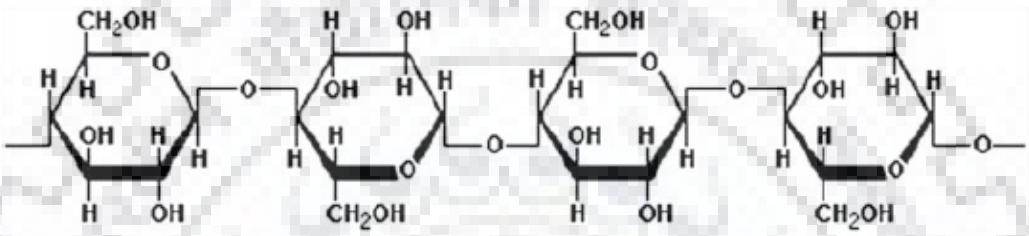

Fig 1.2: Structure of cellulose

3.3.2 Hemicelluloses

Hemicelluloses are a heterogeneous group of polysaccharides with the beta-(1-4)- linked backbone structure of pentose (C5) sugars, such as xylose and arabinose, and hexose (C6) sugars and also includes mannose, galactose and glucose as the repeating units, which have the same equatorial configuration at C1 and C4. The structural similarity of hemicelluloses to the beta-1,4-glycosidic bonds of the cellulose molecule benefits from a conformational homology, which can lead to a strong non-covalent association with cellulose microfibrils. Unlike cellulose which is crystalline and resistant to degradation, hemicelluloses are random and amorphous, and thus easily hydrolyzed to monomer sugars. However, hemicelluloses are embedded and interact with cellulose and lignin, which significantly increase the strength and toughness of plant cell walls. Xyloglucan and xylans are major hemicelluloses in plant biomass. Xyloglucan is abundant in the primary walls, with the oligosaccharide composed of xylose (X) and glucose (G) with various side chains, XXXG or XXGG for vascular plants including grain crops, as the repeating unit. Xylans are polysaccharides with beta-(1-4)-linked xylose residues as a backbone, which are often acetylated at the O-3 position of xylose residues and/or modified by alpha-(1-2)-linked glucuronosyl and 4-O -methyl glucuronosyl residues. Xylans, also known as glucuronoxylans, are the dominant noncellulosic polysaccharide in the secondary walls of dicots. A schematic illustration of xyloglucan and xylans . The major sugars in the hydrolysate of hemicelluloses are therefore xylose, arabinose, glucose and galactose.

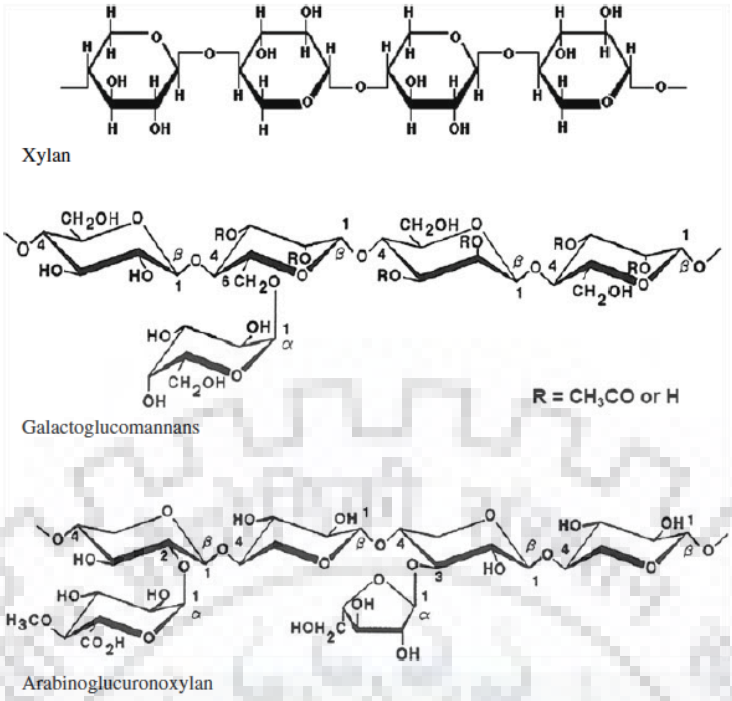

Fig 1.3: Structure of hemicellulose

3.3.3 Lignin

Although lignin is a non-sugar-based polymer and cannot be used as feedstock for ethanol production via microbial fermentation, it exerts a significant impact on the economic performance of the corresponding bioconversion processes, since most inhibitors of microbial growth and fermentation come from this compound during the pretreatment that is needed to render cellulose amenable to enzymatic attack. Meanwhile, as the second most abundant component in biomass after cellulose, lignin yields more energy when burned, and thus is a good selection for combined heat and power (CHP) production in an eco- and environment-friendly mode of the biorefinery [6]. Moreover, lignin is an excellent starting material for various products including transportation fuels and value-added chemicals, which may add credits to bioconversion processes and make bioethanol more economically competitive. It is apparent that understanding the fundamentals of lignin biosynthesis is the prerequisite for developing more efficient pretreatment and conditioning processes and subsequent enzymatic hydrolysis of cellulose, as well as engineering microorganisms with improved tolerance to inhibitors so that they can ferment the hydrolysate more rapidly with high yields. Lignin biosynthesis starts with the deamination of phenylalanine to cinnamic acid, followed by the modification of the aromatic ring by hydroxylation and O-methylation and reduction of the side chain to an alcohol moiety, resulting in the three major monolignols: p-coumaryl, coniferyl and sinapyl alcohols, which are exported across the plasma membrane into the apoplast. The proportion of these monolignols varies substantially among plant species and tissues in the same plant as well as subcellular locations, and is also affected by the developmental stage

and environmental stimuli. In addition to the three canonical monolignols, many other compounds are also involved in the biosynthesis of lignin, particularly ferulates, coniferaldehyde and acylated monolignols [6], which will be liberated during the pretreatment of lignocellulosic biomass [7].

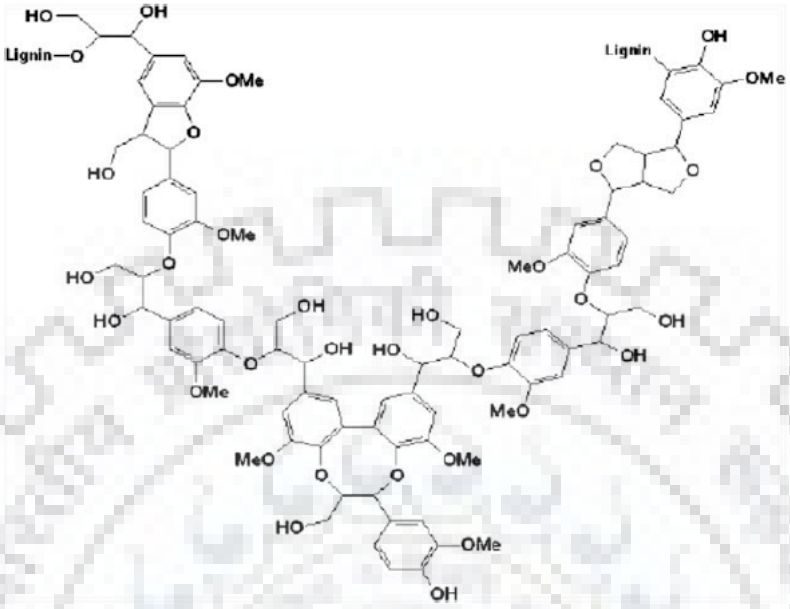

Fig 1.4: Structure of lignin

3.4 Pretreatments

The self-assembly architecture of plant cell walls, having crystalline cellulose microfibrils interacting and entangling with hemicelluloses and lignin, creates ligin carbohydrate complexes (LCCs) [7], which are inaccessible for the enzyme cellulases to bind onto surfaces of cellulose molecules. Therefore, after a preliminary size reduction to 10–30mm through mechanical methods such as chopping, pretreatment is needed to deconstruct LCCs for efficient enzymatic hydrolysis of cellulose [8]. The smaller the size, the more efficient the mass and heat transfer will be for subsequent pretreatment and enzymatic hydrolysis. However, power requirement increases significantly with reduction in size. Therefore, a compromise between size reduction and energy consumption is needed from the economic point of view. Pretreatment technologies can be classified in general into four categories: physical pretreatment, chemical pretreatment, solvent fractionation and biological decomposition [9]. An ideal pretreatment process should maximize sugar yield from cellulose and hemicelluloses, and in the meantime minimize energy consumption and environmental impact. Unfortunately, none of them alone can satisfy all of these criteria.

3.4.1 Physical Pretreatment

Physical pretreatments do not use any chemicals and biological methods. One of them is the size reduction by mechanical methods like grinding or milling, through which the surface area of biomass is increased for better hydrolysis, and the degree of polymerization (DP) and crystallinity of cellulose is decreased to some extent, but the requirement of power for reducing the feedstock from millimeter size to fine particles is extremely high, which is not acceptable from the engineering point of view. Even, radiation such as microwaves are conducted that can penetrate the structure and heat the feedstock instantly has also been studied [10]. However, it is a big problem to process the feedstock in large quantities because it requires a lot of power to generate the radiation. Therefore, more attention regarding physical pretreatment has been focused on the hydrothermal processes such as steam explosion (SE) and liquid hot water (LHW) treatment. SE is the process in which heating of feedstock at elevated temperature and pressure for a short duration which is followed by depressurizing the system to disrupt the structure of LCCs. As it is cost effective, impact on the environment is low, and a very simple process design and operation is involved, the SE process has been done at pilot scales worldwide. The mechanism involved in the pretreatment is assumed to be the partial degradation of LCCs catalyzed by acetic acid released from acetylated hemicelluloses and other organic acids such as formic and levulinic acids, which makes the process autohydrolytic in nature [11]. The major parameters of the SE process that are to be considered are temperature, pressure and holding time, which should be optimized based on the characteristics of feedstocks. In general, a temperature from 160 to 260 C (corresponding pressure of 0.69–4.83 MPa) is applied, with a holding time of a few minutes [8].

LHW is another hydrothermal pretreatment which can enhance sugar extraction [12]. Slurry is pre-heated via a heat exchanger, which not only saves steam consumption for heating the slurry, but also cooling water to cool down the pretreated material. The pre-heated slurry is further heated by steam via another heat exchanger, and passes through the reactor for pretreatment. Theoretically, the reactor should be operated at plug flow. Therefore, tubular reactors are preferred, and residence time and temperatures can be optimized for different types of feedstocks. Compared to SE with high solid uploading, the solid concentration in the slurry for the LHW process is much lower. During the LHW pretreatment, the pH of the biomass can drop below 4, which results in the formation of inhibitors due to the degradation of sugars under acidic conditions [13]. Thus, a pH control strategy can be applied to the system to maintain the pH value above 4, preferably between 5 and 7, by adding a base as needed [14,15]. Since the alkali is not a catalyst as in alkaline pretreatment to be addressed below, this process is termed as pH-controlled hot water pretreatment.

3.4.2 Chemical Pretreatment

Hydrothermal pretreatments under SE and LHW conditions involves the use of high temperature which dehydrate the sugars and produce inhibitors such as furfural from xylose and hydroxymethyfurfural from glucose. To overcome this problem, acids can come into play to

facilitate the deconstruction of LCCs under less severe conditions, where the sugars are not dehydrated. In this pretreatment, lower temperature or shorter reaction time is involved. Among various acids, sulphuric acid is commonly used. Although, the temperatures that is used in concentrated acid pretreatment are much lower, the recovery of the acid presents a big challenge for the economic viability of the process. Therefore, dilute acid is incorporated in the pretreatment which involves the concentration n not more than 2% that can be conveniently neutralized by lime or ammonium during the process of conditioning [16]. Dilute acid pretreatments is a very old method and has been extensively used and studied with various lignocellulose materials and reactors at different levels. [17-20].

Dilute acid pretreatment may seem economical in the industry, some disadvantages of the process like corrosion, which requires expensive acid-resistant stainless steel or coatings, and inhibitors produced during the pretreatment under high temperatures, have caused the exploration of other alternatives. Alkaline pretreatment is one of them. Various alkalis which includes sodium hydroxide, lime and aqueous ammonia have been used for various studies [21-23]. Basically, alkaline pretreatment is a delignification process, and the mechanism is the saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and lignin [24]. In addition, alkaline pretreatment also removes acetyl and other acidic substitutions on hemicelluloses that protect cellulose from attack by cellulase [25].Moreover, alkaline pretreatment causes swelling of the lignocellulosic biomass, leading to the decrease of DP and crystallinity of cellulose and increase of the surface area to facilitate the enzymatic hydrolysis of cellulose. The effectiveness of alkaline pretreatment depends on the characteristics of lignocellulosic biomass and reaction conditions. In general, alkaline pretreatment is more efficient with herbaceous crops and agricultural residues with relatively low lignin content. In comparison with the pretreatment technologies discussed previously, low temperature and pressure, even ambient conditions, can be applied under alkaline pretreatment conditions. However, the time required by the alkaline pretreatment process is hours, days or weeks rather than minutes, making it difficult to achieve the feedstock processing capacity required by commercial production of bulk commodities like ethanol. Moreover, a significant amount of salt produced during the pretreatment is a big problem, which not only affects microbial growth and fermentation, but also raises an environmental concern. Although an alternative strategy using ammonia—for example, the ammonia recycling percolation (ARP) process in which aqueous ammonia is recycled through a column containing lignocellulosic biomass such as corn stover [26]—can overcome these disadvantages, it is not cost-effective due to the high cost of the recovery of ammonia. A modified ARP process operated with low liquid ammonia throughput can address this issue to some extent [27], but is still not practical for commercial application. Ammonia fiber explosion (AFEX) is a hybrid of the SE and ARP processes, in which biomass is pretreated with liquid anhydrous ammonia at mild temperatures (60–100 C) and high pressure [28]. When the pressure is released, the rapid expansion of ammonia gas causes swelling of the biomass, which correspondingly disrupts LCCs and creates more accessible surfaces for enzymatic hydrolysis. Since temperatures in the AFEX process are much lower than those applied to the SE process, not

only can energy consumption be reduced, but also the formation of inhibitory by-products prevented. In addition, washing is not necessary for the process, which benefits for high solid loading hydrolysis. Meanwhile, ammonia remaining in the pretreated biomass facilitates microbial growth and fermentation. However, ammonia recovery by evaporation is needed, which complicates the system design and requires more capital investment and energy consumption [29].

3.4.3 Biological Pretreatment

Compared with physical and chemical pretreatments in which expensive equipment, chemicals and intensive energy consumption are needed, biological pretreatment by solid fermentation employs microorganisms that degrade lignocellulosic biomass at mild conditions without special requirements for equipment. Both bacteria and fungi have been explored, but rot fungi associated with wood decay are the predominant species in lignocellulose degradation for the purpose of biofuel production, particularly white-rot fungi due to their abundant ligninolytic enzymes, including lignin peroxidase, manganese peroxidase, laccases and other enzymes, and better selectivity in lignin degradation [30]. Although biological pretreatment is energy-saving and environmentally friendly, its disadvantages are apparent. Firstly, the extremely low degradation rate requires times as long as weeks for a significant change in the structure of the lignocellulosic biomass, making the process mismatched with the subsequent hydrolysis of cellulose and fermentation of sugars. Secondly, significant biomass is lost during the process, not only the lignin which is mineralized into lowmolecular- weight fragments that might be further catabolized into the useless final product CO2, but also sugars released from hemicelluloses and even cellulose by the hydrolytic enzymes (simultaneous decay with lignin degradation) as a carbon source to support the growth of the microorganisms [31]. Finally, the control of microbial growth and metabolism under open and solid fermentation conditions with mixture species is unreliable, which inevitably affects the subsequent processes such as cellulose hydrolysis and ethanol fermentation. Therefore, biological pretreatment is less attractive from the viewpoint of commercial application.

3.5 Enzymatic Hydrolysis of Cellulose

Following pretreatment, enzymatic hydrolysis is needed to further depolymerize the cellulose component to glucose, which can be used for ethanol fermentation together with sugars released from the hydrolysis of hemicelluloses during Bioethanol from Lignocellulosic Biomass 37 the pretreatment. Despite intensive R & D worldwide for decades, two barriers still remains to be overcome for developing viable processes to make bioethanol economically competitive. Unlike amylases and glucoamylases that are available at low prices for commercial production of various bulk products including ethanol from starch-based feedstocks, cellulases to liberate glucose from cellulose for bioethanol production are more expensive due to the difficulty of their fermentation production as well as the heterogeneous characteristic of the enzymatic hydrolysis which significantly compromises the reaction rate and increases the enzyme dosage. On the other hand, the

ethanologenic species, either S. cerevisiae which has been used for ethanol production from sugar- and starch-based feedstocks since the establishment of the industry, or Z. mobilis which has been intensively studied over the years due to its unique Entner–Doudoroff (ED) pathway for ethanol production with less biomass accumulation [32], cannot ferment pentose sugars in the hydrolysates into ethanol at rates and yields that are acceptable from the viewpoint of industrial production. Although the pentose sugars can be converted into other products like furfural through intramolecular dehydration of xylose by chemical catalysis, and xylitol, lactic acid and 2,3-butanediol by fermentations [33], all these processes seem not to be economically competitive at present, and most effort is still focused on the co-fermentation of the pentose and hexose sugars for bioethanol production by engineered strains.

3.6 Fermentation

Fermentation is a metabolic process that consumes sugar in the absence of oxygen. The products are organic acids, gases, or alcohol. It occurs in yeast and bacteria, and also in oxygen-starved muscle cells, as in the case of lactic acid fermentation. The science of fermentation is known as zymology. In microorganisms, fermentation is the primary means of producing ATP by the degradation of organic nutrients anaerobically. Humans have used fermentation to produce foodstuffs and beverages since the Neolithic age. For example, fermentation is used for preservation in a process that produces lactic acid as found in such sour foods as pickled cucumbers, kimchi and yogurt (see fermentation in food processing), as well as for producing alcoholic beverages such as wine (see fermentation in winemaking) and beer. Fermentation occurs within the gastrointestinal tracts of all animals, including humans.

4. REVIEW OF LITERATURE

4.1 Production of ethanol from lignocellulose biomass

Fossil fuels are depleted globally, rise in fuel prices, concerns in environment and pressures for oil independence are creating a strong market for biofuels [34]. Biofuels have the potential to be domestically and globally available for energy security, with most being carbon neutral, i.e, introducing no additional carbon to the global carbon cycle or potentially carbon negative and supportable within the current agricultural infrastructure [35]. Presently, one of the most promising alternatives for fossil fuels is bioethanol. Ethanol is a simple alkyl alcohol that can be used as a transport fuel in spark ignition engines. It has high octane levels and can be either blended into petrol or used in unmodified vehicles, or run as 100% ethanol in a converted engine [36].

Ethanol production can be carried out from various feedstocks which includes sugar substances such as sugarcane bagasses and molasses, as well as starch-based materials such as wheat and corn [37]. The ethanol production from corn starch is in high demand at the US bioethanol industry [38]. However, this technology of bioethanol production from corn may not be practical in the long run as there is wide planting of corn for ethanol production which will compete for the finite arable land and thus would threaten the national food security [39]. Lignocellulosic raw material has been suggested as the most prominent alternative for the traditional starch feedstock. Lignocellulosic feedstocks have maximum abundance, cost effective and have high polysaccharides (cellulose and hemicellulose) content [40]. Intensive research and developments in the last decades on lignocellulosic materials will most likely make them important feedstock for ethanol production in the future [41].

The currently available approach which converts lignocellulosic feedstock into ethanol involves a complex and expensive multi-step process that is a combination of thermochemical and biological methods [42]. Biomass conversion into ethanol involves three basic steps: (1) Pretreatment of the raw material to increase the accessibility of enzymes to the polysaccharides (cellulose and hemicellulose); (2) enzymatic hydrolysis so that the lignocellulose constituents (polysaccharides) are broken down; and finally (3) microbial fermentation, carried out by bacteria or yeast, which converts these sugars to ethanol. Making the transformation of lignocellulose to ethanol more economical and practical will require the development of molecular redesign of numerous enzymes, biochemical pathways, and full cellular systems.

Ethanol production from lignocellulosic biomass using enzymatic hydrolysis and fermentation can be improved by: (1) effective pretreatment technologies must be developed that do not require expensive chemicals and/or high pressure equipment; (2) a high density of cells are maintained within the reactor which converts sugars to ethanol quickly; (3) integrating enzymatic hydrolysis of cellulose and hemicellulose with fermentation to keep sugar levels low, that improves enzymatic

conversion rates by minimizing the product, i.e, sugar inhibition; (4) conversion of both the cellulose (glucose) and hemicellulose (xylose) to ethanol that increases the overall ethanol yield; (5) co-producing crude cellulase enzyme and/or recycle the enzymes so as to reduce enzyme costs; (6) incorporate low temperature separation of ethanol from the reactor broth so as to keep fermentation reaction rates high and allow recycle of enzymes without thermal destruction [43].

4.2 Lignocellulosic feedstock

Lignocellulosic materials are the renewable resources that can be directly or indirectly used for producing biomolecules and commodity chemicals [44]. Lignocellulose mainly consists of three major components- cellulose, hemicelluloses, and lignin. The cell wall polysaccharides of lignocellulosic biomass are composed of crystalline cellulose fibrils surrounded by a matrix of non-crystalline hemicelluloses, which are a group of hetero-polysaccharides that bind with pectin to cellulose, forming a meshlike network of cross-linked fibres.

Cellulose is a linear polymer chain which is formed by joining the anhydro-glucose units into glucan chains. These anhydro-glucose units are linked together by β-(1, 4)-glycosidic bonds. Due to this linkage, cellobiose is established as the repeat unit for cellulose chains[45]. The degree of polymerization (DP) of native cellulose is in the range of 7,000-15,000 [46].

By forming intra-molecular and inter-molecular hydrogen bonds between -OH groups within the same cellulose chain and the surrounding cellulose chains, the chains tend to arrange in parallel and form a crystalline super molecular structure. Then, bundles of linear cellulose chains (in the longitudinal direction) form a microfibril which is oriented in the cell wall structure[47].
But, hemicellulose has a different structure than cellulose. It consists of different monosaccharide units. The polymer chains of hemicelluloses are short-branched and amorphous. Owing to the amorphous morphology, hemicelluloses are partially soluble in water. The backbone of hemicellulose chains can be a homopolymer, i.e, generally consisting of single sugar repeat unit or a heteropolymer, i.e, mixture of different sugars [48]. These polysaccharides are formed by a wide variety of building blocks including pentoses (e.g., xylose and arabinose), hexoses (e.g., glucose, mannose and galactose) and uronic acids (e.g., 4-O-methyl-glucuronic and galacturonic acids). Generally, these polysaccharides fall into four classes: (a) unbranched chains such as (1-4)-linked xylans or mannans; (b) helical chain such as (1-3)-linked xylans; (c) branched chains such as (1-4)-linked galactoglucomannans; and (d) pectin substances such as polyrhamnogalacturonans. Some hemicelluloses, particularly heteroxylans, also show a considerable degree of acetylation[49]. The most important sugar of the hemicelluloses component is xylose. Hemicelluloses are able to bind to cellulose by multiple hydrogen bonds and to bind to lignin by covalent bonds.

Lignin is a complex hydrophobic network of phenylpropanoid units that is thought to result from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors

[50]. Lignin has been recognized not only to give mechanical strength or rigidity to a plant [51], but also to prevent the invasion by pathogens and pests [52]. Moreover, lignin serves as a disposal mechanism for metabolic waste. Lignin is described as a random, three-dimensional network of phenylpropane units. The precursors of these phenylpropane units are coniferyl, sinapyl, and p-coumaryl alcohols, which are transformed into lignin by a complex dehydrogenative polymerization process. These three aromatic monomers in lignin are referred to as p-hydroxyphenyl, guaiacyl and syringyl residues, respectively [53]. Depending upon the number and type of functional groups on the aromatic rings and propane side chains, the solubility of lignin is highly variable [54]. Lignin in plant cell walls is physically and chemically associated with wall polysaccharides and proteins. The association between lignin and polysaccharides includes glycosidic linkages, ether cross-linkages, ester cross-linkages and cinnamic acid bridges.

Except for the three major components above, lignocellulosic biomass also contains extractives, which refer to the organic substances which have low molecular weight and are soluble in neutral solvents. Resins, fats, waxes, fatty acids and alcohols, phenolics, phytosterols, salts, minerals, and other compounds are categorized as extractives. Moreover, the residue remaining after ignition (dry oxidation at 575 25˚C) of lignocellulosic biomass is ash, which is composed of minerals such as silicon, aluminum, calcium, magnesium, potassium, and sodium[55].

4.3 Pretreatment methods

Lignocellulosic biomass, as the prospective source of fermentable sugars for ethanol bio-production, has great potential use in industry. However, lignocellulosic feedstocks are not easily broken down into their composite sugar molecules. As a result, an effective pretreatment is required to liberate the polysaccharides from the lignin seal and its crystalline structure so as to render it accessible for a subsequent hydrolysis step [56].

Ammonia as a pretreatment reagent has many advantages for an effective delignification as well as swelling of biomass. Meanwhile, ammonia pretreatment does not significantly produce the inhibitors, e.g., furfural and hydroxy methyl furfural (HMF), which are by far considered as the most toxic inhibitors present in lignocellulosic hydrolysate [57] for the downstream biological processes. The presence of inhibitors complicates the ethanol production and increases the cost of production due to required detoxification steps.
The soaking in aqueous ammonia (SAA) at low temperature retains the hemicellulose in the solids by minimizing the interaction with hemicellulose during treatment, which was reported as a feasible approach to increase the ethanol yield based on total sugars and simplify the bioconversion scheme. Retained xylan can usually be hydrolyzed to fermentable pentoses by most commercial cellulase and xylanase mixtures [58]. The fact that 100% of glucan and over 85% of xylan remained in the solids after SAA pretreatment provides the opportunity for increasing the ethanol yield based on totals sugars, i.e., hexose and pentose, and simplifying the bioconversion scheme.

4.4 Bioconversion strategies and process integration

4.4.1 Separate enzymatic hydrolysis and fermentation (SHF)

The terminology ― separate hydrolysis and fermentation (SHF)‖ refers to a process in which enzymatic hydrolysis (saccharification) of polysaccharides and the microbial fermentation are performed sequentially in separate units (Fig. 1.3(a)). The major advantage of this method is that the enzymatic hydrolysis and fermentation can be carried out at their own optimum conditions: 45-50˚C for enzymatic hydrolysis with cellulase and β-glucosidase, and 30-37˚C for ethanol fermentation with fermentative microorganisms.

However, the drawbacks of SHF process include: (1) the inhibition of the released sugars, mainly cellobiose and glucose, on cellulase activity. At a cellobiose concentration as low as 6 g/l, the activity of cellulase is reduced by 60%. Although glucose decreases the cellulase activity as well, the inhibitory effect of glucose is lower than that of cellobiose. On the other hand, glucose is a strong inhibitor for β-glucosidase. At a level of 3 g/l of glucose, the activity of β -glucosidase is reduced by 75% [59]; (2) high probability of contamination since separate vessels are used for hydrolysis and fermentation. The hydrolysis process is long and the released sugars as carbon source provide the opportunity of contamination with naturally-occurring microbes. A possible source of contamination could be the enzyme preparation. In practice, it is difficult to sterilize enzymes in large scale, since it must be filter-sterilized owing to the denaturation of enzymes in an autoclave. In addition, it is not feasible to add antibiotics in hydrolysis reactor because antibiotics may affect the growth and fermentation of microorganisms in the subsequent fermentation step.

4.4.2 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) integrates enzymatic hydrolysis and microbial fermentation into one step: the sugars released from enzymatic hydrolysis are immediately consumed by the fermentative microorganism for ethanol production (Fig. 1.3(b)). By SSF, enzymatic hydrolysis rates can be maximized by reducing the product (sugar) inhibition. SSF gives higher reported ethanol yields from cellulose than SHF and requires lower amounts of enzyme [60]. Another advantage of SSF is that the risk of contamination is reduced due to the low sugar concentration and ethanol accumulation in the fermentation system. Furthermore, the number of vessels required for SSF is reduced in comparison to SHF, resulting in lower capital cost of the process.

SSF has the drawback that the enzymatic hydrolysis and fermentation have to be performed under the comprised conditions, particularly with respect to pH and temperature. The optimum temperature and pH of enzyme activity and fermentation are always different. Hydrolysis is usually the rate-limiting step in SSF, and the optimal temperature of enzyme reaction is typically higher

than that of fermentation. Therefore, several thermotolerant bacteria and yeasts, e.g., *Candida acidothermophilum* and *Kluyveromyces marxianus* have been proposed for SSF in order to raise the temperature close to the optimum temperature of enzyme reactions. Inhibition of ethanol accumulated during the SSF process on enzymes and microorganisms may also be a disadvantage of SSF. It was reported that the enzyme activities are reduced by 25% when ethanol concentration is 30 g/l [61].

4.4.3 Simultaneous saccharification and co fermentation (SSCF)

Basically, simultaneous saccharification and cofermentation (SSCF) has the same mechanism with SSF. Cofermentation refers to the fermentation of both hexoses (C_6) and pentoses (C_5) to ethanol. The hydrolyzed hemicellulose during pretreatment and the solid cellulose are not separated after pretreatment, allowing the conversion of cellulose and hemicellulose to be carried out in a single reactor via a single step [62].

Some conventional wild type fermentative microorganisms, e.g., *Saccharomyces cerevisiae*, are only capable of utilizing glucose as the carbon source for ethanol production, while some can consume both hexoses and pentoses, e.g., *Pichia stipitis*. However, the growth and fermentation of *Pichia stipitis* require expensive media, making the fermentation with *Pichia stipitis* less cost-effective. Therefore, one important requirement is an efficient microorganism capable of fermenting a wide range of substrates (pentoses and hexoses) as well as to tolerate stress conditions. There have been multiple efforts to develop these recombinant microorganisms to allow co-fermentation of both pentose and hexose sugars. Paticularly, three main microbial platforms, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli*, have emerged and their performance has been demonstrated in pilot studies [63].

4.4.4 Consolidated bioprocessing (CBP)

Consolidated bioprocessing (CBP) (Fig. 1.3(c)) combines cellulase production, cellulose hydrolysis and fermentation in one step. CBP is distinguished from other less highly integrated configurations in that it does not involve a dedicated process step for cellulase production. Progress in developing CBP-enabling microorganisms is being made through two strategies: engineering naturally occurring cellulolytic microorganisms to improve product-related properties, such as yield and titer, and engineering non-cellulolytic organisms that exhibit high product yields and titers to express a heterologous cellulase system enabling cellulose utilization [64]. Application of CBP entails no operating costs or capital investment for purchasing enzyme or its production.

4.4.5 Pentose fermentation

Hemicellulose is one of the major components of lignocellulosic biomass. The content of hemicellulose counts for approximately 25 wt. % in lignocellulosic biomass. The conversion of both cellulose and hemicellulose for production of fuel ethanol is being studied intensively with a view to develop a technically and economically viable bioprocess. The ethanol yield in the process is an important parameter with regard to economy both because the cost of the raw material constitutes a major part of the total production cost and also because the processing costs are typically associated with the amount of material passing through the process and not the amount of product made. To achieve maximum ethanol yield, all monosaccharides have to be fermented. The pentose content in the raw material is of importance as pentoses are difficult to ferment to ethanol [65]. The strategies that have been put forward for the conversion of both hexose and pentoses to achieve high ethanol concentration, yield, and productivity mainly include: (1) separate pentose and hexose fermentation with glucose or xylose-fermenting microbes alone; (2) cofermentation of hexoses and pentoses via SSCF with microorganisms that are capable of metabolizing both hexoses and pentoses; (3) coupled isomerization and fermentation with xylose isomerase enzyme as well as microbes.

4.4.5.1 Separate pentose and hexose fermentation

In dilute acid and hot water pretreatment, the hemicellulose component and lignin is solubilized into the liquid fraction, while cellulose remains in the solids. Cellulose is then hydrolyzed with cellulase and β-glucosidase to glucose, which is then fermented to ethanol by yeast or some bacteria. The process can be carried out either with separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). The liquid fraction which contains hemicellulose is subjected to the processes of lignin separation and conditioning before being fermented with pentose-fermenting microorganisms. The fermentation processes on hexose (from cellulose) and pentose (from hemicellulose) are performed in two separate vessels.

4.4.5.2. Coupled isomerization and fermentation

It has been reported that yeasts are able to ferment xylulose to ethanol under anoxic conditions. Therefore, a coupled isomerization and fermentation process is proposed in which xylose is converted with exogenous, immobilized xylose isomerase (already commercially derived from bacteria) to an equilibrium mixture of xylose andxylulose, which would be then be fermented to ethanol and the residual xylose recycled over the xylose isomerase. The process would be continued until all xylose was consumed. Xylose isomerase could be incorporated directly into the fermentation vessel or the xylulose could be produced exogenously and separated from the xylose prior to fermentation [66].

Although sequential xylose isomerization and fermentation is technically feasible, it is impeded by several factors: the cost of the enzymatic isomerization, the formation of xylitol as a by-product, inhibition of xylose isomerase by xylitol, the use of separate optimal pile and temperatures for isomerization and fermentation, and the low rate of xylulose fermentation [66].

4.5 Enzyme Recycling

Despite recent improvement in cellulase enzymes properties, the high cost associated with the hydrolysis step remains a major impediment to the commercialization of full-scale lignocellulose-to-ethanol bioconversion process. Strategies to reduce enzyme cost include increasing enzyme production efficiency, increasing enzyme specific activity and recycling cellulase enzymes to be used in subsequent hydrolysis [67].

During hydrolysis, cellulase enzymes typically are present in the system in two different forms. Some cellulase enzymes remain free in the solution (free enzyme), while others are bound to the residual solids (both cellulose and lignin). An efficient enzyme recycling requires effective recovery of both free and adsorbed cellulase. Previous studies have demonstrated that free enzyme could be recovered potentially by membrane filtration and affinity re-adsorption with fresh substrates. However, there is a lack of effective strategy to recover bound enzyme after lignocellulose hydrolysis. This is primarily due to the non-productive binding between enzyme and residual lignin [68].

4.6 Overcoming the challenges to lignocellulosic ethanol

4.6.1 Challenges with current lignocellulosic ethanol production technologies

Presently, the major challenges in lignocellulose-to-ethanol processes include: (1) the currently available pretreatment technologies are chemical and energy intensive; (2) the consumption and cost of enzymes associated with the hydrolysis step is high [68]; (3) fermentation of pentoses is restricted on real substrates; (4) the concentration of ethanol in final broth is low, contributing to high energy demand in recovery process.

4.6.2 Efforts required to overcome the challenges from lignocellulose to ethanol

Future development of lignocellulose-to-ethanol processes should necessarily involve investigations on cost-effective pretreatment technologies, applications of biotechnology to industrial microorganisms and biocatalysts (enzymes) together with bioprocess engineering to integrate and optimize the production strategies.

4.6.2.1 Development of cost-effective pretreatment

Pretreatment has been viewed as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion with costs as high as 30¢ per gallon ethanol produced [69]. There is great potential to improve the efficiency and lowering of the cost of pretreatment. The improvements in pretreatment technologies are based on better understanding of the chemistry of plant cell walls and the chemical reactions that occur during pretreatment. The future efforts on pretreatment research would be focused on the following areas: (1) developing pretreatment methods that are universally successful with multiple crops, sites, ages, and harvest times; (2) minimizing water and chemical consumption for pretreatment and post treatment; (3) increasing total sugars yields; (4) reducing the generation of inhibitors to eliminate hydrolysate conditioning [70]; (5) minimizing energy requirements.

4.6.2.2 Improvement in enzymatic hydrolysis efficiency and economy

In addition to making more reactive solids through pretreatment, enzymes with better properties are needed to increase reaction rates and achieve high yields of fermentable sugars with much less enzymes, leading to cost reduction in enzymes. Other representative strategies to reduce enzyme cost include increasing enzyme production efficiency and enzyme recycling. Furthermore, the efficiency of enzymatic hydrolysis can also be enhanced by optimizing the composition of enzyme mixtures (cellulase mixtures for optimal synergy) and identifying the role of additional enzymes (hemicellulases, lignin modifying, etc.). Enzyme cocktails that can effectively release the hemicellulose left in pretreated solids are also important for achieving the high yields needed for large-scale competitiveness [71].

4.6.2.3 Increase of overall yield and final concentration of ethanol

The best opportunity for reducing the cost of cellulosic ethanol is, by far, through enhancing sugar yields from cellulose and hemicellulose. Advances in metabolic and genetic engineering have led to the development of microorganisms capable of efficiently converting biomass sugars into ethanol. Generally, such development relies on broadening the substrate range to include other biomass sugars such as arabinose or xylose in strains that cannot ferment sugars other than glucose. On the other hand, the utilization of multiples sugars derived from lignocellulosic biomass can be achieved with appropriate fermentation strategy via single or multiple microorganisms. Further optimization of these strategies is necessary.

Increasing the ethanol concentration in the feed to the distillation reduces the production costs considerably [72]. To lower the cost of ethanol distillation of fermentation broths, a high initial substrate concentration is desirable. However, an increase in the substrate concentration typically reduces the ethanol yield owing to insufficient mass and heat transfer. Therefore, the high-solid fermentation process requires further development and optimization, or an alternative strategy should be necessarily introduced.

4.7 Literature review of different pretreatments

Table 4.1: Different pretreatment methods

Method	Physical conditions	Chemical used	Lignin concentration	Cellulose concentration	Hemicellulose concentration	Sugar	References
Steam explosion	1.3 Map, 190°C, 15 mins	NaOH for alkaline delignification	17% lignin solubilisation from sugarcane bagasse	45%	27.8%		Rocha et al.(2012)
Alkaline	Solid-liquid ratio 1:10(w/v), 100°C, 1 hr	NaOH 1% (w/v)	93% lignin solubilisation from sugarcane bagasse after steam explosion	35%		0.56g of reducing sugar/g of sugarcane bagasse	Rocha et al.(2012)
Alkaline	121°C, 15 mins	2% H_2O_2 and 1.5% NaOH		cellulose level is increased by 1.2 times	decreased hemicellulose content 8.5 times in sugarcane bagasse		Aguiar et al. (2010)
Acid treatment	Enzymatic hydrolysis after microwave - alkali treatment	1% H_2SO_4	45% lignin			0.83g reducing sugar/ dry sugarcane bagasse	Binod et al. (2010)
Ammonia fibre expansion	140C, 30 mins	2kg ammonia+ 1.5 kg water/kg dry bagasse, 140C, 30 mins		85% gluten conversion by celluloses	95-98% xylan conversion by hemicelluloses in bagasse and cane leaf residue		Krishna et al. (2010)
Organosolv	60 mins, 195C	30%(v/v) ethanol with NaOH preceded by dilute acid treatment				Residual solid material from sugarcane bagasse containing 67.3% (w/w) glucose.	Mesa et al. (2011)

Table 4.2: Different pretreatment methods

Organosolv	150 mins, 198.3C	Glycerol 80% in water	Pulp with 7.75% residual lignin (81.4% delignification)			62% of glucose (v/v)	Nova et al. (2011)
Liquid hot water	190-230C, rapid immersed percolation (45s to 4 mins)	Hot water	60% of the acid insoluble lignin	Less than 10% of the cellulose	50% solubilisation of sugarcane bagasse and leaves all hemicellulose		Allen et al. (1996)
Wet oxidation	195C, 15 mins	Alkaline (NaOH)	50% lignin	Solid material with 70% cellulose with solubilization	93% hemicelluloses		Martin et al.(2007)
Solid-state fermentation	2 weeks, 30C	*Phanerochaete chrysosporium*	Reduction of lignin content from 26.5% to 21.3% in chemithermomechanical			52% glucose which converts into ethanol	Pellinen et al. (1989)
Solid state fermentation	2 weeks	*Pycnoporus cinnabarium*	6-10% delignification of *Prosopis juliflora* and *Lantana camara*	Less than 20% of cellulose left			Gupta et al. (2011)
Enzymatic delignification	At 400 IU/ml, 8 hrs	Laccase from *Pleurotus sp.*	84% delignification of *Bambusa bamboo*		Xylose converts into ethanol		Kuila et al. (2011)
Enzymatic delignification		laccase delignification in ionic liquid aqueous media containing 5% ionic liquid	50% delignification of wood biomass after 24 hr	40% cellulose converts into ethanol			Moniruzzaman and ono et al. (2012)

4.8 Physical properties of ethanol

Ethanol is volatile, clear, flammable, colourless liquid. It has a pleasant odour. The physical and chemical properties of ethanol are dependent upon hydroxyl group. This hydroxyl group gives polarity to the molecule and also gives rise to hydrogen bonding.

Table 4.3: Properties of ethanol

Properties of ethanol	Value
Freezing point of ethanol in °C	-114.1
Boiling point of ethanol in °C	78.32
Critical temperature of ethanol in °C	243.1
Critical pressure of ethanol in atm	63.0
Critical volume of ethanol in /mole	0.167
Solubility of ethanol in water at 20 °C	miscible
Lower % by volume	4
Upper % by volume	19
Auto-ignition temperature of ethanol in °C	793
Flash point, open cup, °F	70.0
Specific heat at 20 °C, cal/kg °C	0.579
Thermal conductivity of ethanol at 20 °C	0.00170 J/sec.cm^2

5. MATERIALS AND METHODS

5.1 Raw materials

Two different lignocellulosic raw materials were used in the study. All lignocellulosic material were obtained from different places in Roorkee. The raw materials were wood dust and banana peel.

5.2 Characterisation of the lignocellulosic raw material

The composition of the lignocellulosic raw materials was determined by the methods given by NREL.

5.2.1 Determination of extractives content

3.0g of the raw material sample was taken and dried in the oven at 105°C till the weight is constant. It is followed by setting up of the soxhlet apparatus. 250 ml of acetone is taken and the temperature was set at 75°C. The apparatus was then run for 7 hours to take out the extractives from the sample. After the completion of the process, the raw material sample is again dried in the oven at 105°C until a constant weight of the sample is obtained. The difference in weight before and after the treatment of the raw material gives the amount of extractives (%w/w) present in the sample.

5.2.2 Determination of hemicellulose content

1.0g of extracted dried raw material sample was transferred into a 250 mL flask. 150 mL of 500 mol/m3 NaOH was added to the sample. The mixture was boiled for 2.5 hours with distilled water. It is then filtered after cooling through vacuum filtration and washed with distilled water until pH becomes neutral. The residue was dried at 105°C in a convection oven until a constant weight is obtained. The difference between the sample weight before and after the treatment is the hemicellulose content (%w/w) of dry biomass.

5.2.3 Determination of lignin content

0.3g of dried extracted raw sample material was weighed in glass test tubes and 3 mL of 72% H2SO4 was added to it. The sample was kept at room temperature for 2 hours with carefully shaken at every 30 mins interval to allow complete hydrolysis. After the initial hydrolysis, 84 mL of distilled water was added to the mixture. The second step of hydrolysis was done in an autoclave for 1 h at 121°C. The slurry was then cooled at room temperature. Hydrolyzates were filtered after cooling through vaccum filter. The acid insoluble lignin was determined by drying the residues at 105°C and determination of ash content was done by incinerating the hydrolyzed samples at 575°C in a muffle furnace. The acid soluble lignin fraction was determined by measuring the absorbance of the acid hydrolyzed samples at 320 nm. The lignin content was calculated as the summation of acid insoluble lignin and acid soluble lignin.

5.2.4 Determination of cellulose content

The cellulose content (%w/w) was calculated by difference, assuming that extractives, hemicellulose, lignin, ash, and cellulose are the only components of the entire raw material.

5.3 Pretreatment methods

For the conversion of lignocellulosic material into ethanol, the very first step is to remove lignin from the lignocellulosic material by using various pretreatment methods. In this study, different pretreatment methods were used for the breakdown of the lignocellulose structure.

5.3.1 Biological pretreatment

In the enzymatic pretreatment method, *Phanerochaete chrysosporium* (MTCC 787) was used. The strain produces lignin peroxidase, manganese peroxidase and laccase enzyme which is required for the breakdown of the complex structure of lignocellulose raw materials and separate the lignin from cellulose and hemicellulose. Later, *Saccharomyces cerevisiae* (MTCC 1194) will be used for the process of fermentation. *Phanerochaete chrysosporium* was ordered from MTCC, Chandigarh, as this microbe produces LiP, MnP and laccase enzyme which are required for the breakdown of the complex structure of the lignocellulosic material consisting of cellulose, hemicellulose and lignin. They can also produce cellulase in different conditions. The media prepared for it's growth contains the following ingredients- Glucose- 10g, Malt extract- 10g, Peptone- 2g, Yeast extract- 2g, Asparagine- 1g, KH_2PO_4 - 2g, Thiamin-HCl- 1mg, Agar- 20g, $MgSO_4.H_2O$- 1g. All the constituents are added in 1 litre of distilled water and then plates and slants were prepared which took 2-5 days at 39°C for the microbial growth. Spores (conidia) are prepared by suspension in sterile water followed by passage through sterile glass wool to free it of contaminating mycelia. Spore concentration is determined by measuring absorbance at 650 nm. The culture media had basal III media and trace elements solution. The composition for basal III media is KH_2PO_4 20g, $MgSO_4$ 5g, $CaCl_2$ 1g. The trace elements solution has the following composition- $MgSO_4$ 3g, $MnSO_4$ 0.5g, NaCl 1.0g, $FeSO_4 \cdot 7H_2O$ 0.1g, $COCl_2$, 0.1g, $ZnSO_4 \cdot 7H_2O$ 0.1g, $CuSO_4$ 0.1g, $AlK(SO_4)_2 \cdot 12H_2O$ 10mg, H_3BO_3 10mg, $Na_2MoO_4 \cdot 2H_2O$ 10 mg, Nitrilotriacetate, 1.5 g.

The medium for agitated cultures has 0.05% Tween 80 and the fungus is introduced as a mycelial suspension. The detergent is solubilized and sterilized by autoclaving a 1% solution in distilled water. The mycelial inoculum is prepared by growing the fungus from spore suspension in stationary flasks containing 50 ml of the above medium (without detergent). After 48 hours at 39°C, the mycelium and the medium is blended for 1 minute in a blender.

Agitated cultures are taken in the flask and are grown in 250 ml flasks. The cultures are kept at 39°C for their optimal growth on a rotary shaker and the flasks are kept at about 175 rpm. The rubber-stoppered culture flasks are flushed with 100% O_2 at the time of inoculation, and daily thereafter. Enough cultures are grown to yield approximately 500 ml of culture supernatant. Mycelial growth under the nitrogen-limited conditions stops by day 2 and enzyme activity appears

in the extracellular fluid by day 4, coinciding with development of a brown coloration on the mycelia (Excess trace elements solution gives this colour to the solution). Under both stationary and shaken incubation, activity reaches a maximum on days 5 and 6. After days 5 or 6, the enzyme activity is at it's maximum and the solution is taken for centrifugation at 10000g for 5 minutes at 4°C. The yellow supernatant is then concentrated by ultrafiltration. Now, the supernatant is taken out with the help of vacuum filtration and the mycelial slime is discarded. The yellow supernatant is kept for later use at 4°C.

Enzymatic pretreatment was carried out by adding yellow supernatant to the lignocellulosic raw material. 50ml of different concentrations of the enzyme was added to 5g of the lignocellulosic raw material. The temperature was set at 39°C which is optimum for the activity of lignin peroxidase and manganese peroxidase. DNS method was carried out later for the determination of glucose content in the solution at various time interval.

5.3.1.1 Lignin peroxidase enzyme assay

LiP was measured with veratryl alcohol at 25°C. The reaction mixture contained 0.1 M sodium tartrate buffer (pH 3.0), 0.4 mM veratryl alcohol, and 1.65 ml of culture filtrate in a total volume of 3 ml. The reaction was started by adding H2O2 to a final concentration of 0.2 mM and absorbance at 310 nm was measured. The method was given by Then and Kirk (1988).

5.3.1.2 Manganese peroxidase enzyme assay

MnP activity was monitored with phenol red at 30°C. Reaction mixture contained 25 mM lactate, 0.1 mM MnSO4, 1 mg of bovine serum albumin in 1ml, 0.1 mg of phenol red in 1ml , and 0.5 ml of culture filtrate in 20 mM sodium succinate buffer (pH 4.5) in a total volume of 1 ml. The reaction was started by the addition of H2O2 to final concentration of 0.1 mM and was stopped after 1 min with 0.05 ml of 10% NaOH and absorbance at 610 nm was monitored. The method was given by Then and Kirk (1988).

5.3.2 Acid pretreatment

Dilute as well as concentrated acids were used for this process. Limitation of this process was that acids were very toxic and corrosive. Sulphuric acid was used for the process. Neutralization of the material is required in this process for the next hydrolysis step as the enzymes cannot bear the acidic condition. So, pH is made neutral. We can make it neutral by adding NaOH. In this process, raw material was soaked in acid in different concentrations at 100°C and then DNS method was carried out for determination of glucose content in the solution.

5.3.3 Alkali and acid pretreatment

Alkali pretreatment is used for the lignocellulosic material having high lignin content. During alkali pretreatment, lignin structure is ruptured and bonds between lignin and cellulose is broken. Crystallinity of the material was decreased by this process. Ammonia is also used for the removal of lignin. Various alkali like NaOH, $Ca(OH)_2$, KOH is used for delignification. Alkali pretreatment is mainly used in paper and pulp industry. In this method, NaOH is used. Here, different concentrations of NaOH is used to treat the lignocellulosic material. The temperature was set at 70°C for 24 hours. In this method, ester bonds between xylose and other compounds were broken. After that, different dilute concentration of sulphuric acid was used for the treatment at same temperature. DNS method was used for the determination of glucose content in the solution after every 4 hours.

5.3.4 Pretreatment with sulphuric acid and potassium dichromate

This pretreatment technique is used to increase the efficiency of the acid pretreatment technique. In this, the lignin structure is broken down and the cellulose and hemicellulose is exposed. The technique is used to give better results of delignification. Here, different concentrations of H_2SO_4 and $K_2Cr_2O_7$ are used to treat the lignocellulosic raw material. The temperature was set at 100°C. Later, DNS method was carried out for determination of glucose content in the solution.

5.4 Hydrolysis method

Enzymatic pretreatment was carried out by directly adding pure cellulase enzyme to the lignocellulosic material which converts the cellulose into glucose. 50ml of different concentrations of the enzyme was added to 5g of the lignocellulosic raw material. The temperature was set at 35°C which is optimum for cellulase activity.

5.5 Fermentation

Fermentation is the final stage of bioethanol production. In this stage, the glucose is converted into ethanol. *S. cerevisiae* or *Z. mobilis* is used to convert monosaccharides and disaccharides produced during hydrolysis process into ethanol with the help of the enzymes invertase and zymase present in micro-organisms. *S. cerevisae* is used in this process. The *S. cerevisiae* cells were suspended in deionized water and the pretreated raw material was used as the only carbon source for the yeast cells. Fermentation process was allowed to continue for three days because *S. cerevisiae* reaches it's optimal growth in three days, and finally, the samples were centrifuged and bioethanol was analyzed from the filtrate using chromatography.

6. RESULTS AND DISCUSSION

There is a wide range of lignocellulose biomass which are used worldwide for research to produce bioethanol. In this project work, two were chosen. One being the banana peel and the other being wood dust. These lignocellulosic materials are composed of cellulose, hemicellulose and lignin. These biomass have different composition of cellulose, hemicellulose and lignin which varies with different species, genus, climate, soil, etc. The commonly used biomass used are rice straw, corn stover, sugarcane bagasse, wheat straw etc. According to various literatures, it was found that wood dust contains around 35-45% of cellulose, 15-25% of hemicellulose and 20-30% of lignin. Also, the banana peel's constituents are 30-35% of cellulose, 15-20% of hemicellulose and rest of lignin. It was observed that pretreatment processes were the bottleneck in bioethanol production as these techniques help to expose the lignocellulose structure, removing the lignin which contains furfurals that inhibits the process of bioethanol synthesis.

6.1 Compositional analysis-

The compositional constituents of wood dust and banana peel is given below-

Table 6.1: Constituents in wood dust and banana peel

Constituents in %	Wood dust	Banana peel
Cellulose	33%	32%
Hemicellulose	18%	25%
Lignin	29%	15%
Extractives	12%	18%
Ash and others	8%	10%

6.2 Biological pretreatment

6.2.1 Lignin peroxidase and manganese peroxidase assay

The yellow supernatant was subjected to enzyme assay for lignin peroxidase according to the Tien and Kirk method (1988).

Table 6.2: Delignification enzyme assay

	Lignin peroxidase (mol/l)	Manganese peroxidase (mol/l)
Normal media	1.85*10^-6	2.20*10^-7
Normal media+ 5ml trace elements	5.69*10^-6	1.51*10^-6

	Lignin peroxidase (mol/l)	Manganese peroxidase (mol/l)
Normal media+ 20ml trace elements	8.77*10^-6	6.34*10^-6

It was observed that when trace elements concentration was increased in the media, the enzyme activity also increases to some extent.

The yellow supernatant, containing lignin peroxidase, laccase and manganese peroxidase, was mixed with the lignocellulosic material and kept in the incubator at 39°C. Then, DNS assay was carried out every 24 hours. The results are given below along with a graphical representation.

Table 6.3: Absorbance at 650 nm at different time in case of wood dust and banana peel

Lignocellulosic material	24 hrs	48 hrs	72 hrs
Wood dust	0.393	0.439	0.488
Banana peel	0.815	0.971	0.982

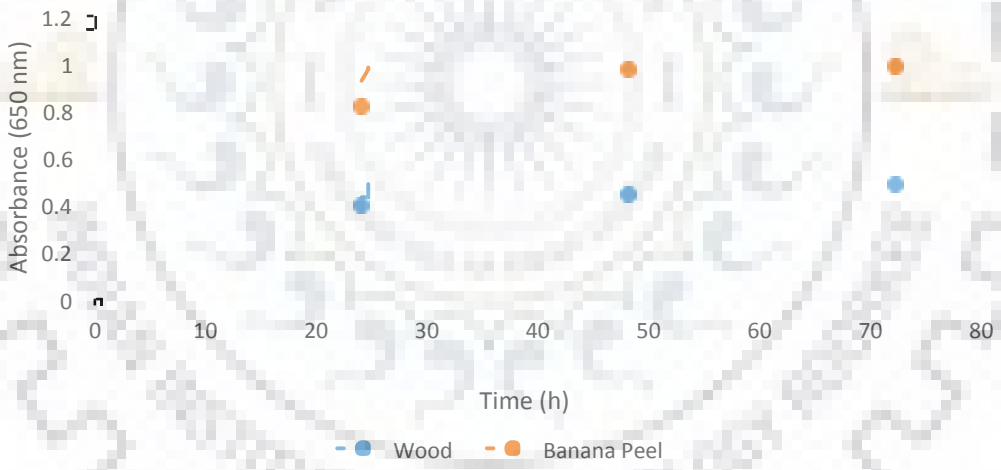

Fig 6.1: Graph for biological pretreatment on banana peel and wood dust.

In the biological pretreatment of wood dust and banana peel with the enzymes produced by *Phaenerochyta chrysosporium*, it is found that degradation of lignin structure occurs and it increases with course of time but it comes to a constant by around 72 hours which shows that the enzyme has shown it's maximum activity.

6.3 Acid pretreatment-

Sulphuric acid, at different concentrations was used on banana peel and temperature was kept at 70°C. Then, DNS assay was carried out every 4 hours.

Table 6.4: Absorbance at 650 nm at different time and concentration in banana peel

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs	20 hrs
1%	0.02	0.03	0.03	0.03	0.03
2%	0.02	0.02	0.02	0.03	0.04
3%	0.01	0.01	0.02	0.02	0.02
4%	0.01	0.04	0.04	0.04	0.05
5%	0.01	0.05	0.05	0.05	0.09
6%	0.03	0.04	0.04	0.04	0.05
7%	0.04	0.04	0.04	0.05	0.06
8%	0.02	0.03	0.04	0.05	0.06
9%	0.03	0.04	0.05	0.06	0.08
10%	0.04	0.07	0.08	0.11	0.16
11%	0.01	0.02	0.06	0.07	
12%	0.02	0.04	0.07	0.12	
13%	0.02	0.03	0.11	0.13	
14%	0.01	0.05	0.14	0.15	
15%	0.03	0.09	0.25	0.28	

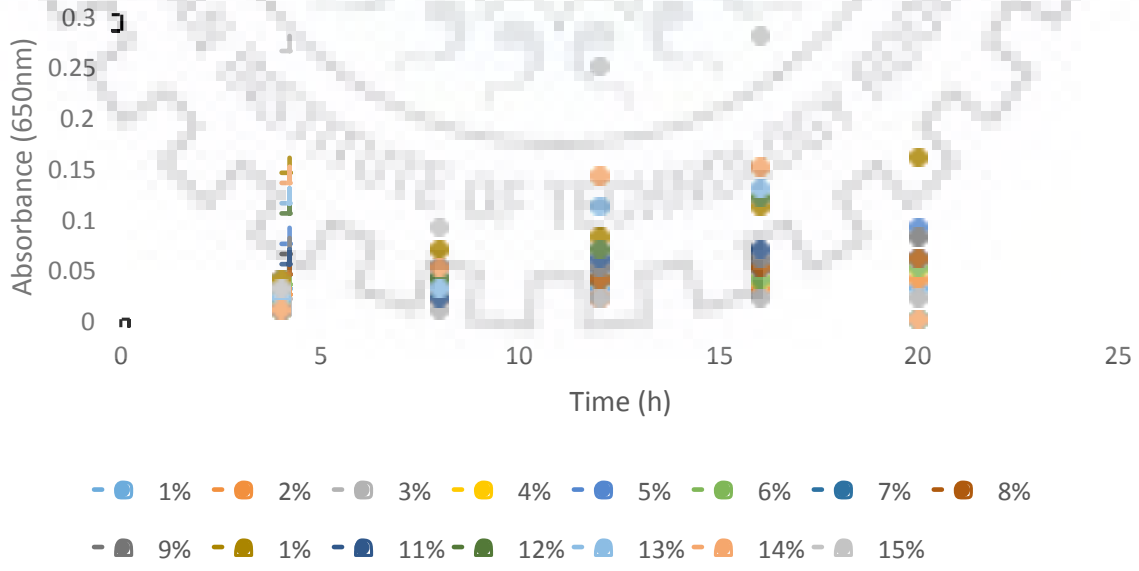

Fig 6.2: Graph for acid pretreatment on the banana peel

Sulphuric acid, at different concentrations was used on wood dust and the temperature was maintained at 100°C. Then, DNS assay was carried out every 4 hours.

Table 6.5: Absorbance at 650 nm at different time and concentration in wood dust.

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs	20 hrs
6%	0.03	0.04	0.03	0.04	0.04
7%	0.01	0.02	0.03	0.04	0.04
8%	0.02	0.03	0.04	0.03	0.03
9%	0.03	0.04	0.05	0.06	0.08
10%	0.06	0.07	0.06	0.06	0.12
11%	0.01	0.01	0.06	0.07	
12%	0.00	0.04	0.07	0.12	
13%	0.01	0.03	0.11	0.13	
14%	0.00	0.05	0.13	0.15	
15%	0.03	0.09	0.25	0.28	

Concentration of H_2SO_4	2 hrs	4 hrs	6 hrs	8 hrs	10 hrs
1%	0.01	0.02	0.03	0.03	0.03
2%	0.01	0.02	0.01	0.01	0.02
3%	0.00	0.01	0.01	0.02	0.02
4%	0.00	0.01	0.03	0.04	0.05
5%	0.01	0.02	0.01	0.04	0.05

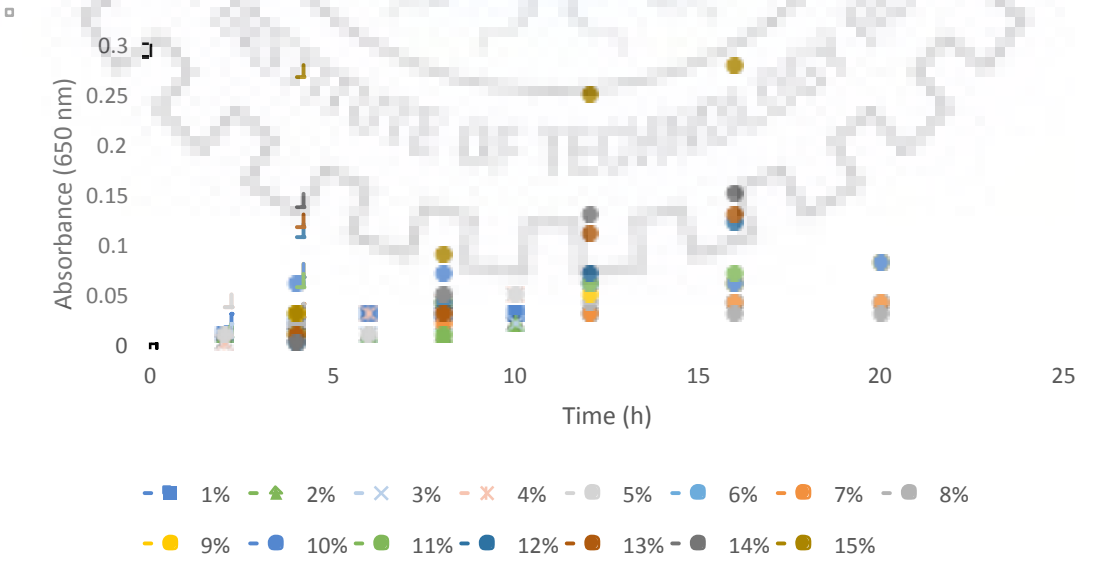

Fig6.3:Graph for acid pretreatment on the wood dust

In the acid treatment, the concentration of the acid was gradually increased to break the complex lignocellulose structure and expose the cellulose and hemicellulose for further hydrolysis. It was observed that as the concentration of the acid increases, the breakdown of the structure also increases but the use of high concentration of acid is not advisable as it is not cost effective and moreover, it is a problem to remove the acid which is required for the later stages of the hydrolysis of the biomass.

6.4 Alkali and acid pretreatment-

Sodium hydroxide, at different concentrations was used on wood dust and temperature was kept at 70°C for 24 hours. Later, sulphuric acid was added at different concentrations. Then, DNS assay was carried out every 4 hours.

1M NaOH

Table 6.6: Absorbance at 650 nm at different time and concentration of alkali (1M) and sulphuric acid in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
0.5%	0.03	0.03	0.08	0.07
1%	0.01	0.02	0.02	0.02

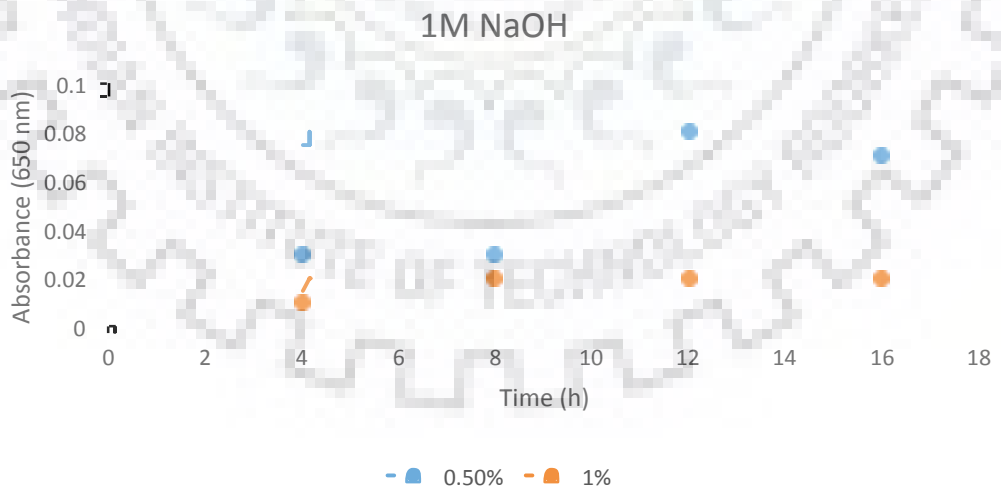

Fig 6.4: Graph at different time and concentration of alkali (1M) and sulphuric acid in wood dust

1.5M NaOH

Table 6.7: Absorbance at 650 nm at different time and concentration of alkali (1.5M) and sulphuric acid in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
0.5%	0.09	0.12	0.17	0.19
1%	0.04	0.08	0.05	0.06

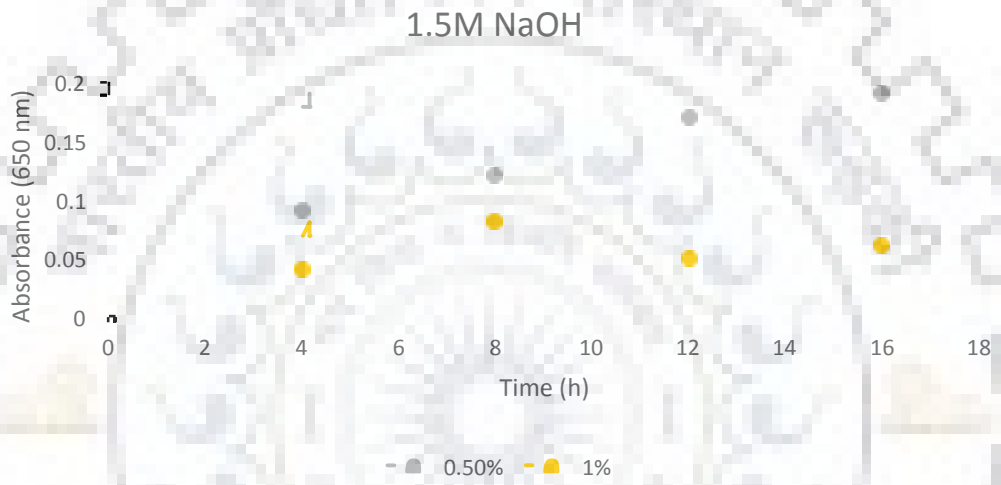

Fig 6.5: Graph at different time and concentration of alkali (1.5M) and sulphuric acid in wood dust

2M NaOH

Table 6.8: Absorbance at 650 nm at different time and concentration of alkali (2 M) and sulphuric acid in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
0.5%	0.02	0.05	0.06	0.16
1%	0.02	0.01	0.02	0.05

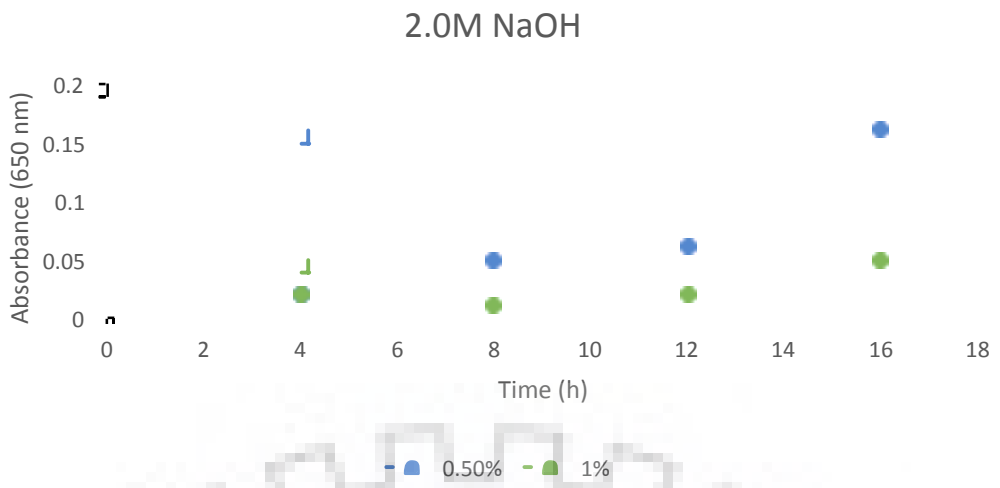

Fig 6.6: Graph at different time and concentration of alkali (2 M) and sulphuric acid in wood dust

2.5M NaOH

Table 6.9: Absorbance at 650 nm at different time and concentration of alkali (2.5 M) and sulphuric acid in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
0.5%	0.05	0.06	0.11	0.08
1%	0.02	0.01	0.07	0.06

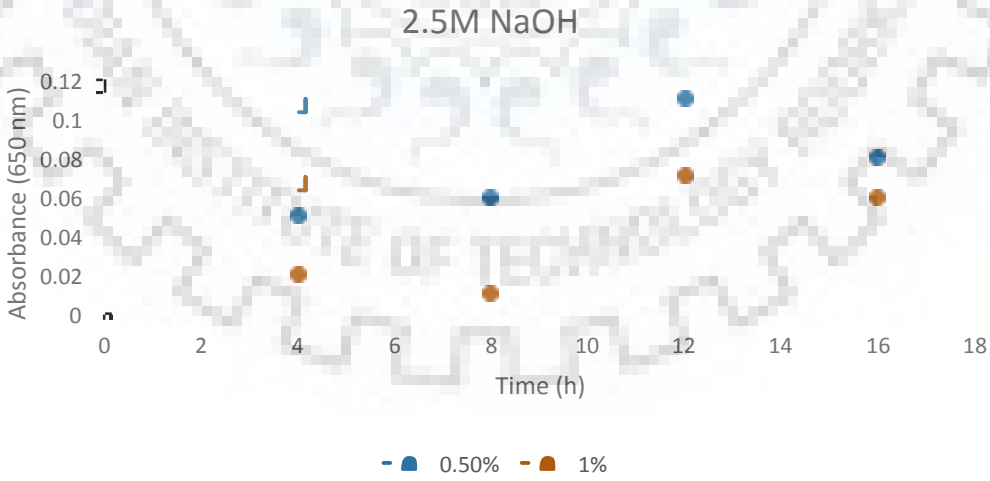

Fig 6.7: Graph at different time and concentration of alkali (2.5 M) and sulphuric acid in wood dust

In the pretreatment involving H_2SO_4 and NaOH, different concentrations of both alkali and acid was used and it was found that with increase in the concentration of alkali, increase in the disruption of

the lignin structure was observed. In this method, there is formation of inhibitors which can cause inhibition of the process of bioethanol production in the hydrolysis stage.

6.5 Pretreatment with sulphuric acid and potassium dichromate

A mixture of sulphuric acid and potassium dichromate at various concentrations were used on the lignocellulosic material and then the solution was kept at 100°C. Later, DNS assay was used for the determination of glucose at various time intervals.

8% $K_2Cr_2O_7$

Table 6.10: Absorbance at 650 nm at different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (8%) in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
4%	2.40	2.16	2.38	2.84
5%	2.18	2.36	2.67	2.44
6%	2.59	2.46	2.43	2.43
7%	2.19	2.52	1.95	2.12

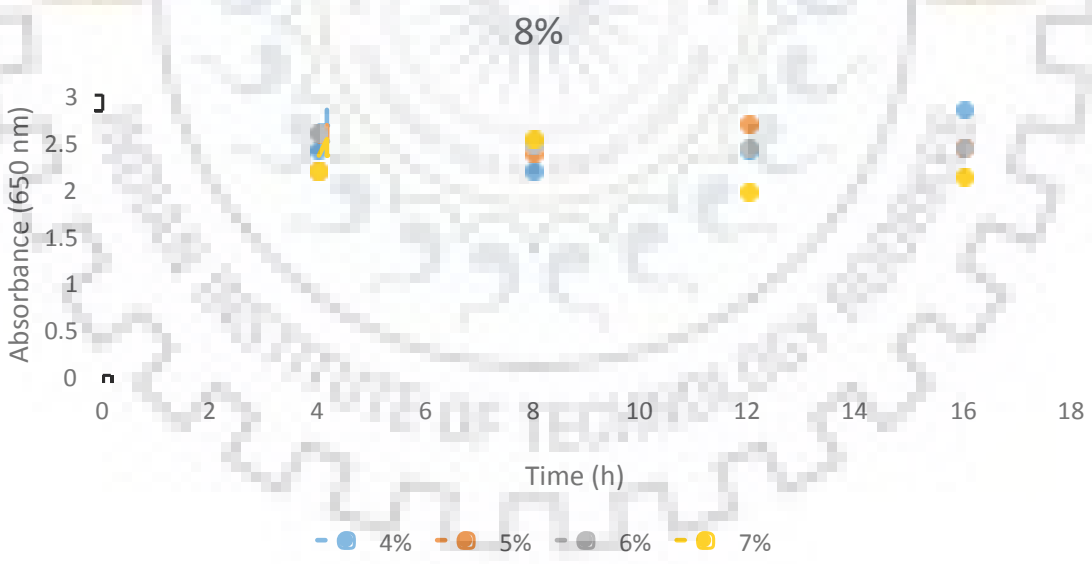

Fig 6.8: Graph for different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (8%) in wood dust

6% $K_2Cr_2O_7$

Table 6.11: Absorbance at 650 nm at different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (6%) in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
4%	1.71	1.67	1.73	1.85
5%	1.99	1.99	1.80	2.02

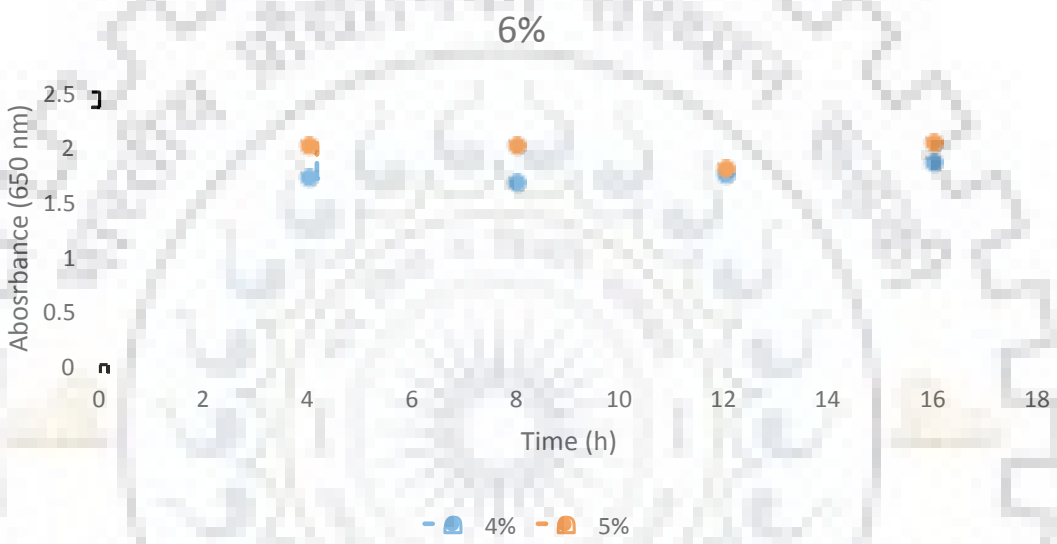

Fig 6.9: Graph for different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (6%) in wood dust

4% $K_2Cr_2O_7$

Table 6.12: Absorbance at 650 nm at different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (4%) in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
2%	1.64	1.68	2.30	2.74
3%	1.76	1.78	1.69	1.72
4%	1.23	1.26	1.21	1.07
5%	1.07	1.12	0.95	0.93

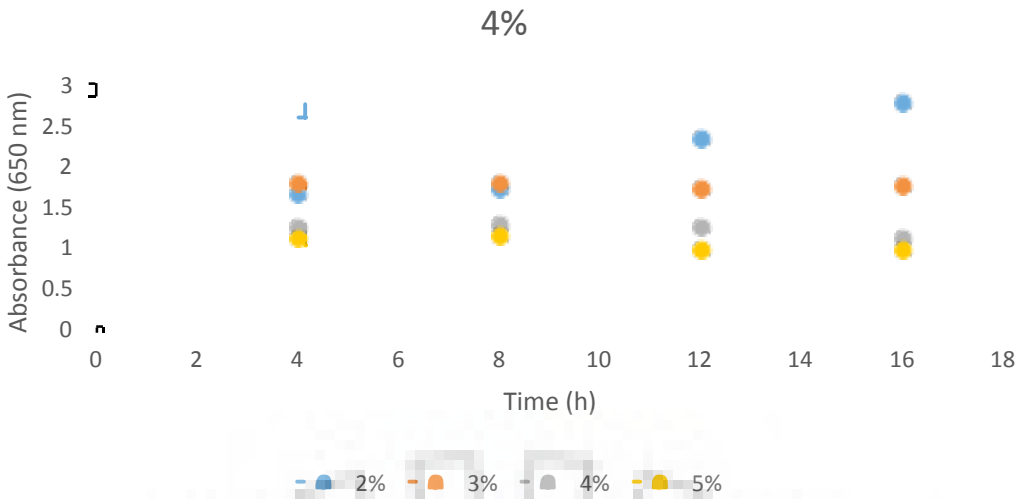

Fig 6.10: Graph for different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (4%) in wood dust

2% $K_2Cr_2O_7$

Table 6.13: Absorbance at 650 nm at different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (2%) in wood dust

Concentration of H_2SO_4	4 hrs	8 hrs	12 hrs	16 hrs
1%	0.38	0.41	0.37	0.40
2%	1.16	1.23	1.56	1.99
3%	0.63	0.64	0.62	0.63

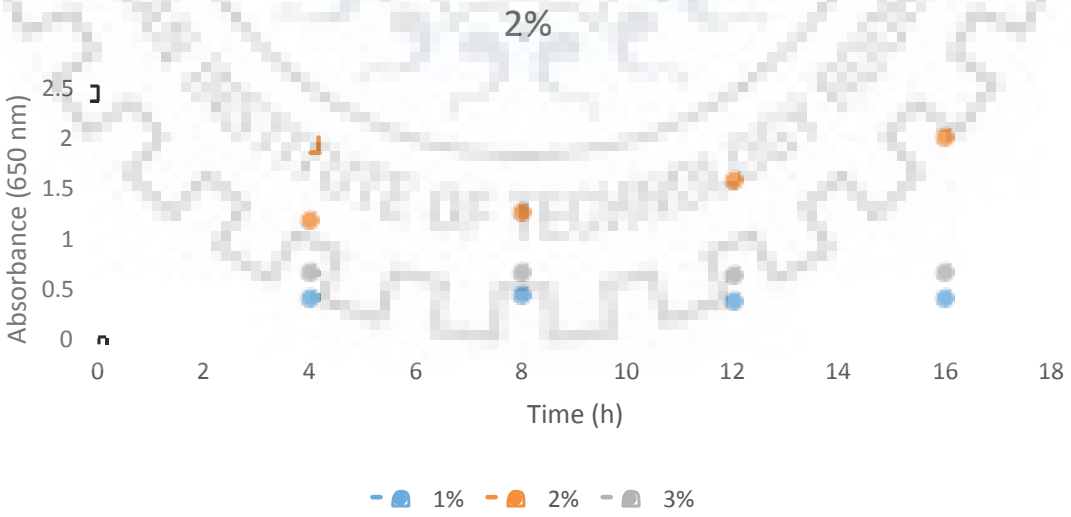

Fig 6.11: Graph for different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (2%) in wood dust.

The results are given for the treatment on banana peel.

3% $K_2Cr_2O_7$

Table 6.14: Absorbance at 650 nm at different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (3%) in banana peel

Concentration of H_2SO_4	6 hrs	12 hrs	24 hrs	30 hrs	36 hrs
5%	1.083	1.152	1.121	0.795	1.118
4%	1.127	1.174	1.137	1.036	1.202
3%	0.706	0.708	0.741	0.842	0.926
2%	0.808	0.850	0.873	0.884	1.186

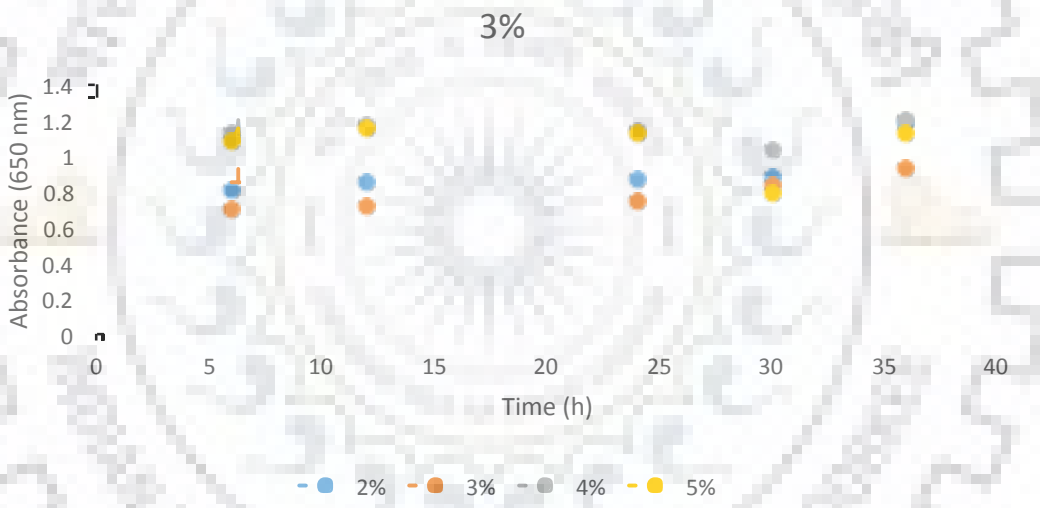

Fig 6.12: Graph for different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (3%) in banana peel

2% $K_2Cr_2O_7$

Table 6.15: Absorbance at 650 nm at different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (2%) in banana peel

Concentration of H_2SO_4	6 hrs	12 hrs	18 hrs	24 hrs	30 hrs
5%	0.798	0.811	0.834	0.813	0.858
4%	0.811	1.060	0.828	0.789	0.845
3%	0.798	0.825	1.169	0.848	0.827
2%	0.544	0.576	0.737	0.870	0.904

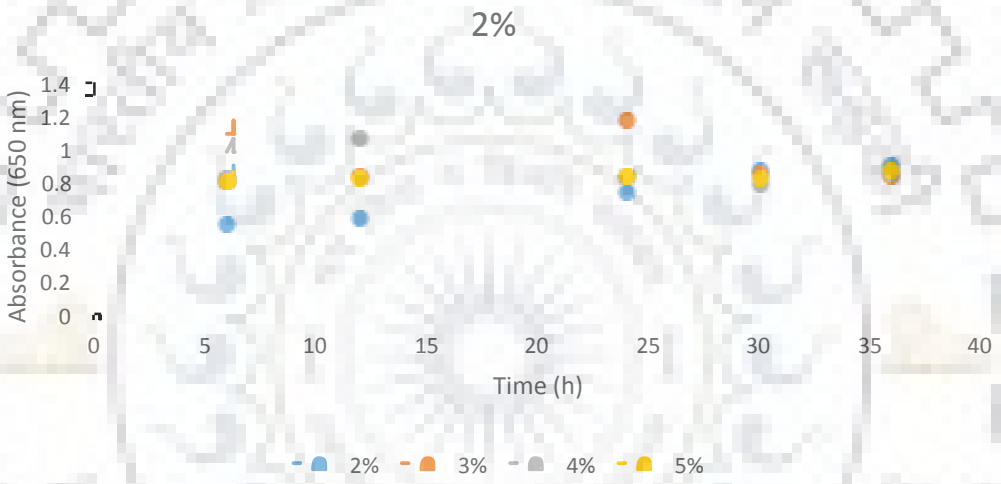

Fig 6.13: Graph for different time and concentration of sulphuric acid and $K_2Cr_2O_7$ (2%) in banana peel

This is a new technique where firstly, higher amount of H2SO4 and K2Cr2O4 are used for the pretreatment process. As usual, there was high disruption of the structure of lignin. Later, to optimise the technique, the concentration of both H2SO4 and K2Cr2O4 were reduced gradually. It was found that there was considerable amount of the disruption of the lignin structure in smaller concentrations of H2SO4 and K2Cr2O4 too in both the lignocellulose biomass.

6.6 Enzymatic hydrolysis-

Cellulase enzyme, at different concentrations was used on banana peel and temperature was kept at 35°C. Then, DNS assay was carried out every 4 hours.

Table 6.16: Absorbance at 650 nm at different time and concentration of cellulase enzyme on banana peel

Concentration	4 hrs	8 hrs	12 hrs	16 hrs	24 hrs	28 hrs	32 hrs	36 hrs
1 g/l	0.23	0.21	0.24	0.29	0.37	0.45	0.51	0.54
2 g/l	0.28	0.34	0.39	0.41	0.49	0.53	0.57	0.62
3 g/l	0.19	0.22	0.22	0.25	0.32	0.40	0.46	0.55
4 g/l	0.25	0.28	0.26	0.32	0.33	0.38	0.47	0.57
5 g/l	0.20	0.23	0.25	0.25	0.45	0.50	0.59	0.70

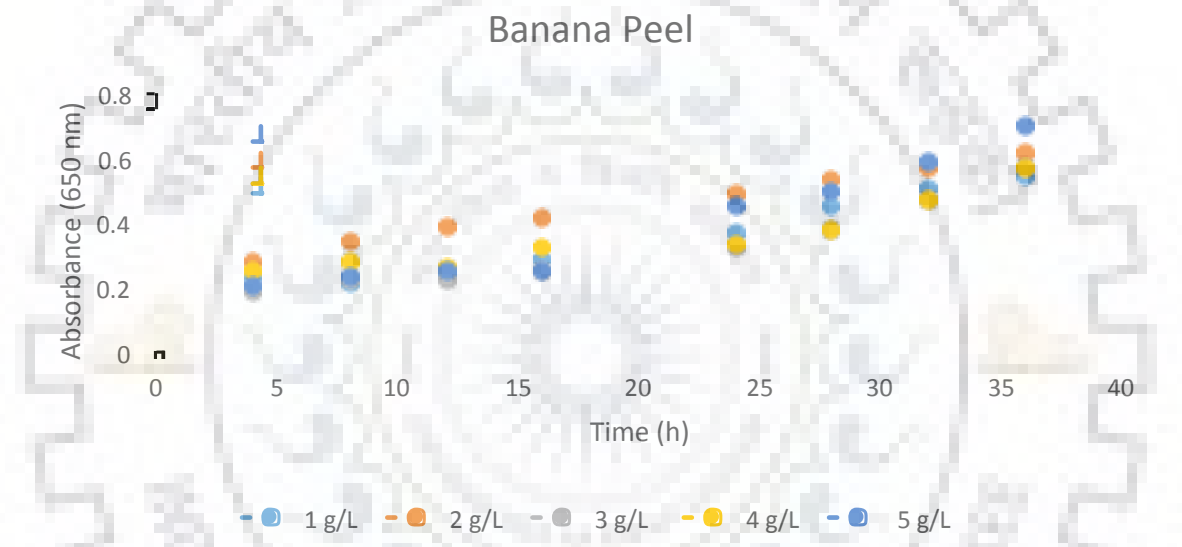

Fig 6.14: Graph for different time and concentration of cellulase enzyme on banana peel

Cellulase enzyme, at different concentrations was used on wood dust and temperature was kept at 35°C. Then, DNS assay was carried out every 4 hours.

Table 6.17: Absorbance at 650 nm at different time and concentration of cellulase enzyme on wood dust

Concentration	4 hrs	8 hrs	12 hrs	16 hrs	24 hrs	28 hrs	32 hrs
10 g/l	0.31	0.35	0.33	0.39	0.36	0.46	0.55
15 g/l	0.31	0.33	0.34	0.36	0.41	0.42	0.44
20 g/l	0.33	0.39	0.39	0.38	0.51	0.52	0.59
25 g/l	0.38	0.39	0.42	0.43	0.48	0.50	0.58

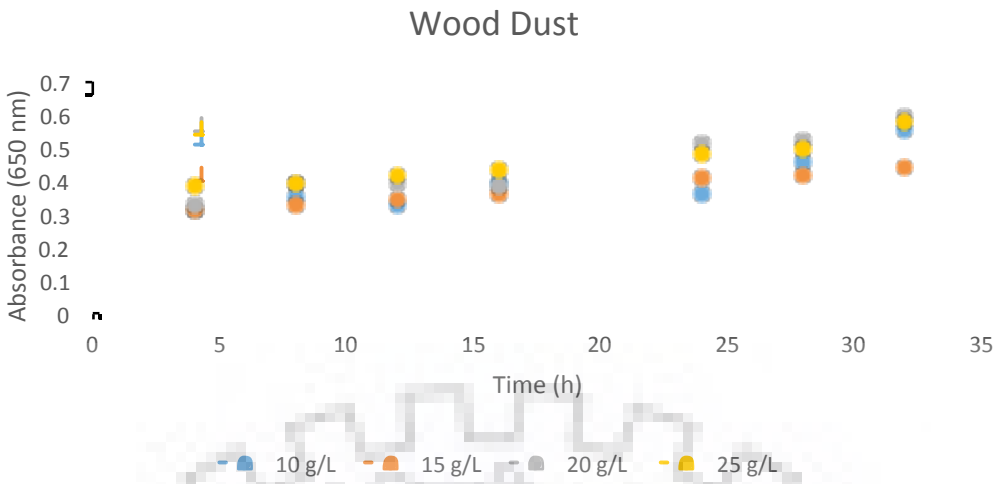

Fig 6.15: Graph for different time and concentration of cellulase enzyme on wood dust

The enzymatic hydrolysis gives good results but its time consuming. So, the aim is to pretreat the raw materials chemically first and then subject to enzymatic hydrolysis for better result. Moreover, the acidic and alkali pretreatment are to be optimised as they are not cost effective and can cause degradation of the cellulose in the lignocellulosic material too.

Moreover, the pretreatment process where sulphuric acid and potassium dichromate is used, it is found that the delignification process of the lignocellulosic raw material is better than the other methods where only acid or both alkali and acid are used for the pretreatment.

At lower concentration of sulphuric acid and potassium dichromate, it is observed that there is a considerable amount of disruption in the lignocellulose structure which exposes the cellulose and hemicellulose, ultimately giving a greater hydrolysis rate of these polysaccharides to monosaccharides.

7. CONCLUSION

The aim is to pretreat the lignocellulosic raw materials in such a way that maximum amount of cellulose is extracted from the raw material by breaking the complex structure of lignin. Lignin is the unwanted constituent among the main constituents of lignocellulose biomass. The lignin is composed of phenolic groups which causes the formation of furfurals. As a result, for the next step of hydrolysis, exposing the cellulose and hemicellulose is very important. In this work, various pretreatment processes are used like acid pretreatment, acid and alkali pretreatment and biological pretreatment. Among them, the use of sulphuric acid and potassium dichromate emerged out to be very promising with destructing the lignin structure to a great extent. Moreover, very less concentration of the chemicals is used. As a result, efficiency of the process is high with low cost. This will help in the maximum yield of glucose from the cellulose and other monosaccharides whose further fermentation will give rise to the bioethanol.

8. FUTURE PROSPECTS

Currently, the transport system worldwide is mainly based on the availability of cheap crude oil, which is highly unlikely to continue in the future. Further, the overwhelming dependence on oil causes serious problems ranging from vulnerability of supply, exposure to high prices, monopolistic abuses, to serious health hazards and other environmental impacts, such as global climate change. The energy transition in the transportation system will be gradual and will occur over a long period. This transition will shape both the nature of the fuel and the automobile industry. The factors shaping this transition are too many and varying in nature to make any prediction with an acceptable degree of certainty. Bioethanol has a great efficiency in replacing the fossil fuels and it carries great potential as it does not involve in climate problems like global warming as it is a clean and green energy. As a result, further and efficient research is required in the production of bioethanol from lignocellulose waste and in the pretreatment steps of the biomass in order to expose the complex structure of the biomass.

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