Optimisation of Ethanol Yield by Fermentation Using Garden Waste and the Study of the Kinetics of the System

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"Statement of Originality"

I..... SUJATA SARDAR .....registered on 02.01.2015 do hereby declare that this thesis entitled **"Optimisation of Ethanol Yield by Fermentation Using Garden Waste and the Study of the Kinetics of the System"** contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I havefully cited and referred all materials and results that are not original to this work.

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PREFACE

The purpose of this dissertation is to introduce and demonstrate a suitable approach to utilise three novel biomasses for the generation of value-added products such as bioethanol. The methodology investigated different attributes of the process as well as the morphological changes that occurs due to the process. The efficacy of the process as well as the kinetic behaviour to produce the desired product is also assessed. Spanning over five chapters this dissertation presents theoretical and experimental works on bioethanol production. Chapter 1 and 2 includes a brief introduction and literature review, chapter 3 and chapter 4 focuses on the pretreatment, hydrolysis and fermentation performed on the biomasses and the results obtained. The overall conclusion of the study is expressed in chapter 5.

The dissertation should be of interest to the scholars working on biofuel as well as professionals working in industrial research and development. The research was partially funded by Jadavpur University and Ministry of Human Resource Development, Govt of India under RUSA (R-11/600/19 dated 11.06.2019) scheme.

ABSTRACT

In this study, *Sterculia foetida, Eucalyptus grandis* and garden waste were studied as a possible source for bioethanol production. Two pre-treatment processes i.e. alkaline treatment and alkaline peroxide treatment, assisted by autoclave heating and conventional heating were investigated for *Sterculia foetida, Eucalyptus grandis* and garden waste. In addition to that deep eutectic solvent was also used for *E.grandis*. The structural and chemical changes occurred during pretreatment was observed through FTIR, XRD analysis and DNS assay. The effects of NaOH & H_2O_2 concentration along with temperature on lignin removal efficiency and production of reducing sugar were studied. The result showed that alkaline treatment alone was not significant enough for reduction in lignin content (14-15%) and must be accompanied by peroxide. When treated under autoclave assisted heating, for *S.foetida* a sharp decrease in reaction time (from 3 hours to 45 minutes), peroxide requirement (2-5%) and lignin concentration (80%) were observed. Moreover, in case of autoclave assisted heating less percentage of peroxide was able to remove same amount of lignin compared to that of conventional heating. Upon enzymatic hydrolysis for *S. foetida* the combination of 5%NaOH and 3%H_2O_2 gives best results with glucose concentration of 18.59g/L at substrate conc of 50g/L and enzyme conc of 1.5g/L. The enzymatic hydrolysis kinetics of delignified biomass shows decreased product inhibition with increased substrate concentration under a particular enzyme loading. Dried bark of Eucalyptus grandis (EG) was treated in alkaline (NaOH) and alkaline peroxide (NaOH along with H_2O_2) solution at 60°C (AHP-60), 80°C (AHP-80) and in an autoclave (AAHP). Kinetic parameters of enzymatic hydrolysis and fermentation indicated removal of lignin and easy accessibility of cellulose as crystallinity of the pretreated Eucalyptus grandis increased. The lignin removal was highest (73.20%) for AAHP. Maximum reducing sugar yield of 215.5 mg/g was also obtained from the same pretreatment conditions confirming recalcitrance nature of lignin is the key inhibitory factor for production of reducing sugars. During enzymatic hydrolysis, glucose concentration is observed to be increased over time with the increase in substrate concentration for a particular enzyme loading. EG in upon enzymatic hydrolysis generates highest amount of reducing sugar and yields better ethanol conversion (9.941g/g). The kinetic parameters from hydrolysis and fermentation indicates no inhibition of enzymes by reducing sugar and glucose inhibition.

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INTRODUCTION

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INTRODUCTION

After the industrial revolution in the 18th century, exponential growth was observed all over the world in every sector. With the growth of transport, energy and industrial sectors demand and cost of fossil fuels also has augmented significantly. Search for an alternative energy source has been increased in the last decade due to increase in academic and public awareness of greenhouse gas (GHG) emissions and global warming (Kopetz, 2013).

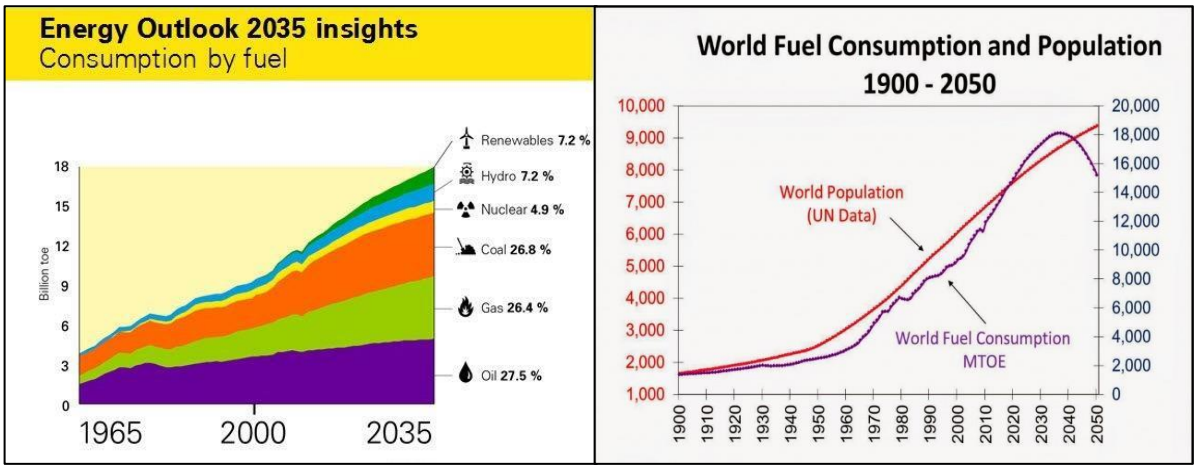


Figure 1: The population and consumption scenario of the world energy [Source: United States Census Bureau (USCB) and United States Department of Energy (USDOE)]

Figure 1 shows a concerning picture where the world population is projected to escalate from 6.7 billion to 8 billion by 2030 and the world fuel consumption is also anticipated to increase from 1500 MTOE to 1800 MTOE by 2040. Ever growing demand of fuel in the transport sector, fast depletion of fossil fuel and pollution associated with it has brought forth the concept of renewable biorefinery. The concept states production of fuel from renewable resources such as waste from food crops, lignocellulosic materials, waste oil etc., while in the production route of biofuel from lignocellulosic residues, a number value added by-

products with environmental benefits are produced and the improved techno economic scenario makes biofuel a promising substitute of fossil fuel (Fairley, 2011). Based on the source material, biofuel is classified into three categories: first generation, second generation and third generation biofuel. Food crops based such as sugarcane, corn, wheat, barley-based biofuel is termed as first generation. Brazil and USA are the leading countries in producing biofuel from food crops available there in large quantities such as corn and sugarcane. In the 1970's an initiative called 'Pro-alcohol program' was initiated by Brazil which introduces blending of ethanol and fuel and successfully implemented the technique in transport sector. Presently, Brazil, USA and UK are the countries that participates in extensive research on biofuel from food crops (Oberling et al., 2012). However, the complete dependency on first-generation biofuels from food crops clashes directly with the food and fodder supply and demand conflict. This conflict leads to the generation of concept of second-generation biofuel which is based on availability of lignocellulosic waste rather than food sources. For second generation biofuel the focus was on lignocellulosic materials including agricultural wastes, forest residues and organic wastes due to its abundant availability in nature. The conversion of biomass to biofuel facilitates by eliminating the oxygen from carbohydrates in order to obtain hydrocarbons. Third generation biofuels were focused on overcoming the conflict between food, fodder, and non-food sources. It also caters to the problem of unavailability of farmland for non-food sources. Third generation ethanol uses algal biomass with high yield which includes microalgae, macroalgae, and cyanobacteria. First and second-generation biofuel depends on the availability of plant derived feedstock while third generation biofuel are being produced from algal biomass and does not have conflict of interest with the availability of agricultural land. The algae cultivation mostly done in open ponds, attached growth systems, and closed photobioreactors but are not only limited to these

sources. High growth rate of algae is one of the main advantages of third generation biofuel over first and second generations of biofuel.

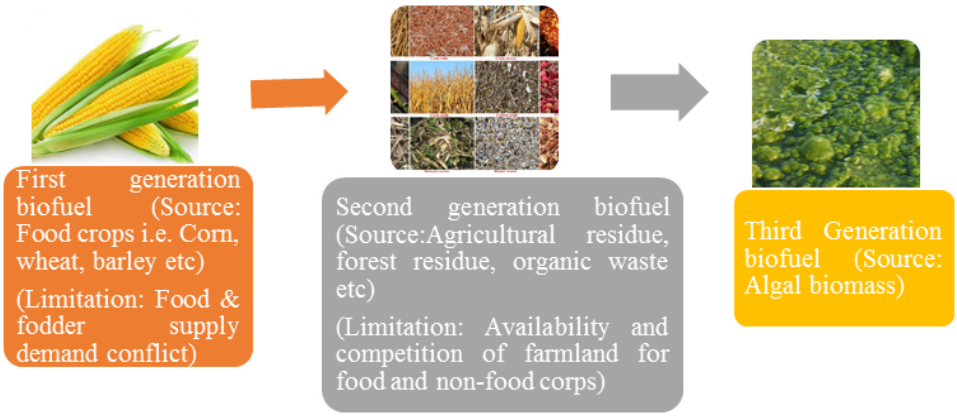


Figure 2: Evolution of biofuel and their sources

Figure 2 represents the different types of biofuels and their sources. Large scale production facilities along with strong energy policies are of utmost importance for biofuel to become a substitute for fossil fuel which is scarce in many countries. Countries like Germany, USA and Brazil (Köhler et al., 2014) has a great hold in deciding the energy policies and for growing interest towards the global market. One of the initiatives to curb the dependency towards fossil fuel currently adopted by many countries are to blend ethanol with fossil fuel. The blending concentration of biofuel in petrol and diesel varies by country. Table 1 represents the mandate for ethanol blend in different countries. For example, ethanol is blended at 10 and 85% concentrations with petrol and can be termed as E10 and E85, respectively. Similarly, when blended with biodiesel at a concentration of 2, 5, 20 and 100%, can be termed as B2, B5, B20 and B100 blends, respectively (Escobar et al., 2009).

Table 1 : Ethanol blending scenario in various countries (NITI Ayog, 2021)

Country	Mandate for ethanol blends	Program	Implemented by
Brazil	18-27.5% of Ethanol in gasoline starting at 2015. Currently at 27%.	National biofuels policy (Dec 2017)	Ministry of mines and energy (MME)
United States	Volume requirements for ethanol blending are being updated by EPA each year based on fuel availability according to clean air Act.	Renewable fuel standard (RFS) program	Environmental protection agency (EPA)
European Union (EU)	EU aims for 32% renewable energy for EU's energy consumption by 2030	Renewable energy directive	European Commission
China	In 2017, ethanol in fuel can be used in all of China with the target of 10% ethanol blending.	Fuel quality standards	National energy administration
Thailand	Alternative Energy Development Plan (ADEP) aims 25% share of renewable and alternative energy within 2036	ADEP	Ministry of Energy
India	Target is to blend 20% ethanol (E20) with petrol	E20	NITI Ayog, Govt. of India

In India the roadmap for ethanol blending includes implementation of E20, E10 as well. The conversion process of biomass to biofuel comes with a lot of technological barriers. The price of biofuel depends on the source material used, location of the cultivation and the processes adopted. The greenhouse gas (GHG) balancing also depends on the feedstock used and whether the feedstocks are carbon neutral. The selection of biomass, pretreatment of the biomass and subsequent enzymatic hydrolysis and fermentation are key steps to produce ethanol from lignocellulose.

1.1. Global status of biofuel production and Indian perspective

The International Renewable Energy Agency (IRENA), an intergovernmental organization was formed in 2009, by 75 countries signing the charter of IRENA, to promote the adoption of renewable energy worldwide. It aims to provide concrete policy advice and facilitate technology transfer. As of April 2019, IRENA has 160 member states. In September 2011, the United Nations' Secretary-General launched the "UN Sustainable Energy for All" initiative for the improvement in accessibility, efficiency and the distribution of renewable energy. The 2015 "Paris Agreement" on climate change inspired other countries to improve or frame policies for renewable energy. In 2017, no less than 121 countries have come under the renewable energy policy. Moreover, there is also an extensive range of policies already implemented at state and/or provincial levels including implementation of the concept of "Green bank". A green bank is a quasi-public financial institution that uses public capital to leverage private investment in clean energy technologies and to bridge gaps with the market for smooth distribution of clean energy. Climate neutrality is also an important concept and also the main goal of European Green Deal for the year 2050. For the European Union to reach their target of climate neutrality, one way is to decarbonize the energy system by achieving 'net-zero greenhouse gas emission' by 2050.

While Brazil and the USA have already expertise on the technologies to convert lignocellulose to biofuel and are considered global pioneer for biofuels from food and non-food lignocellulosic crops, Asian countries have also emerging biofuel industries. In the early 2000's China had invested in ethanol producing technologies vastly and emerges as the world's third largest biofuel producer(Huang et al., 2012). Southeast Asian countries such as Malaysia, Indonesia and Thailand, uses primarily palm oil and Jatropha for biofuel

production. Usage of Miscanthus for fiber and pulp production is also a trend in many South Asian countries. In Pakistan biofuel production is focused on sugarcane, maize, soybean and rapeseed (T. Ali et al., 2013).

According to India's Biofuel Policy 2018, India has set up a goal to reach E20 by 2025 while maintaining its immediate objective of E10 blending by 2022.Bioethanol production initiative was first adopted by Hindustan Petroleum Corporation (HPCL), Indian Oil Corporation (IOCL), Bharat Petroleum Corporation Limited (BPCL) and Praj Biofuels. IOCL recently announced a strategy to build a 63-million-litre cellulosic ethanol facility using dilute acid pretreatment. However, the expenses associated with the building a 2G ethanol plant ($136 million or INR 1,000 crores) vs. a 1G facility ($13–27 million or INR 100–200 crores) are main limitations to advance biofuel production in India. Indian infrastructure company TATA has also planned to install a 100,000 L ethanol plant at Bargarh, Odisha, India (IEA Bioenergy, 2021). Figure 3 depicts India's ethanol blending scenario.

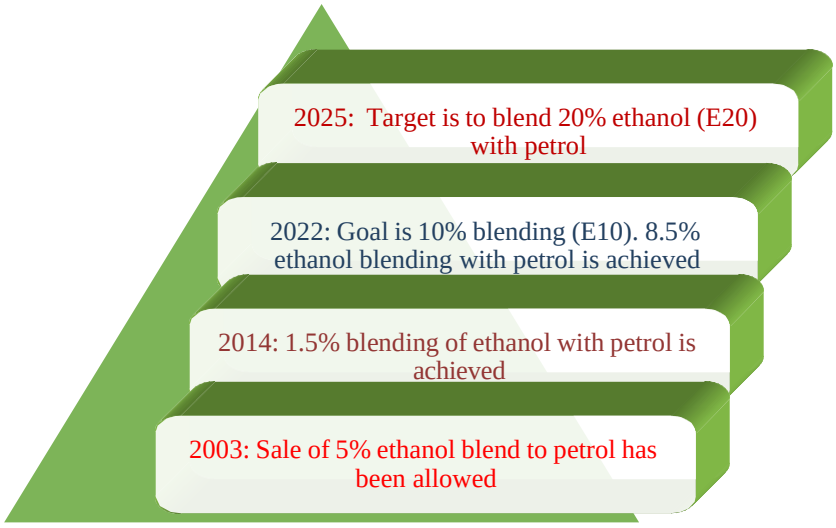


Figure 3: India's roadmap for ethanol blending (NITI Ayog, 2021)

In India, Department of Food and Public Distribution (DFPD) is the nodal department for promotion of fuel grade ethanol producing distilleries in the country. Government has allowed ethanol production or procurement from sugarcane-based raw materials viz. C & B heavy molasses, sugarcane juice, sugar, sugar syrup, surplus rice with Food Corporation of India (FCI) and Maize (NITI Ayog, 2021). NITI Aayog, the public policy making section of Government of India, estimated that sugarcane and paddy combined are using 70% of the country's irrigation water, depleting water availability for other crops. Hence there is a need for change in crop pattern, to reduce dependence on one particular crop and to move to more environmentally sustainable sources for ethanol production. Cereals, particularly maize, and Second Generation (2G) biofuels with suitable technological innovations offer promise of a more environmentally benign alternative feedstock for production of ethanol.

1.2. Challenges and prospects in bioethanol production

Globally sources of second generation are available abundantly. The deciding factor is the utilization and processing technology which in the long run impacts production cost, and quality.

Gaps in the research include proper supply chain management and analysis of the same. Strong knowledge base for optimized and controlled techniques such as bioprocessing, fermentation technology for setting up large biorefinery is required under one roof. This provides job opportunities and research over a wide range of disciplines.

Lignin is primarily obtained as a co-product of lignocellulosic based ethanol industries. Research is needed to valorize lignin commercially. Transgenic plants which has modified or altered lignin content serves as improved feedstock in lignocellulose based industries.

Lignin chemistry and its utilization as a value added component is an emerging field of research.

1.3. Research Aims & Objectives:

The global objective of this present work is to explore a facile and viable route to valorize garden waste for efficient production of ethanol and to study kinetics of the system. In this present study three types of garden waste namely, bark of Eucalyptus, fruit shell of Java olive or wild Indian almond tree (*Sterculia foetida*), and mixed garden waste consists of dried leaves and twigs are valorized for possible production of bioethanol, a greener alternative to fossil fuel via hydrolysis and fermentation route, optimization of process parameters thereof and to study the kinetics of the system. The specific objectives to attain the global objectives are

i) To identify novel biomass for bioethanol production.

ii) To enhance the monomeric sugars by facilitating higher lignin removal and minimizing formation of sugar degradation products using various pretreatment of lignocellulosic biomass considered as garden waste.

iii) To study and compare the degradation pattern of the biomass and the structural alteration of these compounds during the pretreatment.

iv) To determine effective pretreatment methods for garden waste for successive enzymatic hydrolysis and fermentation.

v) To enhance ethanol production yield and study the kinetics of enzymatic hydrolysis.

vi) To determine suitable fermentation technique to obtain high ethanol yield.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2
REVIEW OF LITERATURE

Generally, substances with high glucose contents (to name a few, sugarcane, corn and grains) are used as a source of bioethanol production. But due to "food vs. fuel" debate, fueled by European commission's policy to bar the use of food or fodder in production of biofuel, significant research has been devoted to valorize lignocellulosic biomass obtained from agricultural residue (corn cob, rice straw, sugarcane bagasse, reed and rapeseed stover), fruit wastes (shells of pistachio, kinnow waste and banana peels), forest residues (oil palm empty fruit branches, Eucalyptus globulus, Bermuda grass) (Demiral et al., 2009; Jung et al., 2013; H. Li et al., 2009a; Martín-Sampedro et al., 2012; Rajvanshi, 2006; Sharma et al., 2007; Velmurugan & Muthukumar, 2012a) as feedstock for second generation of biofuel. Lignocellulosic materials can be arranged into four key sources: forest products and residues, agricultural residues, municipal paper waste, and dedicated energy crops (Agbor et al., 2011; T. Guo et al., 2013). The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin. Cellulose is a polyacetal of cellobiose which is a polymer of glucose as cellobiose consists of glucose. The β-(1-4)-glycosidic bonds between glucose molecules helps the polymer to be arranged in a straight chain. Hemicellulose consists of polysaccharides such as xylans, glucomannans and galactans. Hemicellulose is insoluble in water at low temperature, but presence of acid improves the solubility. Lignin is a complex amorphous polymer with phenylpropane units, mainly p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin represents an insoluble 3D network. At elevated temperature softening of lignin takes place. The composition of lignocellulose depends largely on its source. For example, percentage of cellulose and

hemicellulose is more in hardwood compared to softwood. This is due to the fact that the complex carbohydrates of hemicellulose e.g. xylose, mannose, galactose forms non-covalent bonds with cellulose fibrils and serves as a mesh around cellulose and provide strength and resilience to the cell wall.

Moreover, Lignocellulosic biomass has a strong resilience towards microbial damage because of the presence of covalent cross linkage of lignin -hemicellulose in the cell wall (C. Li et al., 2011; Min et al., 2013; Xu et al., 2013). It has been shown that low lignin containing biomass is more digestible than the high lignin containing biomass (Chang & Holtzapple, 2000; Kikas et al., 2016) due to recalcitrant nature of lignin. The main part of the cell wall consists of three macromolecules; cellulose, hemicelluloses and lignin. Figure 4 represents a schematic representation of the lignocellulosic structure.

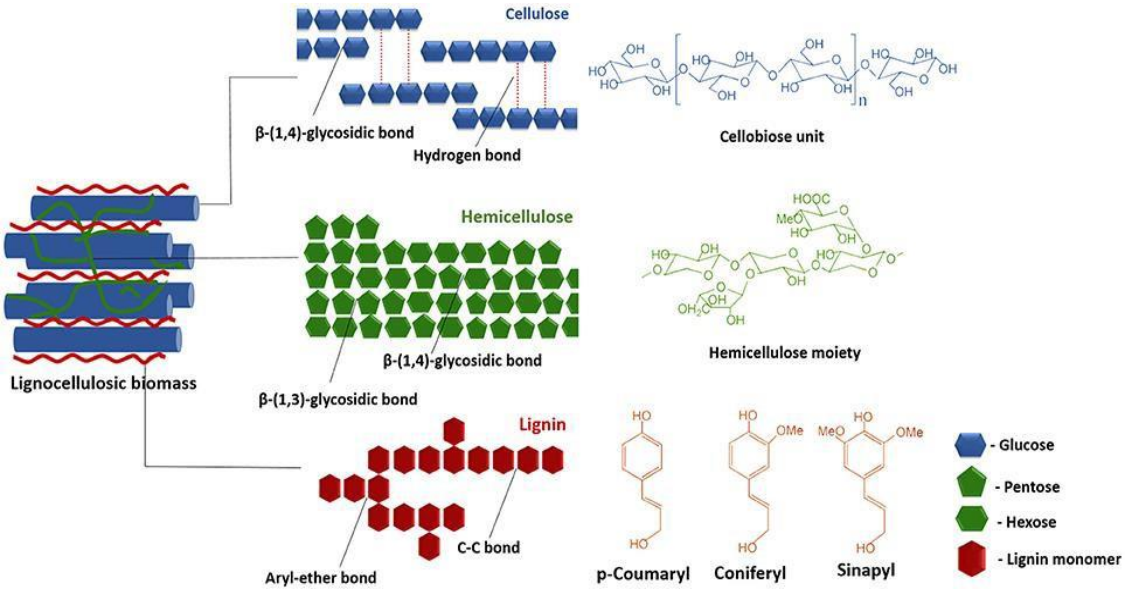


Figure 4: The schematic of the structure of lignocellulosic biomass (Baruah et al., 2018).

Cellulose is said to be the most abundant polymer available on the earth and constitutes the largest reservoir of organic carbon (Festucci-Buselli et al., 2007a). However, the options to choose a particular feedstock (s) vary with the regional availability pattern of the biomass, the cost of procurement and to some extent socio-political judgements (B. Palmqvist, 2014). Compositions of several lignocellulosic biomasses are summarized in Table 2 depending on their origins.

Table 2 : Source based composition of lignocellulose on dry basis (wt %)

Lignocelluloseic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Hardwood (oak, teak, maple etc.)	40-55	24-40	18-25	(Y. Sun & Cheng, 2002)
Softwood (Pine, Cedar etc.)	45-50	25-35	25-35	(Y. Sun & Cheng, 2002)
Nut shell	25-30	25-30	30-40	(Y. Sun & Cheng, 2002)
Leaves	15-20	80-85	0	(Y. Sun & Cheng, 2002)
Corn Stover	40.8	20.6	21.3	(L. Liu et al., 2009)
Rice straw	41.7	18.3	16.6	(I. Kim et al., 2014)
Wheat straw	35.2–43.5	19.0-28.3	5.72-20.5	(J. Chen et al., 2021; Jiang et al., 2016; Linde et al., 2008)
Sugar cane bagasse	41	31	20	(Yoon et al., 2011)
Rapeseed	27.6	20.2	18.3	(X. Yu et al., 2016)
Cotton stalk	36.3-42	18.7-20	28.7-30	(Gaur et al., 2017)
Mustard stalk	39.04–42.0	22.3–23.5	20.8–22.0	(Maiti et al., 2007; Pal et al., 2013)

Ethanol from lignocellulosic route mainly comprises of four steps: pretreatment, acidic/enzymatic hydrolysis, fermentation and product separation. Pretreatment plays a key role in the process of turning waste into wealth. For the past years, many pretreatment methods have been proposed for efficient hydrolysis of the pretreated

biomass. In general, these pretreatments can be divided into physical, chemical, physico-chemical, biological pretreatments and combinations thereof. Figure 5 represents various type of pretreatment available to pretreat lignocellulose.

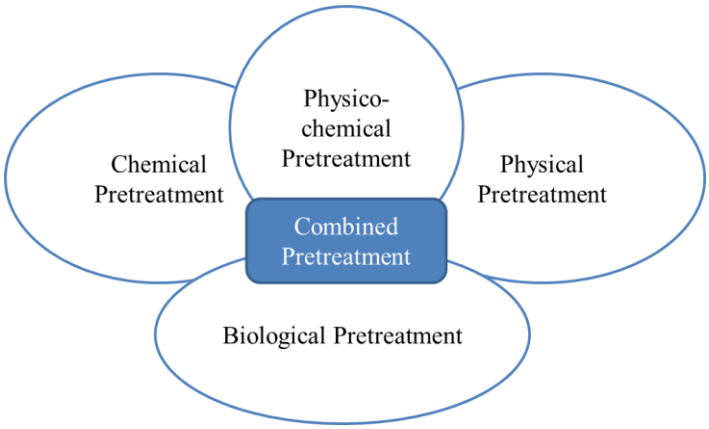


Figure 5: Types of pretreatment available to pretreat lignocellulose

Each pretreatment method has its own merits and demerits; thus, the selection of pretreatment method should be in synchronization with the selection of hydrolysis process. Also, techno-economic feasibility is an important factor for scaling up of processes which is lacking in many pre-treatment methods making them unsuitable for large scale operations (Gırio et al., 2010). The yield and rate of production monomeric sugars during enzymatic hydrolysis are highly dependent on efficacy of pretreatment which enables easy accessibility of cellulose and hemicellulose to the enzymes. Removal of hemicelluloses increases pore size of the substrate and hence increases the probability of cellulose to be hydrolyzed. The rate of degradation of lignocelluloses by enzymes in subsequent hydrolysis process depends on the crystallinity of cellulose, accessible surface area, degree of cellulose polymerization (lower the degree of polymerization, more the biomass is accessible for enzymatic hydrolysis), and degree of acetylation of hemicelluloses (C. Wyman, 1996). The type of pretreatment to be applied depends

largely on the structure and composition of the biomass and on the type of the subsequent hydrolysis process.

2.1 Pretreatment

Pretreatment is required to alter the external and internal structure of lignocellulose as well as its chemical composition so that subsequent hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields (Mosier et al., 2005; C. E. Wyman et al., 2005). Thus, the main aims of pretreatment are to (Converse et al., 1990; Rollin et al., 2011; G. Shen et al., 2011; Y. Sun & Cheng, 2002; X. Zhao et al., 2012):

- Break the lignin-hemicellulose-cellulose network and make the cellulose accessible to chemicals and enzymes which convert the carbohydrates into monomeric sugars in a way such that formation of inhibitory byproducts during subsequent hydrolysis and fermentation processes can be avoided.

- Separate hemicellulose from cellulose in order to break the linkages between lignin and cellulose for efficient removal of lignin from the substrate by avoiding degradation or loss of carbohydrate.

- Disrupt and/or remove lignin components from plant cell wall to decrease recalcitrant nature of the lignocellulose,

- Increase the reaction surface and pore size of the substrate for better penetration of hydrolyzing agents by reducing particle size or increasing porosity.

Severity of pretreatment conditions need to be optimized as the harsh pretreatment conditions (long residence time, high temperature, high acid/salt concentrations) have negative impact on the monomeric sugars which tend to degrade as aldehydes and organic acids, many of them have shown to be highly inhibitory for most fermenting microorganisms(C. E. Wyman et al., 2005). Inhibitors are originated from lignocellulose during pretreatment (Gırio et al., 2010) and, based on the operating conditions, inhibitors can be formed from carbohydrates (furan derivatives and organic acids) or from lignin (phenolic compounds) (Cybulska et al., 2014; Phitsuwan et al., 2013). Depending on the raw material and the severity of the pretreatment, the type and amount of the generated compounds can differ (Alvira et al., 2010a). It has been observed that since different lignocellulosic biomass have different physicochemical characteristics, it is necessary to adopt suitable pretreatment technologies based on the biochemical composition of the lignocellulosic biomass (Koullas et al., 1992; Puri, 1984).

Pretreatments that have the ability to remove lignin sufficiently are more effective (Arantes & Saddler, 2010; Chang, Nagwani, et al., 2001b; Cordero et al., 2001; Hendriks & Zeeman, 2009; Kikas et al., 2016; Teymouri et al., 2005a); Also mechanical pretreatment prior to chemical pretreatment such as ball milling, can enhance digestibility of lignocellulose by reducing the size and crystallinity(Chang, Nagwani, et al., 2001a). The products obtained during pretreatment, which show inhibitory effects, can be classified as: carboxylic acids, furans, phenols and inorganic salts. The formation of these products depends on biomass as well as pretreatment conditions and can be removed by ion exchange, active coal treatment, laccase and peroxidase treatment and/or over liming, which is especially effective for the removal of furan and phenol (Klinke et al., 2004a).

2.1.1 Mechanical Pretreatment:

For every pretreatment, particle size reduction is necessary for efficient handling of material as well as to increase surface area. By mechanical pretreatment, using grinding, milling, reduction of particle size and crystallinity is achieved. According to a study by Palmowski et al., reduction in particle size not only increases the surface area but also decreases the degree of polymerization (Palmowski & Müller, 2000). Depending on the type of biomass, decrease in degrees of polymerization can increase yield of enzymatic hydrolysis by 5-25% (Delgenes et al., 1996).

Mechanical pretreatment is usually the first step for all other pretreatment methods. But an innovative approach by Motte et al., shows that wheat straw upon biological treatment (dry dark fermentation) followed by mechanical treatment (centrifugal milling and ball milling for 5min at room temperature) improves the ethanol yield by 83% as well as increases the overall substrate conversion (Motte et al., 2015). However, operating cost and depreciation of equipment plays a key role in mechanical pretreatment. Table 3 shows usage of mechanical pretreatment in aiding subsequent pretreatments.

Table 3: Usage of mechanical pretreatments prior to other pretreatments

Biomass	Mechanical pretreatment	Subsequent pretreatment	Reaction conditions	Results	References
Biologically treated wheat straw	Centrifugal milling & ball milling	-	Time:5min Temp: Room temperature	Ethanol yield: 83%	(Motte et al., 2015)
Wheat straw	Drying and milling in hammer mill	Alkaline peroxide	Temp:25-35°C Time: 3-24h	Total Sugar: 53%	(Saha & Cotta, 2006)
Corn Stover	Grinding	Ammonia Recycle Percolation (ARP)	Temp:170°C Time: 20min Ammonia concentration: 15wt%	Cellulose yield: >95%, Hemicellulose removal: 60% Lignin removal: 70-85%	(T. H. Kim & Lee, 2005)
Empty fruit branch of palm tree	Grinding and screening (7-14 mesh)	Acid [acetic acid]	Temp: 170-190°C Time: 10-20 min Acid concentration: 3-7%	Lignin removal: 59.5% Hemicellulose hydrolysis: 53.6%	(D. Y. Kim et al., 2016)

2.1.2 Chemical Pretreatment:

Chemical pretreatments involve processes using various chemicals such as acids, alkalis, and organolsolvs to pretreat biomass under various reaction conditions. The mechanisms of these pretreatments depend on the chemicals used and pretreatment conditions. Apart from minor drawbacks in some chemical pretreatments, it is highly efficient tool of pretreatment which alters the structure of lignocellulose.

Acid pretreatment:

Diluted sulphuric acid (W. H. Chen et al., 2011) has been used widely for acid pretreatment of lignocellulosic biomass while other diluted acids such as hydrochloric acid (Z. Li et al., 2014; Q. Liu et al., 2016), nitric acid (I. Kim et al., 2015) and phosphoric acid (Avci et al., 2013; Geddes et al., 2010; H. Li et al., 2009a) have also been explored. Generally acid treatment breaks down the glycosidic bonds of

polysaccharides, freeing individual monosaccharides thereafter. Use of these acids like H_2SO_4, HCl even in their diluted form can be hazardous as these acids are highly corrosive in nature and therefore in case of scaling up the process, material of construction for handling the acids can be expensive.

Dilute acids mainly sulphuric and hydrochloric acids are used at temperatures of 160-220ºC for several minutes to significantly disrupt and redistribute lignin in cell wall of lignocelluloses. There are primarily two types of dilute acid pretreatment processes: high temperature (Temperature >160ºC), and low temperature (Temperature <160ºC). Dilute acid treatment under high temperature can be done in continuous flow processes whereas in case of low temperature it can be done in batch process with high solids loading (10-40% (Alvira et al., 2010b; Festucci-Buselli et al., 2007b; Koullas et al., 1992; Puri, 1984; Y. Sun & Cheng, 2002)). Both the procedure has some drawbacks which includes recovery of the acid from effluent and neutralization of pH of the system and energy requirement, especially for pretreatment involving high temperature.

Phosphoric acid and volatile solvents like acetone are also used recently (Klinke et al., 2004b; H. Li et al., 2009b; Teymouri et al., 2005b). The process involves mixing of sample with phosphoric acid followed by incubation at certain time and temperature followed by centrifugation. The result showed that phosphoric acid can remove sufficient amounts of hemicellulose and can remove lignin partially. Also, there is a significant decrease in production of inhibitory and harmful compounds. Moreover, acetone extracts almost all organic matter leaving only cellulose, referred as ‘clean’ cellulose. The use of aldonic acid is also reported to enhance the hydrolysis of lignocellulose (Cannella et al., 2012). Table 4 represents the performance of different acids in pretreatment of lignocellulose materials.

Table 4: Performance of various acids in pretreatment lignocellulose materials

Biomass	Initial mechanical pretreatment	Acid Used	Process Parameters	Results	References
Wheat straw	Grinding & screening (Size: 0.80-0.91mm)	Sulfuric acid	Temp: 140°C Time: 30min Acid concentration: 10 dm^3m^{-3}	Glucose yield: 89.25% Xylose yield: 47.18%	(Rajan & Carrier, 2014)
Corn Stover	Grinding & screening (40 mesh)	Hydrochloric acid & subsequent lime treatment	Temp: 120°C Time: 40min For Lime: Temp: 60°C Time: 12 h L/S ratio: 10:1	Total glucose yield: 85.9% Xylose yield: 97% Glucose yield increases upon enzyme loading.	(Z. Li et al., 2014)
	Milling & screening (1.27 mm screen)	Phosphoric acid	For maximum glucose yield Temp: 180°C Time: 15min Acid concentration: 0.5% For maximum xylose yield Temp: 160°C Time: 10 min Acid concentration: 1%	Max glucose yield: 85% Max xylose yield: 91.4%	(Avci et al., 2013)
Reed Stover	Chopped	Phosphoric acid	Temp: 50°C Time: 1h Acid concentration: >85%	Ethanol yield: >98.7% Ethanol concentration: 50.5g/L	(H. Li et al., 2009a)
Rice straw	Grinding & screening (<2mm)	Nitric acid	Temp: 158.8°C Time: 5.86 min Acid concentration: 0.65%	Xylose yield: 86.5% Enzymatic digestibility: 83.0%	(I. Kim et al., 2014)
Empty fruit branch of palm	Grinding & screening (7-14 mesh)	Acetic acid	Temp: 170-190°C Time: 10-20 min Acid concentration: 3-7%	Lignin removal: 59.5% Hemicellulose hydrolysis: 53.6%	(D. Y. Kim et al., 2016)

Alkaline pretreatment:

This process utilizes lower temperatures and pressures compared to other pretreatment technologies as it can be carried out at ambient conditions. Morphological data showed an increase in external surface area and porosity for better exposure to the microorganism or enzymes for an effective hydrolysis. In some cases, air incorporation in the system can be beneficial for delignification (Chang, Nagwani, et al., 2001a; Q. Z. Zhang & Cai, 2008).

Calcium or sodium hydroxide hydrolysis: Lime (calcium hydroxide) treatment is a way to improve alkaline treatment by heating at an elevated temperature using lime. In lime pretreatment solution of lime and water is sprayed onto the biomass material before storing the material in a pile for a period of several hours to weeks. Elevated temperatures reduce contact time which also strongly depend on the type of lignocellulose (i.e., 3 h at 85 ºC for wheat straw and 1 h at 120ºC for bagasse) (Mosier et al., 2005; G. Shen et al., 2011; Zaldivar et al., 2001).Various studies reported previously showed that time and temperature influence significantly whereas lime loading, water loading has little to almost no impact (Hendriks & Zeeman, 2009; Holtzapple et al., 1992; Kaar & Holtzapple, 2000; P. Kumar et al., 2009; R. Sun et al., 1995a; C. E. Wyman et al., 2005) on pretreatment efficacy. Lime treatment able to increase digestibility of lignin and at the same time pretreated sample does not show any inhibitory effect on subsequent hydrolysis. Lime is easily available and relatively cheap which is an added advantage towards economic pretreatment. Moreover, calcium can be recovered as calcium carbonate and reused as lime using lime kiln technology (Chang, Nagwani, et al., 2001c; Hendriks & Zeeman, 2009). Sodium hydroxide is another alkaline substance which is widely used in pretreating

lignocellulose biomass. In many cases NaOH in combination with dilute acid or hydrogen peroxide was observed to increase delignification (I. Kim et al., 2015; P. Kumar et al., 2009). Required reaction time is less for sodium hydroxide compared to lime. A study by Yoon et al shows a yield of 92.8% reducing sugar when sugarcane bagasse is treated with NaOH at 115˚C for 38min (Yoon et al., 2011).

Ammonia pretreatment: Pretreatment using ammonia includes ammonia fibre explosion (AFEX), ammonia recycle percolation (ARP), etc. Ammonia fibre explosion (AFEX) will be addressed in physico-chemical pretreatment section. In ARP aqueous ammonia is charged into a slightly pressurized flow through a reactor column packed with biomass. After reaction, lignin along with ammonia is recovered from liquid fraction and ammonia is recycled back to the rector. The cellulose and hemicellulose rich solid fraction is treated further after thorough washing. Aqueous ammonia can also be used effectively by soaking the biomass at low temperature. Ammonia removes lignin efficiently and significantly increases surface area (T. H. Kim & Lee, 2005). This treatment is not cost effective because of the high cost of ammonia itself and the cost associated with ammonia recovery. But the total sugar yield often balances the cost of pretreatment (C. E. Wyman et al., 2005).

Alkaline peroxide is another effective method for pretreatment of biomass. In this method, the lignocelluloses are soaked in pH-adjusted water (pH 11-12) using NaOH, containing H_2O_2 at room temperature for 6-24h (M. Taherzadeh & Karimi, 2008). The process can increase reducing sugar content, to be utilized in the enzymatic hydrolysis by delignification. Furfural and hydroxy methyl furfural (HMF) content in the treated biomass is also negligible in this process, which makes it preferable for digestion

compared to pretreatments involving dilute acid. There is also a process which uses alkaline peroxide to further treat steam exploded biomass (X. F. Sun et al., 2005). In the process wheat straw was used and the operating conditions for steam explosion was 200-220 °C temperature and 15-22 bars pressure. The alkaline peroxide treatment was performed using 2% H_2O_2 at 50°C for 5h under pH of 11.5. As a result of steam explosion loss in hemicellulose, and about 11-12% lignin removal was observed, while the alkaline peroxide post-treatment resulted in 81-88% removal of the original lignin, which sums up to 92-99% removal of the original lignin from wheat straw.

Organosolv Pretreatment:

Many organic solvents such as alcohols, esters, ketones, glycols, organic acids, phenols, and ethers can be used in this process. Organic or aqueous organic solvents can be used with or without addition of catalysts such as oxalic, salicylic, and acetylsalicylic acid in temperature range of 90-250°C(M. Taherzadeh & Karimi, 2008; X. Zhao et al., 2012) to treat lignocellulosic material. The processes involve mixing of solvents and water to various proportions and adding them to the biomass, which is heated to dissolve lignin to leave a reactive cellulosic cake (M. Taherzadeh & Karimi, 2008). In this pretreatment, operating temperature ranges between 90 and 120ºC for grasses and 155 and 220ºC for woods, with a residence time ranging from 25 to 100 min. Depending on the substrate, catalyst concentration can vary from 0.8-1.7% and alcohol concentration from 25-74% (v/v) (da Costa Sousa et al., 2009). Easy recovery techniques of organic solvents may leads to isolation of lignin as a solid material which is a benefit of organosolv pretreatment (Lora & Aziz, 1985).

However, to make the pretreatment more cost effective, the costs of solvents as well as the costs of recovery should also be considered. The solvents should be alienated by means of evaporation and condensation and recycled to cut down total operational costs of the process. Presence of solvent residue in the pretreated cellulose could be harmful to the enzymatic hydrolysis and fermentation because of inhibitory nature of some solvents. For economic reasons, alcohols having relatively low molecular weight such as ethanol and methanol could be preferred over alcohols with higher boiling points, e.g. ethylene glycol. Ethanol should also be removed from the solid fraction before enzymatic hydrolysis due to its inhibitory nature towards enzymatic hydrolysis.

However further research is needed to know how organosolv pretreatment (condition, solvent type) affects the crystallinity and also the degree of cellulose swelling (G. Shen et al., 2011). As lignin is oxidized, inhibitors can be formed and lots of carbohydrates as sugar can be lost.

2.1.3 Physico- chemical Pretreatment:

Similar to chemical treatment, physico-chemical treatment also aids in increasing accessible surface area, partial or nearly complete delignification as well as partial or complete hydrolysis of hemicelluloses. These treatments also decrease cellulose crystallinity and degrees of polymerization. For these reasons these methods are among the most effective and most promising processes for industrial applications (Phitsuwan et al., 2013; C. E. Wyman et al., 2005). But there are various limitations associated with these processes as well. Many of these treatments need harsh conditions. Also inhibitory products such as furfural are often formed which affect negatively the subsequent hydrolysis and fermentation processes.

Steam explosion

Steam explosion removes the major part of hemicellulose and makes the cellulose more susceptible to hydrolysis. In this method, biomass is treated with high-pressure saturated steam followed by sudden release of the pressure, which make the biomass undergo an explosive decompression. Steam explosion is in general initiated at a temperature of 160-260°C (corresponding pressure 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure (Y. Sun & Cheng, 2002; M. J. Taherzadeh & Karimi, 2007; M. Taherzadeh & Karimi, 2008; C. E. Wyman et al., 2005). At high temperatures the addition of water to H_2SO_4 or SO_2 or CO_2 [typically 0.3-3% (w/w)] in steam explosion may affect negatively and it can lead to complete removal of hemicellulose (Brownell & Saddler, 1987; P. Kumar et al., 2009; M. Taherzadeh & Karimi, 2008; Weil et al., 1997).

The operating conditions for steam explosion should be chosen in such a way that excessive degradation of cellulose could be avoided. Use of very harsh conditions, could be resulted in lower enzymatic digestibility of lignocelluloses after steam explosion. Severity of the pretreatment can be expressed by the severity factor as:

‘‘log = log (t* e^ ((T-100)/14.75))”;

where ‘t’ is in minutes and ‘T’ is in degrees Celsius.

Apart from steam explosion, the severity factor can also be applied to hydrothermal acidic pretreatment and conversion as well. The application of steam explosion is limited to hardwood and agricultural residues. The main disadvantages of the steam explosion process are the partial degradation of hemicellulose and formation of

toxic compounds such as furfural, phenolic compounds etc., which adversely affect the subsequent hydrolysis and fermentations processes (Palmowski & Müller, 2000). The type and amount of toxic compounds produced during steam explosion are decided by the type of feedstock and severity of pretreatment. A study by Ballesteros et al. showed that the use of thermotolerant yeast strain can curb the problem to a certain level (Ballesteros et al., 2004).

Ammonia Fiber Explosion (AFEX)

Ammonia Fiber Explosion or AFEX is almost similar to steam explosion. In this pretreatment biomass with desired moisture content, subjected to treatment with liquid anhydrous ammonia at high pressure (17-21 bar) and moderate temperature (60-100°C) for a short time span (5-8mins) followed by rapid release of the pressure (Teymouri et al., 2005b). In AFEX process, also known as ammonia recycled percolation (ARP), ammonia solution (5-15%) is passed through a column reactor packed with biomass at elevated temperatures (160-180ºC) and a fluid velocity of 1 m^3/s for 2min with residence times of 14 min inside the reactor. After reaction cellulose and hemicellulose rich solid fraction is separated and liquid fraction is transferred to a steam heated evaporator for ammonia recovery as well as lignin and sugar separation. This method is also known as ammonia recycled percolation (ARP) process since ammonia is separated and recycled. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses (Alizadeh et al., 2005; Dale et al., 1999; Delgenes et al., 1996; Mosier et al., 2005; Y. Sun & Cheng, 2002; Teymouri et al., 2005b). The cost of ammonia and especially of ammonia recovery drives the cost of this pretreatment (Holtzapple et al., 1992). However, its ability to minimize the formation of sugar degradation products at moderate

temperature and pH value makes it preferable compared to other processes. Moreover the treated biomass does not have to be neutralized for enzymatic hydrolysis as large amount of ammonia can be recovered from the process and the remaining ammonia can act as nitrogen source for the microbes (Teymouri et al., 2005b) . Modifications of the processes are sometimes made to improve the delignification and to achieve fractionation of biomass.

CO_2 Explosion

This method is similar to AFEX and steam explosion except that in CO_2 explosion pressurized CO_2 is charged into the reactor and after a few seconds, pressure is suddenly reduced subsequently. As a result, carbonic acid is produced and the rate of hydrolysis is improved although yield is significantly lower than that of steam explosion or AFEX (X. F. Sun et al., 2005).

Another way to use CO_2 to pretreat lignocellulose is as a solvent in extraction at supercritical condition. Supercritical fluid shows properties of both liquids and gases above critical temperature and pressure. Water present in the biomass in combination with supercritical CO_2 produces carbonic acid mixture and aids hemicellulose hydrolysis by providing the weak acid environment. The important parameters for this pretreatment other than temperature and pressure are moisture content and pretreatment time. Study shows rice husk with 25% moisture content when treated with supercritical CO_2 at 80°C temperature and 270 bar pressure for 10 min leads to reduction of lignin content upto 90.6% (Daza Serna et al., 2016). According to the techno-economic study presented in this study, this method is proved to be more effective and economic than acid pretreatment. Though energy needed for CO_2 conditioning increase the cost of utilities

but in dilute acid pretreatment cost of raw material, operating cost are much higher due to the inability to recycle the reagents, costly biomass recovery and detoxication process (Daza Serna et al., 2016; Gu, 2013; Narayanaswamy et al., 2011).

Liquid hot water (LHW)

Liquid hot water (LHW) treatment can be categorized as the hydrothermal pretreatment. In this process there is no requirement of corrosion resistant biomass for hydrolysis reactors and no addition of chemicals is needed. In LHW pretreatment, no size reduction is required. The process requires a much lower chemical usage for the neutralization of the produced hydrolyzate which contains liberated organic acids (Hendriks & Zeeman, 2009). LHW pretreatment enlarges the accessible and susceptible surface area of the cellulose and makes it more accessible for hydrolytic enzymes. LHW is said to be advantageous over steam pretreatment as LHW offers high pentosan recovery and chances of inhibitor formation is also less (Y. Kim et al., 2011; Kobayashi et al., 2009; Mosier et al., 2005; G. Shen et al., 2011; Tsao et al., 1981; Q. Yu et al., 2010). In this process biomass is treated in hot water for about 15 min at elevated temperature in the range of 200-230°C. Approximately 40-60% of the total biomass is dissolved in this process, with 4-22% of the cellulose, 35-60% of the lignin, and all of the hemicellulose being removed. Three types of liquid hot water reactor configurations are used, namely, co-current, countercurrent, and flow-through. In co-current pretreatment, (Biomass in liquid slurry form) of approximately 16% solids is heated to 140–180ºC, and held for 15–20 min followed by cooling and heat recovery. In a flow-through reactor, hot water is made to pass over a stationary bed of lignocellulose at 180–220ºC and about 24–28 bar pressure. Temperatures, pressures and residence times are similar in case of flow-

through and countercurrent mode. Flow through systems removed more hemicelluloses and lignin from corn stover than batch system at same severity factor (Hendriks & Zeeman, 2009; P. Kumar et al., 2009; C. E. Wyman et al., 2005)

2.1.4 Combination of pretreatments:

The conventional pretreatments mentioned earlier have some limitations and to combat the drawbacks some improvised and effective techniques have been adopted recently. These techniques include sono-assisted, microwave assisted treatment as well as incorporation of nanoparticles and usage of activated carbon via photolysis. Thermal pretreatment in combination with acid, alkali, oxidative or alkaline oxidative treatments have proved to be highly effective tool of pretreatment.

Ultrasonic-assisted pretreatment

The use of ultrasound as a pretreatment tool is a comparatively new approach and considered as potential one to enhance the yield of fermentable sugars. Depending upon the frequency it can be divided into three frequency ranges: power ultrasound (16–100 kHz), high frequency ultrasound (100 kHz–1 MHz) and diagnostic ultrasound (1–10 MHz) (Nikolić et al., 2010). If a low frequency wave propagates in an aqueous medium, it generates large bubbles due to cavitation and a hydro-mechanical shear forces generates in the bulk liquid due to the rapid collapse of the bubbles resulting in the disruption of the coarse particles present in the liquid into finer particles. If enough energy generated, the hydrogen bonds present in the crystalline structure of cellulose can get ruptured (García et al., 2011; Nikolić et al., 2010; Nitayavardhana et al., 2010).

Sono-assisted alkali pretreatment involves dilution of dried and sieved samples into 2% NaOH solution followed by ultrasound treatment for an optimum time span. The contents were then filtered and the solid residues are subjected to vigorous rinsing to achieve neutral condition. This technique increases accessibility of enzymes during saccharification as it can degrade lignin sheath better than steam explosive alkaline treatment and also the energy requirement is lower (Velmurugan & Muthukumar, 2011, 2012b, 2012a) .

So far batch mode experiments with ultrasonic-assisted proved to be promising for ethanol production in small scale when used to pretreat cassava chip. This pretreatment when used followed by simultaneous saccharification and fermentation (SSF) has proved to be useful in disrupting corn starch structure, to increase glucose concentration before or after liquefaction under certain conditions for sonication. It reduces the fermentation time by almost 24 hours and significantly increases ethanol production. However, keeping in mind the energy consumption scenario of both batch mode and continuous flow system, continuous flow system is found to be more suitable for industrial scale application. Scale up can be a cause of concern as several aspects like experimental design and the cost of pretreatment can play havoc on overall plant economics (Nikolić et al., 2010; Nitayavardhana et al., 2010; Niu et al., 2009).

Microwave assisted pretreatment

Compared to the conventional heating process microwave heating is shown to be an effective way to enhance enzymatic hydrolysis. The process is more effective than chemical pretreatment with conventional irradiation (Zhu, Wu, Yu, Chen, et al., 2006; Zhu, Wu, Yu, Wang, et al., 2006). Xylose cannot be recovered during the microwave

assisted alkali pretreatment process but could be recovered as crystalline xylose during microwave assisted acid, alkali, and H_2O_2 pretreatment (Zhu et al., 2005). It is performed in many ways such as microwave assisted alkali, microwave assisted acid and alkali treatment, and microwave assisted acid, alkali and hydrogen peroxide combined pretreatment (J. Liu et al., 2010; Zhu et al., 2005; Zhu, Wu, Yu, Chen, et al., 2006; Zhu, Wu, Yu, Wang, et al., 2006).

The structural changes in the bagasse actually caused due to the combination of microwave disruption and chemical dissolution of hemicellulose by dilute sulfuric acid (W. H. Chen et al., 2011). Microwave treatments also offer much higher digestibility of cellulose than conventional heating, but unable to completely break down recalcitrant structures like switchgrass. Owing to that fact fermentable sugar yield falls down comparatively (Hu & Wen, 2008).

A study of Zhu et al shows that as compared to conventional alkali pretreatment, microwave assisted alkali pretreated substrate requires lower enzyme loading, shorter fermentation time, and offers high ethanol yield (Zhu, Wu, Yu, Chen, et al., 2006). To pretreat rice-straw microwave assisted acid, alkali and hydrogen peroxide combined pretreatment is more effective than microwave assisted alkali pretreatment as it has the highest hydrolysis rate, and also high glucose and low xylose content. This xylose can be recovered in this pretreatment whereas in some cases it cannot be recovered (Zhu et al., 2005).

Pretreatments such as steam explosion or AFEX involve elevated temperature (Brosse et al., 2009; Chang, Kaar, et al., 2001; Dale et al., 1999) which exploits energy

sources and thereby increases cost. To overcome that limitation an additional procedure is adopted which uses a compound (such as glycerine) with high dielectric constant which upon addition in water, can absorb the microwave irradiation as well as depress the vapor pressure of the system. This system allows performing the experiment in atmospheric pressure (Kitchaiya et al., 2003).

Activated carbon approach

Activated carbon is a micro porous form of carbon, activated by a controlled oxidation process having high surface area for adsorption and chemical reaction. Use of activated carbon in pretreatment of lignocellulosic material is an innovative approach. Activated carbon can be used to modify acid or alkaline treatment for better yield. It is also very effective in removing inhibitors generated during the pretreatment. The process involves washing and drying of activated carbon granules, addition of activated carbon to the liquid fraction at room temperature, and separation of activated carbon with adsorbed inhibitors from the liquid fraction (Jung et al., 2013). Amount of one of the inhibitors of hydrolysis, furfural can be reduced significantly by activated carbon treatment thereby increasing the ethanol yield. If fermentation can be done on the activated carbon pretreated whole slurry the utilization of sugar increases and the production cost will reduce (Jung et al., 2013).

There is also a unique pretreatment method reported that use alkali solution assisted by photo catalysis (Niu et al., 2009). It is a modified alkaline treatment where nano-TiO_2 upon irradiation emits hydroxyl and superoxide radicals which forms radical site on the substrate and results in better degradation. Photo catalysis time is a very important factor in this treatment. As a result of this process the cellulose content of the

pretreated sample increases, and a decrease in alkali dosage required for the pretreatment is observed. But this technology should be explored more for further improvement (Niu et al., 2009; Tanaka et al., 1997, 1999).

Ionic liquid approach

Ionic liquids contain mostly ions and have low melting points (below 100ºC), high thermal stability, and negligible vapor pressure (van Rantwijk & Sheldon, 2007). The ionic liquids containing halide, acetate, formate, and phosphate anions can be used as a media for the dissolution of carbohydrates including cellulose (Ohno & Fukaya, 2009; Swatloski et al., 2002). Polar solvents have the ability to absorb microwave irradiation and therefore can be heated rapidly than non-polar solvents having less or no ability to absorb microwave radiation. High polarity of ionic solvents acts as an added advantage as microwave heating functions on the basis of the polarization of the molecules.

Recent study shows that a solution with ILs can be significantly increased when ionic liquid treatment is assisted with microwave heating. The cellulose can be regenerated by the addition of an anti-solvent such as water, alcohol, or acetone which leads to an eco-friendly and low energy consumption compared to other dissolution processes (Hu & Wen, 2008; Kuo & Lee, 2009). These properties make ionic liquids such as 1-butyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium acetate, an excellent alternative tool to pretreat lignocellulose. The experimental data shows that cotton celluloses regenerated from microwave assisted ionic liquid pretreatment, can be hydrolysed much faster than those without microwave irradiation and the degree of polymerization of cellulose is decreased significantly (Kappe & Dallinger, 2009).

Ionic liquid can also be used to incorporate nanoparticles into the cell walls of lignocellulosic biomass, which can later be functionalized chemically (Lucas et al., 2010). Incorporation of nano particles having a high surface to volume ratio can open up whole new possibilities in pretreatment. This method involves swelling of woods by an ionic liquid at room temperature followed by an exposure to an aqueous suspension of nano particles (Abraham et al., 2014). So far silver and gold nano particles have been explored for this purpose (Lucas et al., 2010). The expansion of wood cells by using an ionic liquid and contraction of the cells by simply rinsing with deionized water is the basis of this pretreatment. This overall expansion and contraction by using ionic liquid disrupt the hydrogen-bond network, the backbone of lignin structure.

ILs are qualified as green solvents due to affluence of reprocessing and reuse due to low vapor pressure and high boiling point. However, toxic nature and poor biodegradability of most ILs is also reported (Romero et al., 2008). Presence of small amounts of ILs can be detrimental as impurities, even in trace amounts, affect their physical properties. Additionally, their synthesis process can also be contested on the ground of environment friendliness, since it generally requires a large quantity of salts and solvents in order to completely exchange the anions. These drawbacks combined with the high price of common ILs acts as a setback for their industrial usages and new concepts are the need of the hour in order to utilize these systems in a more rational way. To overcome the high price and toxicity of ILs, a new generation of solvent, named Deep Eutectic Solvents (DES), has emerged.

Deep eutectic solvents (DESs):

Formation of Deep eutectic solvents (DESs) can easily be attained by mixing two components, economically affordable, renewable and at the same time biodegradable, which can form a eutectic mixture. Two solid phase chemicals at a certain molar ratio (eutectic composition) can form super-joint lattices leading to a homogeneous mixture. The joint super-lattice usually melts at a lower temperature than individual components melting point, known as eutectic temperature, leading to formation of homogeneous colorless mixtures, DESs. DES are called deep because the melting point curve has a particularly deep crevice at the eutectic point (Lynam et al., 2017).

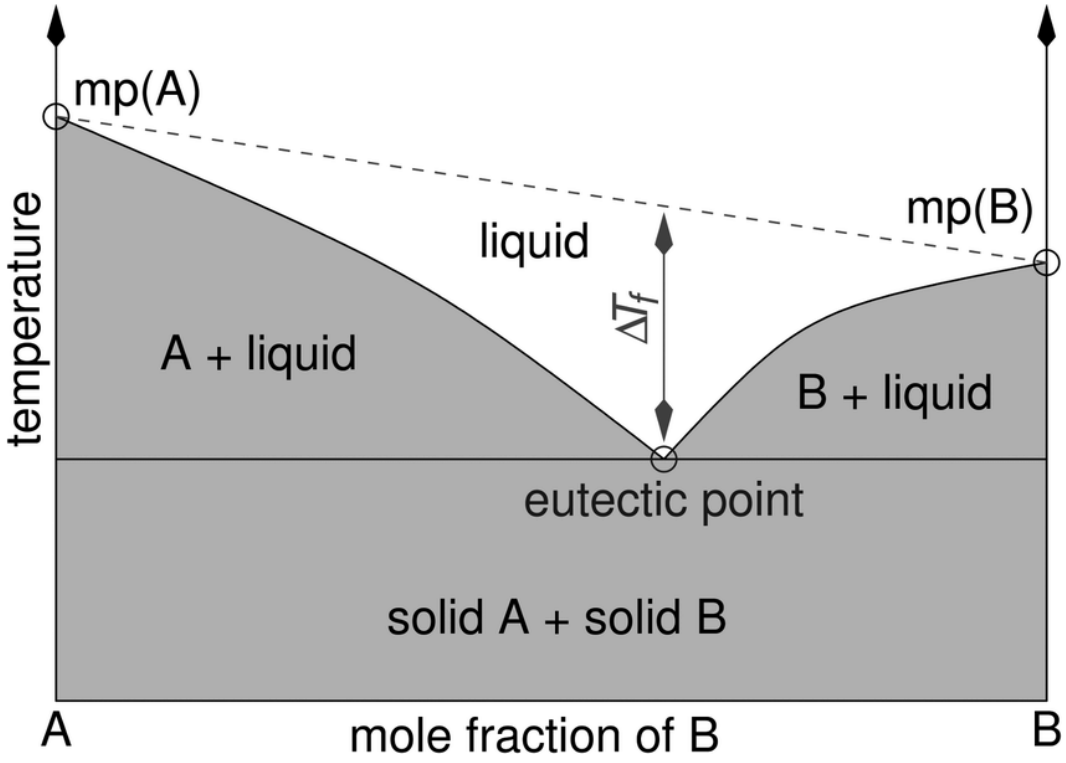


Figure 6: Schematic representation of a eutectic point on a two-component phase diagram (Smith et al., 2014).

Figure 6 represents the eutectic point of a binary mixture of A and B. The difference in the freezing point at the eutectic composition of the binary mixture compared to that of a theoretical ideal mixture, ΔT_f, is related to the magnitude of the interaction between A and B. The larger the interaction, the larger will be ΔT_f (Smith et al., 2014).

As compared to traditional organic solvents, DESs are not considered as volatile organic solvents and not flammable which makes their storage convenient. From the green, pro-environment stand point, these DESs are even more attractive since some of them have been proven to be biodegradable and compatible with enzymes making them more interesting. Additionally, synthesis of DESs is 100% atom economic, easy to handle and no purification is required, thus making their large-scale use feasible. As synthesis of DESs are 100% atom economic, the components can be simply mixed to form the eutectic mixture and all the subsidiary steps in the preparation such as purification and waste disposal is not required and thus, large quantities of DESs with little to no waste can be formed (González et al., 2020; Z. Zhang et al., 2012). The easy availability of the raw components (quaternary ammonium salts and metal salts) reduces their production cost and their chemical inertness makes it easier to store it efficiently and conveniently. Abbott et al. first reported 1:2 mol fraction mixture of choline chloride with crystalline urea, with eutectic temperature of 12˚C. Abbott et al. explained the nature of DESs based on hydrogen bond through which species of DESs are aggregated (Abbott et al., 2003). They defined this mixture to consist of hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs). The Hydrogen bond interaction between these two types of species in DESs imparts very low melting point of eutectic mixture formed thereafter. DES thus obtained by mixing a quaternary ammonium salt (i.e, ChCl) with metal salts or

a hydrogen bond donor (HBD) possesses the ability to form a complex with the halide anion of the quaternary ammonium salt. Different quaternary ammonium salts that are used frequently in combination with various HBDs in the formation of DESs can be observed at figure 7.

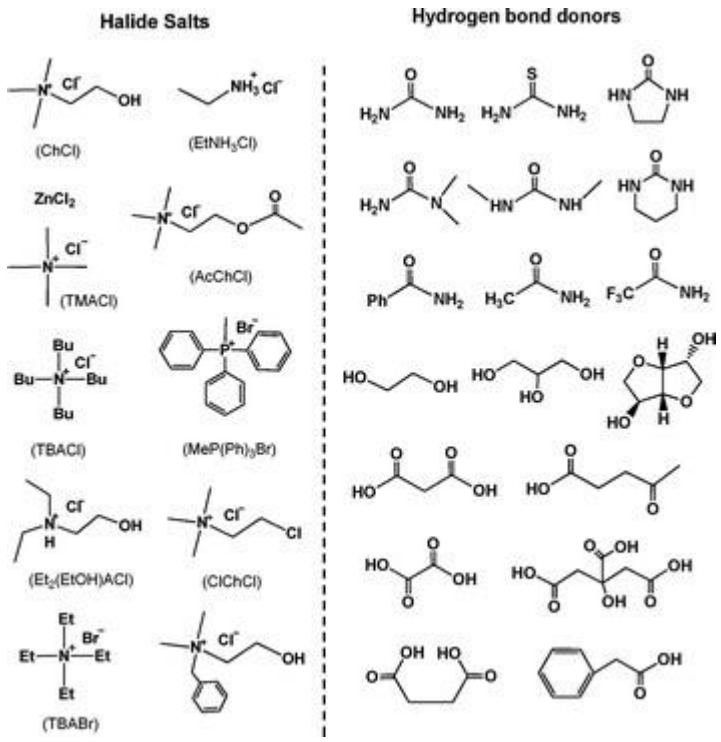


Figure 7: Structures of halide salts and hydrogen bond donors used for DES synthesis (Q. Zhang et al., 2012)

In early 2007, Abbott and group also defined DESs using the general formula $R_1R_2R_3R_4N^+X^-\cdot Y^-$ (H. Yang, 2015),

Type I, DES Y = MCl_x, Where, M = Zn, Sn, Fe, Al, Ga

Type II, DES Y = $MCl_x\cdot yH_2O$, Where, M = Cr, Co, Cu, Ni, Fe

Type III, DES Y = R_5Z, Where, Z = $-CONH_2$, -COOH, -OH

They also mentioned another type of DES which consists of metal chlorides (e.g. $ZnCl_2$) in combination with different HBDs such as urea, ethylene glycol, acetamide or hexanediol, known as type IV DES.

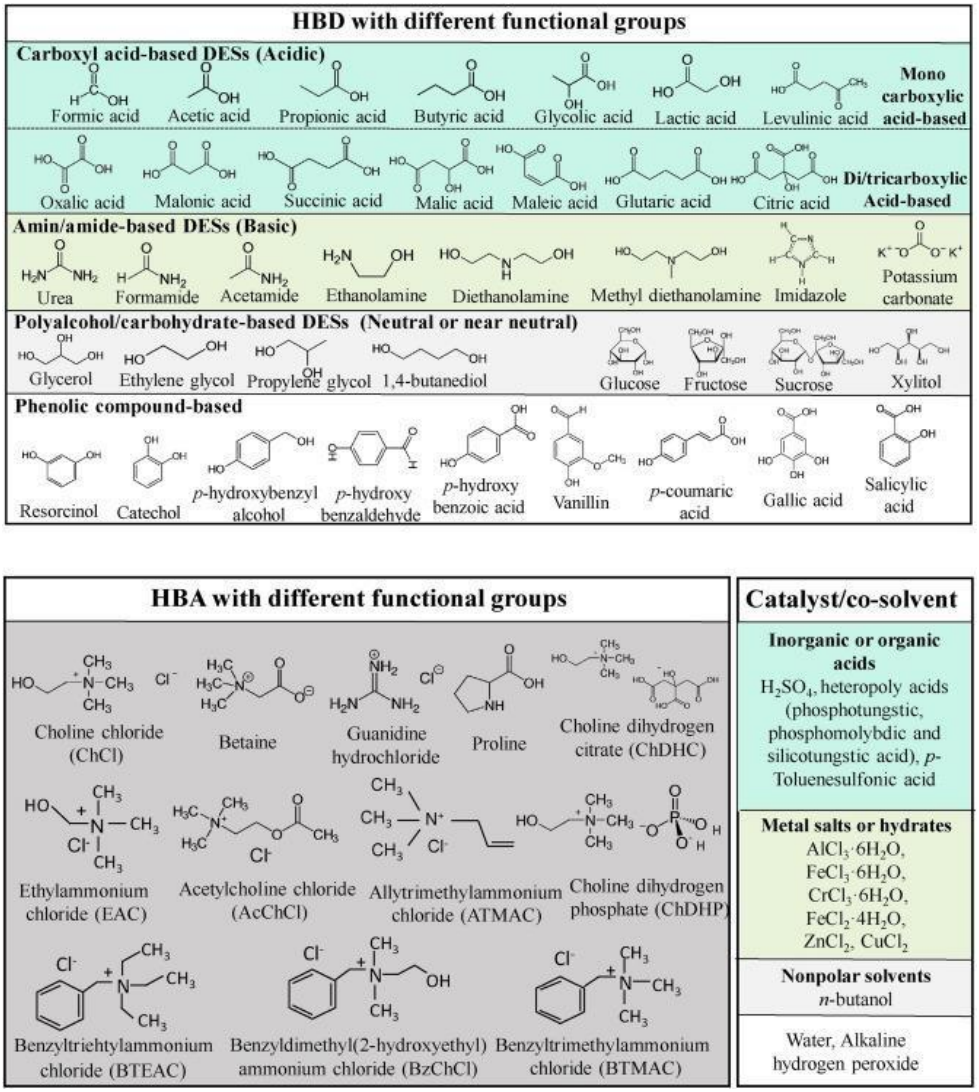


Figure 8: Structures of HBD and HBA of DES as well as catalysts/co-solvents (X. Zhang et al., 2022).

Figure 8 represents the structure of HBD and HBA of DES. ChCl is a quaternary ammonium salt which can be extracted either from lignocellulosic materials or can be synthesized easily. It is biodegradable and non-toxic in nature. ChCl can easily form DES

with hydrogen bond donors such as urea, renewable carboxylic acids (e.g., oxalic, citric, succinic, or amino acids) or renewable polyols (e.g., glycerol, carbohydrates. Physico-chemical properties of DESs (toxicity, density, viscosity, refractive index, ionic conductivity, hydrogen bonding, surface tension, chemical inertness, etc.) are almost similar to those of common ionic liquids. DES have become the point of attraction recently both at academic and industrial levels because of their low environmental footprint and striking cost. The number of publications exploring the use of DESs has increased rapidly in the last 2-3years. There are different applications which are being explored for DESs, to name a few, pretreatment of lignocellulose, purification of biodiesel, bio-molecule stabilization etc. (M.B. Singh Mr et al.,2021). Figure 9 shows different applications of DES.

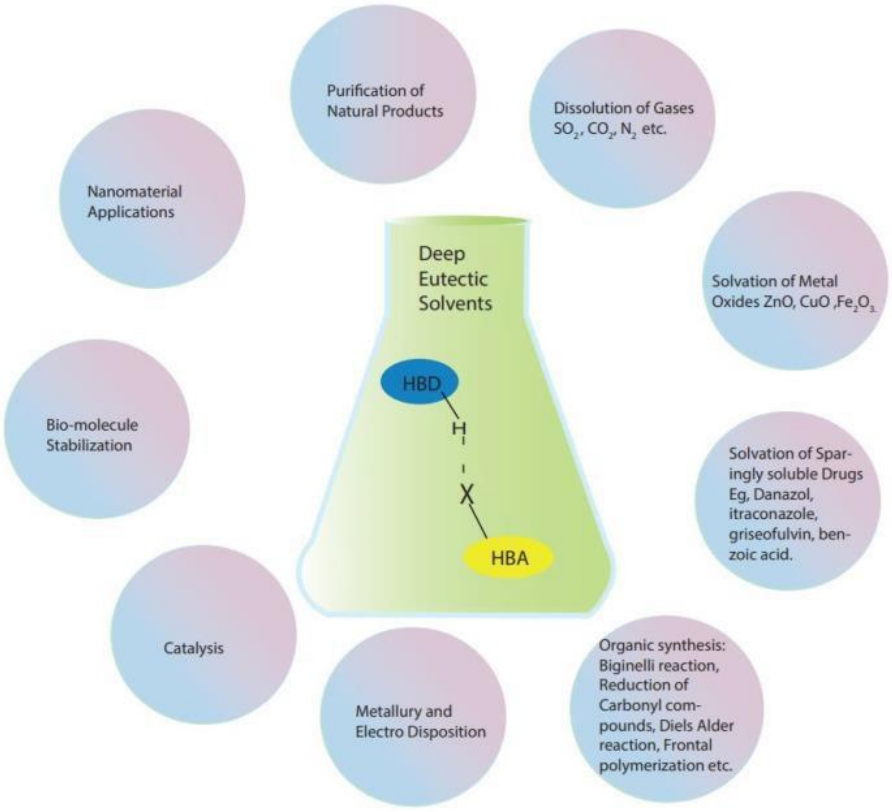


Figure 9: Applications of deep eutectic solvents (M. B. Singh et al., 2021)

In the present study, DES is used as a pretreatment method for lignocellulose material. The combination of DESs mostly used in handling lignocellulosic material is presented in Table 5.

Table 5: DES with different hydrogen bond donors and molar ratios

Hydrogen bond acceptor (HBA)	Hydrogen bond donor (HBD)	Molar ratio of choline chloride and HBD	pH	References
Choline chloride	Citric acid	1:2	1	(Lynam et al., 2017; Pan et al., 2017; Yoon et al., 2022)
	Glycerol	1:2	5	
	Malonic acid	1:1	1	
	Urea	1:2	7	
	Ethylene glycol	1:2	5	
	Formic Acid	2:1	1	
	Lactic acid	10:1	1	
	Acetic acid	2:1	1	

The molar ratios of choline chloride and HBD mentioned in table 5 is in standardized form but depending on the material to be treated and reaction conditions the ratios can differ. The performance of various DES on lignin removal or delignification method is shown in table 6.

Table 6: Performance of different DES on lignin removal

DESs (HBA: HBD)	Lignocellulosic sources	Operating conditions: Temperature (°C), Time (h) and Solid loading	Delignification (%)	References
ChCl:LA (1:2)	Douglas fir	145 ˚C, 6 h, 10%	58.2	(Alvarez-Vasco et al., 2016)
	Poplar		78.5	
	Sorghum		78	(Das et al., 2018)
	Walnut		64.3	(H. Li et al., 2023; W. Li et al., 2018)
ChCl:LA (1:10)	*Eucalyptus camaldulensis*	130˚C, 6 h, 10%	93.2	(X.-J. Shen et al., 2019)
ChCl:urea (1:2)	Oil palm empty fruit branch	120˚C, 8h, 10%	34	(Tan et al., 2018)
	Wheat straw	90˚C, 12h, 5%	27.7	(Z. Zhao et al., 2018)
	Sweet corn cob	80˚C, 15h, 16%	83	Procentese et al,2015

Combined pretreatment approach

Thermal pretreatment in combination with alkaline oxidative pretreatment increases the enzymatic digestibility 13 times more than that of the untreated biomass. About 21% of the lime used during treatment could be recovered by carbon dioxide carbonation after the oxidative lime pretreatment. Oxygen needs to be used as an oxidative agent as thermal lime pretreatment alone is not capable to remove lignin from high lignin containing biomass to enhance the enzymatic digestibility. Low sugar degradation is also observed in the process because of low operating temperature (150ºC) (Chang, Kaar, et al., 2001).

Biological pretreatment in combination with dilute acid pretreatment has also been explored and it has proved to be an effective tool for pretreatment. Conventional acid pretreatments are in general hazardous and not very feasible economically. On the other hand less energy consuming biological treatments involving fungi requires mild condition and significantly decreases lignin content by disrupting hemicellulose-lignin bond (H. Yu et al., 2009; X. Zhang et al., 2007). Study of Ma F et al shows raw and bio-pretreated water hyacinth with *E. taxodii* or *Antrodia sp*. 5898 in combination with sulfuric acid at diverse temperature (25 ºC, 80 ºC, and 100ºC) for 15–60 min. It has enhanced ethanol production from water hyacinth by improving enzymatic hydrolysis. It was further reported an increase in 1.13–2.11-fold reducing sugar formation from enzymatic hydrolysis of co-treated sample compared with that of acid-treated sample at the same conditions (Ma et al., 2010).

2.2 Hydrolysis and Fermentation of Pretreated Biomass

Ethanol can be produced by hydrolyzing the reducing sugar generated through pre-treatment followed by fermentation of the hydrolyzed biomass. The pretreatments loosen up or modify the structure of lignocellulose material by bond dissolution which results in the breakage of the compact lignin, cellulose and hemicellulose matrix.

Hydrolysis of lignocellulose has been studied for about 200 years (Binod et al., 2019). In acid hydrolysis catalysts can infiltrate in the lignocellulosic cell wall without any delignification or any specific pretreatment, and for some cases the rate of acid hydrolysis is much faster than that of enzymatic hydrolysis. Acid catalyzed hydrolysis for both its concentrated and dilute form has been proved to be effective in yielding sugars from the lignocellulosic materials. Dilute acid hydrolysis (0.7-3.0%) although less toxic

for the equipment to be used, involves high operating temperatures (200–240˚C) while concentrated acid hydrolysis necessitates higher acid concentration (El-Zawawy et al., 2011). Acid recycling also involves considerable costs (Banerjee, Mudliar et al. 2010). On the contrary, enzymatic hydrolysis uses specific enzymes for cellulose for specific temperatures. Therefore, enzymatic hydrolysis is the most promising alternative to acid hydrolysis, but it is certainly not a replacement process (El-Zawawy et al., 2011). There are several factors which influences the hydrolysis of lignocellulose which includes reactivity, solubility of carbohydrates, structure of lignin and substrates. Reactivity of carbohydrates is closely related to their structures (Zhou et al., 2021). The amorphous regions can be easily penetrated by chemical reagents/enzymes due to the lower cohesive energy density associated with it compare to the crystalline regions leading faster hydrolysis in amorphous regions. The amorphous part of microcrystalline cellulose requires lower temperature compare to the crystalline part for disruption glycosidic bonds to produce glucose monomers. Figure 10 depicts the factors influencing hydrolysis.

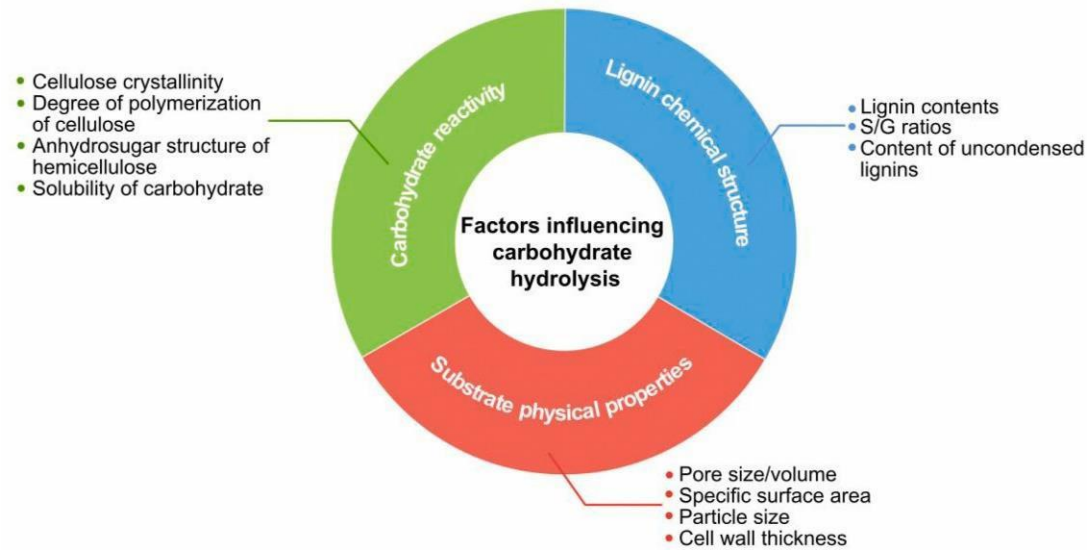


Figure 10: Factors influencing the hydrolysis of polysaccharides (Zhou et al., 2021).

There are two different ways practiced for the hydrolysis of lignocellulosic materials for ethanol production, namely, acid hydrolysis and enzymatic hydrolysis.

2.2.1 Acid Hydrolysis

Depending on the form of the acid used in the process, acid hydrolysis can be divided into three categories, namely, concentrated acid hydrolysis, hydrolysis by using dilute acids, hydrolysis by using solid acids. Sulphuric and hydrochloric acids are the most used acids for hydrolysis of lignocellulosic biomass. The 10-30% concentrated acids are used in the concentrated acid hydrolysis process. High cellulose yield is obtained at low temperature due to high acid concentration (H. Li et al., 2009b). However, this process requires high concentration of acids which leads to corrosion of the equipment. On the other hand, main advantage of the dilute acid hydrolysis over concentrated acid hydrolysis is the lower concentration of required acid (2-5%). However, for substantial cellulose yield the process needs to be carried out at high temperature. The high temperature leads to the formation of toxic furfural and 5-hydroxymethyl-furfural (HMF) by decomposing hemicellulose derived sugars (Y. Zhang et al., 2020). These compounds inhibit the growth of yeast cells and the subsequent fermentation stage, causing a lower ethanol production rate.

Concentrated Acid Hydrolysis (CAH):

The acids mostly used are sulfuric acid (50-80%), hydrochloric acid (37-42%) and phosphoric acids (82-84%), or the mixture of acids in a specific ratio. The process occurs in two steps, at normal atmospheric pressure and temperatures below 100˚C (Harmer et al., 2009; Heinonen et al., 2012; Saxena et al., 2009). At first, concentrated acid was used to disrupt the crystalline structure of cellulose present in the raw material and dissolving

the fibers. In the next step, diluted acid is used to hydrolyze the glycosidic bonds for formation of monosaccharides. Sometimes a pre-hydrolysis is also included to remove hemicelluloses. Several processes have been developed and patented for hydrolysis of lignocellulosic materials by concentrated acids such as, Hokkaido process, Arkenol process, Bergius-Rheinau process etc. However, no substantive progress on hydrolysis technology has been made in recent decades (Zhou et al., 2021). The Hokkaido process involves three major reaction steps which includes a pre-hydrolysis of hemicelluloses with the help of steam at 140-150˚C to prepare the raw materials for hydrolysis. Next, cellulose is infiltrated with 90% sulfuric acid at ambient temperature and finally, dilution of the solids and acid is facilitated to post-hydrolyze the material at 100˚C and the residual acid is neutralized by using lime. The Hokkaido process majorly used to produce crystalline glucose, furfural, methanol, acetic acid, and gypsum. The Hokkaido process claimed a glucose yield of about 83–85% of the theoretical, but the acid recovery was only 80% (Clausen & Gaddy, 1993; O'Neil et al., 1979). Arkenol process involves decrystallization with 70%-77% sulfuric acid followed by dilution of the acid for second-step hydrolysis. The sugars and acid can be separated by a chromatographic column to achieve a high yield and acid recovery (Farone & Cuzens, 1996; Wijaya et al., 2014). The separation of acid and sugar is the main concern for the economic feasibility of CAH process. Where acid loss is higher than 10% the process is not considered as economically feasible. Figure 1 showed the schematic diagram of reactions involved in the hydrolysis of cellulose by concentrated acids.

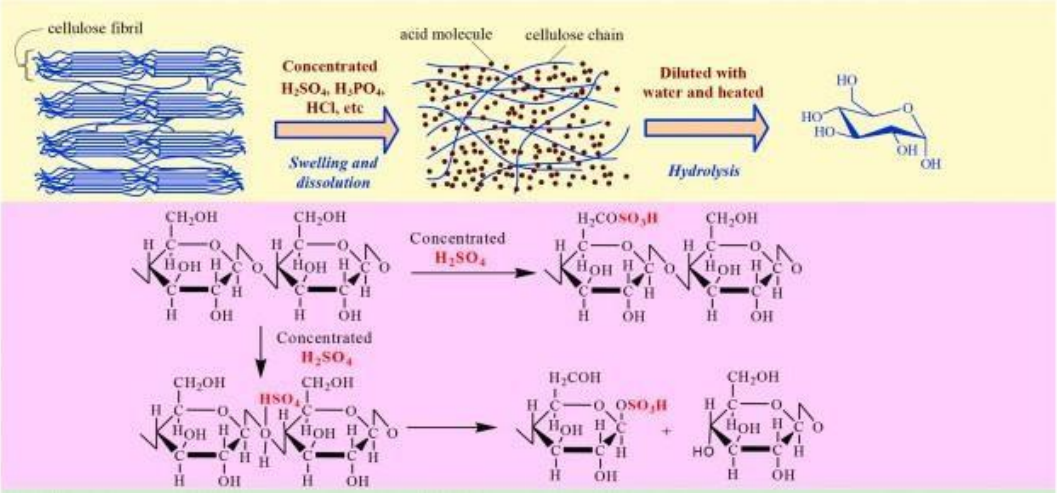


Figure 11: Schematic diagram of reactions involved in the hydrolysis of cellulose by concentrated acids i.e, cellulose decrystallization, hydrolysis and esterification of cellulose by concentrated H_2SO_4 (Zhou et al., 2021).

Dilute Acid Hydrolysis (DAH):

Dilute acid hydrolysis (DAH) is considered to be more economically feasible than that of concentrated acid hydrolysis because of the usage of acid in lower concentration. In DAH the tedious processes of separating acid and sugar can be avoided as the hydrolysate obtained by DAH can be directly utilized after neutralization with lime. The sugar yield depends on temperature, concentration of acid as well as residence time. The hydrolysis process can be functioned in batch or continuous models. Primarily, DAH processes are of two types: continuous flow process for solid loading of as low as 5-10% at high temperature i.e. greater than 160˚C, and batch process for handling high solid loading in the range of 10-40% at low temperature. The degradation of sugar is more prominent in a batch process than that of in continuous flow process, as cellulose and hemicelluloses reacts differently under reaction temperature during hydrolysis. Sugar yield of about 85-90% can be accomplished from hemicellulose fraction at 160˚C when treated with 0.7% concentration of acid for about 10 min. However, about 10% of

glucose yield can be obtained when α-cellulose is hydrolyzed in presence of 0.05 M H_2SO_4 at temperature of 175˚C and 40 min reaction time (Zhou et al., 2021). Temperatures more than 200˚C are mostly used in traditional process of diluted acid hydrolysis although elevated temperature may cause degradation in pentose. Two-stage process can be applied in order to curb the sugar degradation. At first, lignocellulose is hydrolyzed at a low severe reaction condition in order to maximize the recovery of hemicellulosic sugars. At the nest step, the water insoluble solids are infiltrated with dilute acid and then kept in contact with more severe conditions to aid in hydrolyzation of cellulose .Due to the decrease in sugar degradation, formation of inhibitors could also be reduced. By controlling the flow rate and the residence time, the sugar recovery can be maximized. Figure 12 depicts conversion of lignocellulose biomass in to fermentable sugar by diute acid hydrolysis (Loow et al., 2016).

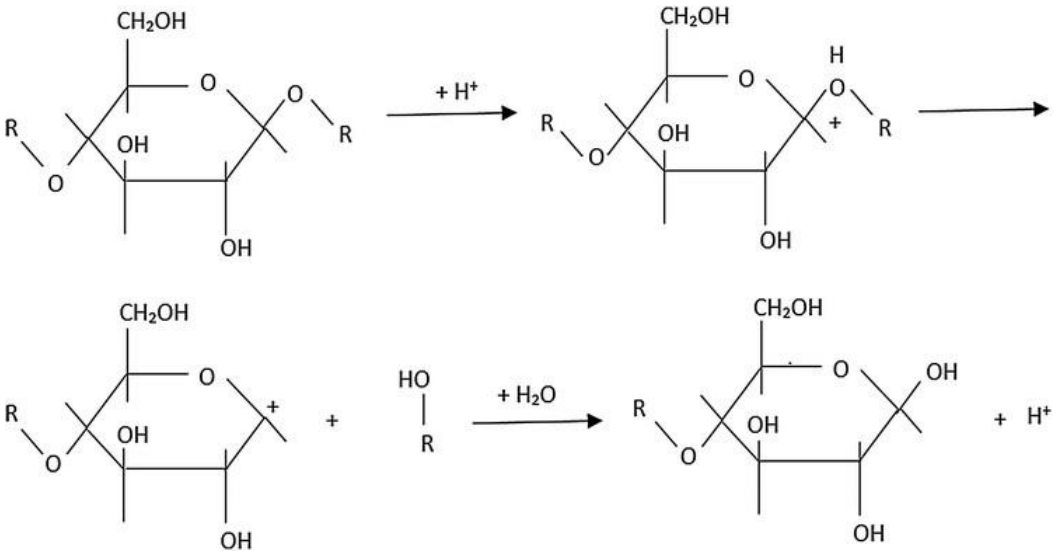


Figure 12: Conversion of lignocellulose biomass in to fermentable sugar by dilute acid hydrolysis (Loow et al., 2016)

Solid acid hydrolysis:

Homogenous acids are very effective to aid in catalyzing hydrolysis of lignocellulose. However, these processes come with limitations in terms of toxicity, possibility of corrosion and limitations with the separation and recovery of the acids. Currently, solid acids have acquired much interests for the hydrolysis of lignocellulose due to some promising characteristics such as activity, high selectivity, extended lifespan of catalysts, environmentally favourable properties, and ease in recovery and reuse (F. Guo et al., 2012; Y. Zhang et al., 2020). The solid acids used in hydrolysis usually are metal oxides, polymer based solid acids, sulfonated carbonaceous solid acids (CSAs) with strong acidity and high specific surface area. Also, it should have tolerance to water and should have access to the active sites in the materials. However, the efficiency is comparatively lower for cellulose hydrolysis. This is due to the water insoluble nature of cellulose, and due to the limitation exists in case of contact between the substrate with the catalyst. To improve the catalytic performance, solid acids are mostly prepared to increase the porosity with high specific surface areas. Moreover, the ease involved in adjusting the ratio of Lewis and Brønsted acids sites added to the advantage of using metal oxide solid acids. The increase in the acid site density and the available surface area of the transition-metal oxide benefits in the conversion of cellulose (Zhou et al., 2021). Figure 13 is a brief schematic representation of the solid acid catalysts used for cellulose to glucose conversion.

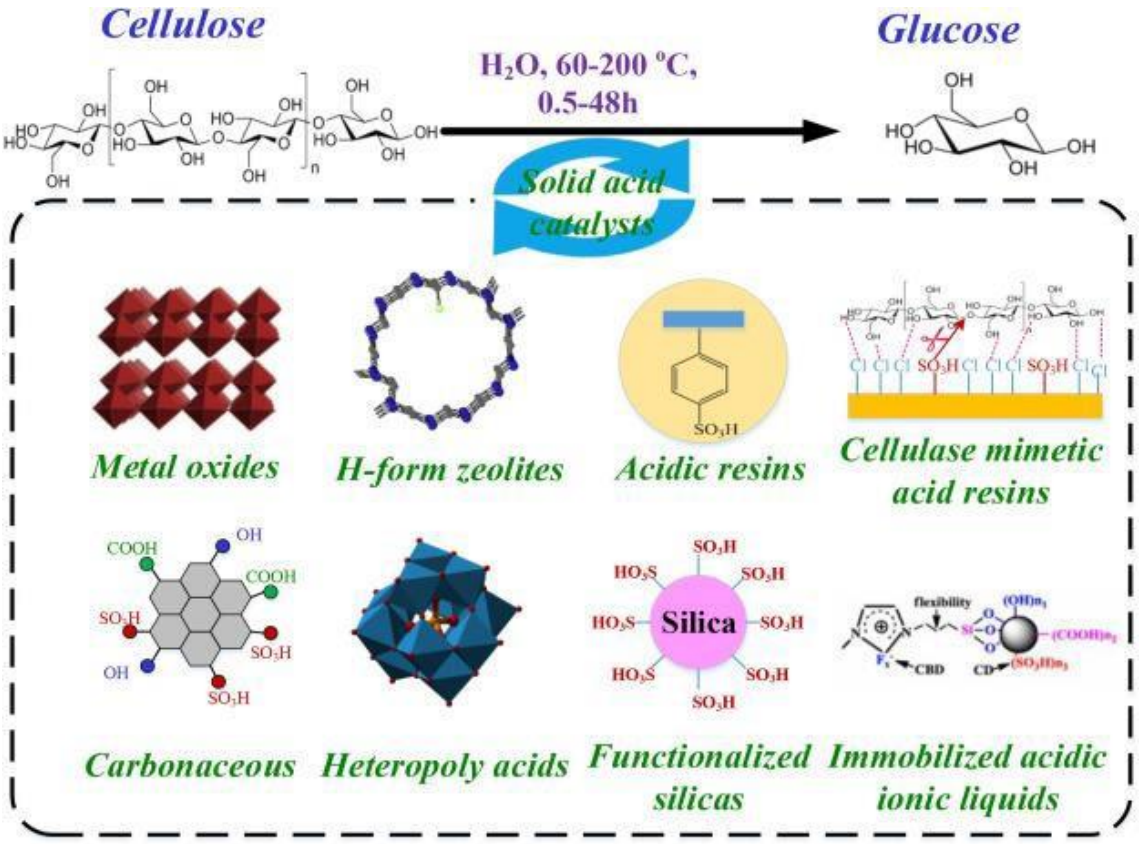


Figure 13: Solid acids used for hydrolysis (Zhou et al., 2021)

2.2.2 Enzymatic hydrolysis

Production of fermentable sugar for ethanol production can be done by employing different enzymes or a multi enzyme complex. The efficacy of pretreatment and the enzymes used are important factor for digestibility of the substrate and the end product formed. There are a range of strategies, and methods available to choose suitable biomass and enzyme loading concentrations. To perform hydrolysis of cellulose, cellulotytic enzymes are required separately or in synergy with other enzymes such as endo-β-1, 4-glucanase (EG), cellobiohydrolase (CBH), and β-glucosidase. β-(1-4) bonds of cellulose got hydrolysed by Endo-cellulase / Endo-glucanase (EG), whereas Exo-cellulase / exoglucanase (EXG) forms cellobiose by acting upon linear bonds. The cellobiose

finally got hydrolysed by cellobiase / β-D-glucosidase (CB) in order to produce single glucose units (X. F. Sun et al., 2005). Figure 14 represents the steps involved in enzymatic hydrolysis. This reaction proceeds through different stages such as transfer of the enzyme to the cellulose environment, reaction between the enzyme and substrate through adsorption, hydrolysis of cellulose, transportation of hydrolysed units from cellulosic particles to aqueous phase, and finally hydrolyzed into the aqueous phase to form glucose units (X. Li et al., 2022; Martín-Sampedro et al., 2012). The cellulases cleaved cellobiose to form glucose by in the presence of β-glucosidase. There is always a chance of product inhibition of the enzymes which may in turn decreases the efficiency of hydrolysis. However, enzymatic hydrolysis offers higher yields of monosaccharides than acid hydrolysis, as cellulase enzymes does not participate in sugar degradation reaction, rather acts as catalyst for hydrolysis reaction. Moreover, enzymes are naturally sourced and bio degradable which made it environment friendly. Pretreatment is needed prior to hydrolysis so as to make the structure accessible by enzymes.

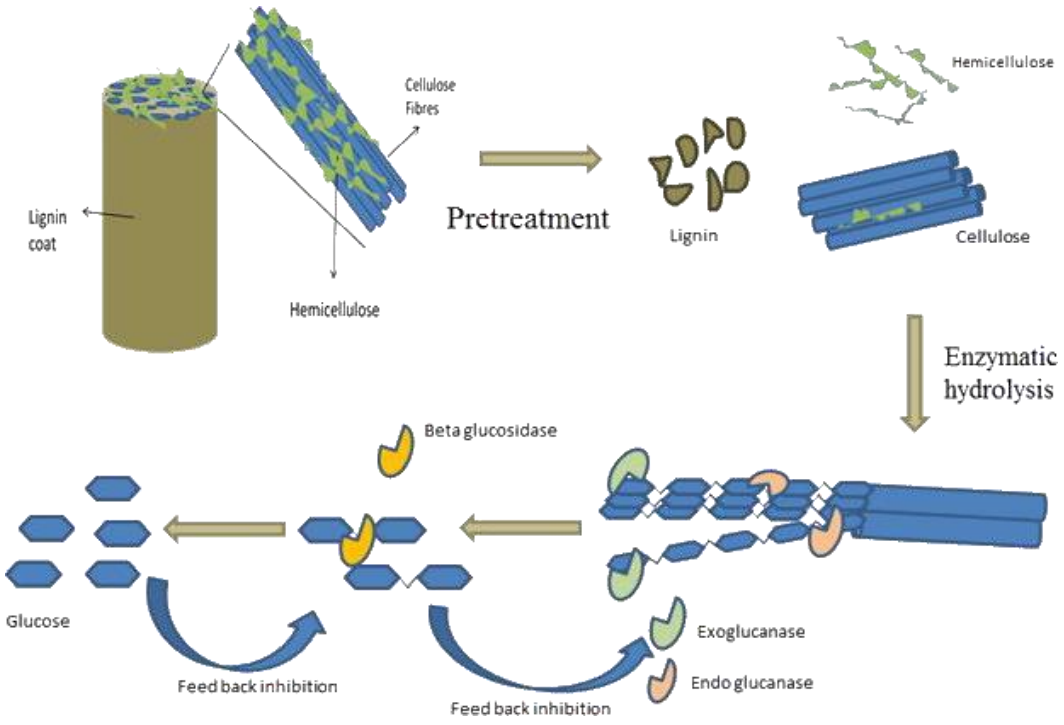


Figure 14: Enzymatic hydrolysis of lignocellulose (Source: Celignis Biomass Analysis Laboratory)

The ratio between substrate loading and enzyme loading as well as ratio between reaction volume are significant factor for product formation. Enzyme loading is also an important factor for product formation and it can alter the cost of production in a great extent. Therefore, enzye loading should be reduced and at the same time making enzyme more efficient by employing advanced biotechnology such as immobilization of cellulase. Lack of proper delignification can also leads to huge enzyme loading requirement (Teymouri et al., 2005b; Q. Z. Zhang & Cai, 2008). Table 7 demonstrates the effect of hydrolysis and fermentation of different biomass at different reaction conditions.

Table 7: Effect of hydrolysis and fermentation on different biomass

Raw material	Pretreatment	Enzyme used and Operating Condition	Microorganisms and Conditions	Findings	References
Eucalyptus globulus	Autohydrolysis (Hydrothermal process)	Cellulases, β-Glucosidase, 120 rpm,35®,96 h Ph=5	Saccharomyces cerevisiae,32 ° C,24h, Medium: glucose, peptone, malt extract, yeast extract	High ethanol conversion 92% and high volumetric conc: 68 g/l	(Romaní et al., 2012)
Eucalyptus grandis	Autohydrolysis pretreatment, Thermal treatments, kraft pulping	Citric acid-sodium citrate buffer (pH 4.85), cellulases Cellic Ctec2, sodium azide, 150 rpm, 48 °C, 96 h	(Pre-hydrolysis) pH 4.8, 48 °C,150 rpm, 24 h, *Saccharomyces cerevisiae*, SSF at 35 °C, 100 rpm, 48 h.	Recovery of 75% of xylan ,90% of acetyl groups. High recovery of lignin, greater than 90%.	(Guigou et al., 2019)
E. globulus	Steam explosion	cellulases (Cellulast 1.5 L) and β-glucosidase (Novozym 188), 50 °C, pH = 4.85, 150 rpm	*Saccharomyces cerevisiae*, SSF, 100 rpm, 35 °C, pH = 5	Ethanol yield of 91% of the theoretical yield at a concentration of 51 g/L	(Romaní et al., 2013)
Para rubber wood sawdust (PS)	Organosolv fractionation	The optimized conditions were at 160°C for 40 min with MIBK,ethanol, water in the ratio of 0.25:0.42:0.33 and 0.025 M of H_2SO_4,		Klason lignin (%) 91.7±1.3 87.6±1.5 Sugar (%): Glucose 0.92±0.2 0.2±0.1 Xylose 0.12±0.2 1.0±0.2 Arabinose 0.12±0.02 0.02±0.02 Ash (%) 0.86±0.1 0.4±0.3	(Inkrod et al., 2018)
Eucalyptus grandis sawdust	Steam explosion pretreatment with and without a previous NaOH impregnation	Cellic CTec 2, The solid loading and enzyme dosage of 15% (w/w) and 30 FPU/g glucan,	*Saccharomyces cerevisiae* at 30 °C with orbital agitation at 100 rpm for 24 h.	High hydrolysis efficiencies at high solids loading (27%). High ethanol concentrations of 75.6 g/L and yields of 259 L per ton of dry raw sawdust	(Rochón et al., 2022)
Eucalyptus	Choline chloride-formic acid (ChCl-FA) deep eutectic solvent pretreatment	Cellic, CTec2 At pH = 4.8, 10% eucalyptus loading, 50 °C, 72 h,	*Saccharomyces cerevisiae* 35 °C, pH = 5.5 (adjusted with 5 mol/L sodium hydroxide), and 180 rpm for 72 h	Maximum yield of glucose of 35.3 %. Ethanol yield of 16.5 g/L, which corresponded to 74.5% theoretical ethanol yield.	(X. Zhang et al., 2022)

A lot of research still needs to be done for the optimization of microbial and enzymatic detoxification of lignocellulosic hydrolysate. The enzymes are still a costly commodity due to their production techniques. However, as the technology advances and comparatively cheaper substrates are emerging as a result of extensive research, the cost is expected to be decreased in future. As a result, commercial development to produce enzyme commercially can be produced on a large scale (X. Li et al., 2022).The results obtained from enzymatic hydrolysis at different pretreatment and hydrolysis conditions. Keeping on mind the aim of present study the information is focussed on *Eucalyptus*. The table also includes results obtained after fermentation, the subsequent step to produce bioethanol.

In order to model the complexities involved in enzymatic hydrolysis of a lignocellulosic material, the key element is the attack of cellulose by enzymes to release glucose.Adsorption models have been developed in enzymatic hydrolysis, which can be related to the concentration of accessible substrate, cellulase enzyme, lignin, enzyme–substrate complexes, and enzyme–lignin complexes. Langmuir-type adsorption has been shown to represent this relationship in various studies (Baksi, Sarkar, et al., 2019; Teoh & Mat Don, 2011). A kinetic model incorporating dynamic adsorption, enzymatic hydrolysis and product inhibition was performed for enzymatic hydrolysis of pretreated fibers, wherein the dynamic adsorption was modeled by Langmuir-type isotherm while a first-order reaction was applied to hydrolysis process with consideration of the product inhibition. Li et al proposed an enzymatic hydrolysis using the Michaelis–Menten kinetic model (C. Li et al., 2004) with and without inhibition.

2.2.3 Fermentation:

A number of processes like, Separate hydrolysis and fermentation (SHF) process, and simultaneous saccharification and fermentation (SSF), Simultaneous saccharification and co-fermentation (SSCF), Consolidated bioprocessing (CBP) are available as fermentation technologies. Figure 15 demonstrates the types of fermentation and hydrolysis techniques available.

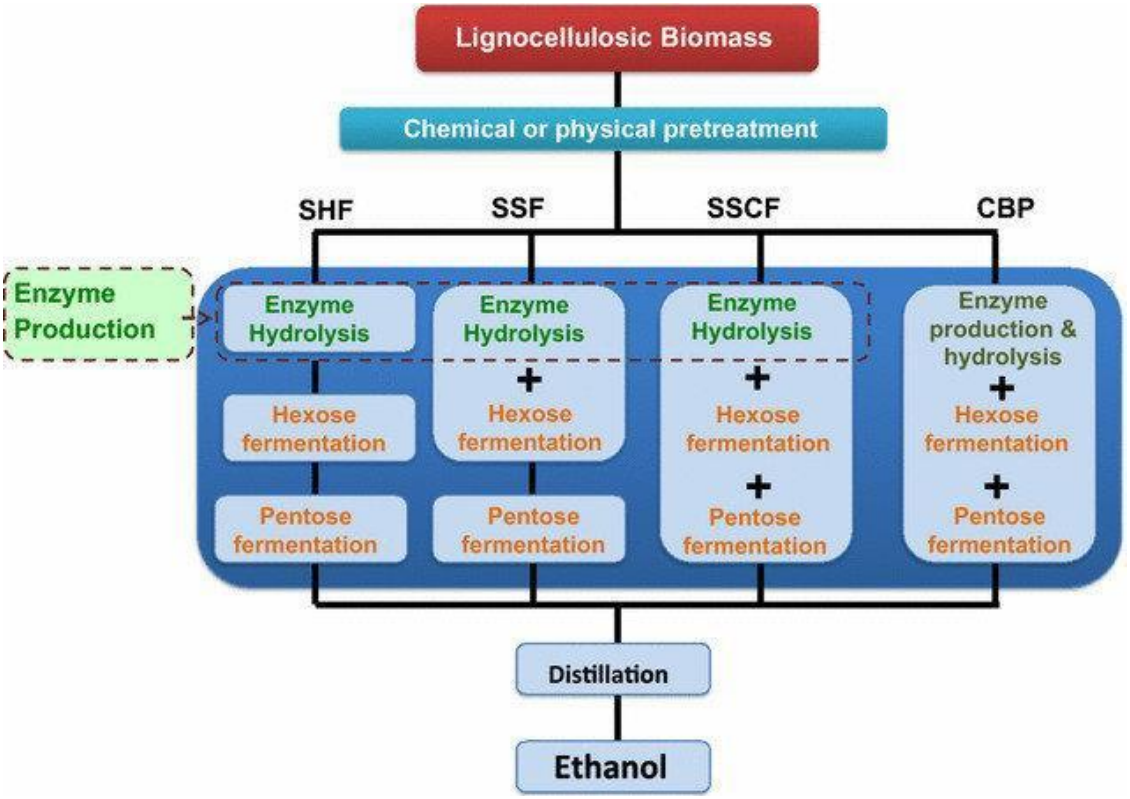


Figure 15: Schematic of different fermentation technologies (S. S. Ali et al., 2016)

Based on our present study, the focus will be on SHF and SSF process. Separate hydrolysis and fermentation (SHF) involves saccharification and fermentation in a separate step, whether, for simultaneous saccharification and fermentation (SSF) the simultaneous saccharification of biomass and fermentation happens at a single step.

During fermentation often formic acid and acetic acid forms as a byproduct which may in turn inhibit the process (Ballesteros et al., 2004; Wirawan et al., 2012).

The ethanol yield is dependent on microorganism used, their growth condition, production of hydrolysable sugar and inhibitors generated. The microorganisms generally used for the process are *S. cerevisiae, Z. mobilis* and recombinant *E.coli*. They used different pathways, such as *S. cerevisiae* uses the Embden-Meyerhof pathway (glycolysis), *Z. mobilis* uses the Entner Doudoroff pathway. The simultaneous saccharification and fermentation (SSF) or separate hydrolysis and fermentation (SHF) are two of the preferred techniques for fermentation (Amândio et al., 2023a; Chang, Kaar, et al., 2001; Delgenes et al., 1996; Jung et al., 2013; Rahnama et al., 2013; Sharma et al., 2007; Wirawan et al., 2012). There is a chance of generation of inhibitors such as formic acid, acetic acid, furfurals etc. has an inhibitory effects on fermentation as they tend to make the fermentation media acidic which affects the microorganisms.

In the present study, *Saccharomyces cerevisiae* was used to utilize the sugar hydrolysate obtained after the enzymatic saccharification of pre-treated *Sterculia foetida* and *Eucalyptus grandis* for the production of biofuel.

CHAPTER 3

PRETREATMENT

CHAPTER 3

PRETREATMENT

Ethanol production from lignocellulosic route consists of four main steps, namely, pretreatment, acidic/enzymatic hydrolysis, fermentation and separation of product. Pretreatment plays a key role in the process of turning waste into wealth. Pretreatment is the process to reduce recalcitrant nature of lignocellulosic materials by delignification, hemicellulose preservation, reduction in cellulose crystallinity, increase in biomass porosity leading easy accessibility of cellulose and hemicellulose for subsequent enzymatic hydrolysis and fermentation for biofuel production. The pretreatment process is effective in improving the overall efficiency biofuel production but could be expensive. There is always a trade-off between efficiency and cost of the process. This chapter will focus on pretreatment technologies explored and applied during the course of this study on various lignocellulosic materials, their efficacy and mechanism to valorize lignocellulosic materials.

There is no universal pretreatment technology available due to variations in composition between different lignocellulosic materials. In this particular study alkaline, alkaline peroxide pretreatments are mainly applied to various lignocellulosic materials such as fruit shells of *Sterculia foetida*, bark of *Eucalyptus grandis* and mixed garden waste which contains leaves and twigs of plants. Alkaline pretreatment in general uses sodium hydroxide and calcium hydroxide and requires lower temperatures and pressures compared to other pretreatment technologies due to its advantage of carrying out the experiment at ambient conditions. Morphological data showed an increase in external surface area and

porosity for better exposure to the microorganism or enzyme (Chang, Nagwani, et al., 2001b; Q. Z. Zhang & Cai, 2008). Sodium hydroxide in combination with dilute acid or hydrogen peroxide was observed to increase delignification (Kaar & Holtzapple, 2000; R. Sun et al., 1995b) and required reaction time is less for sodium hydroxide compared to lime. Alkaline peroxide pretreatment can be categorized into the thermo-chemical methods and is an effective technique for pretreatment of lignocellulosic biomass. Hydrogen peroxide is supposed to solubilize about half of lignin and most of the hemicellulose. Alkaline pretreatment in combination with peroxide on the other hand more efficiently solubilizes lignin and improves digestibility than alkali treatments (C. E. Wyman et al., 2005). Through oxidative lime pretreatment about 80% of the lignin can be removed from lignocellulose material with high-lignin content such as hardwoods (Curreli et al., 1997). A two stage treatment of alkaline and alkaline peroxide treatment has been recommended to perform hemicellulose solubilisation in the first stage pursued by lignin removal (Banerjee et al., 2012).

DES synthesized of ChCl/urea was reported by Abbott et al. at the year 2003 (AybenKılıç-pekgözlü, 2019) and also extensive research on ChCl-derived DES was performed. The freezing point of ChCl/urea at a molar ratio of 1:2 is 12˚C. ChCl/urea has also been used as catalysis (Z. Zhang et al., 2012).

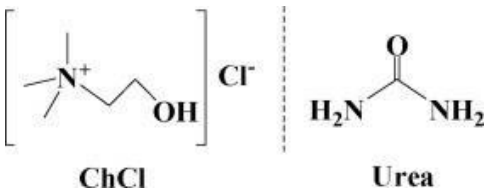


Figure 16: Structure of ChCl and urea

However, there are few reports on delignification of Eucalyptus grandis using DES of ChCl/urea. To better evaluate the transformation mechanism during ChCl/urea pretreatment on Eucalyptus the chemical fractions acid insoluble lignin (AIL) as well as acid soluble lignin were isolated. The structural and physicochemical properties present in *Eucalyptus grandis* and the chemical fractions observed during ChCl/urea pretreatment were comprehensively determined with the help of scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FTIR) and Thermo gravimetric analysis (TGA) respectively.

To produce second generation biofuel agricultural waste, softwood (pine, cedar etc.), hardwood (eucalyptus, acacia/sonajhuri etc.) and industrial waste can be used as raw material. The choice of lignocellulosic raw material to be utilised in the biofuel industry is largely dependent on the substantial availability of the biomass in the specific country (Y. Sun & Cheng, 2002).Countries with agriculture based economy usually prefers agricultural residue as source material for ethanol production. Some of the widely used lignocellulosic biomass for biofuel production includes but not limited to straw of rice and wheat, corn stover, sugarcane bagasses, sawdust and bark of pine, and eucalyptus. High cellulose content and low lignin content aids in production of high concentration of reducing sugars as lower lignin content facilitates cellulose to be accessible for high cellulose and in turn produces high quantities of hexose and pentose sugars.

Sterculia foetida is a tropical dioecious and deciduous tree of *Malvaceae* family growing in tropical regions including East Asia, Australia, some parts of Africa (Tanzania, Somalia, and Ghana) and United States, mainly Hawaii. From timber, leaves, the fibre extracted from the bark to seeds as well as the oil extracted from the seed has various

usages ranging from medicinal (bark, leaves, seeds, the oil extracted from seeds), edible (seeds, leaves, rootstocks) to interior and other stationary works (timber, fibre obtained from bark, gum extracted from trunk and branch). The large green fruits in general ripen and turn red in colour 11 months after the flowering and the shell of the fruit eventually becomes fibrous. Upon collection of the seeds the shell is discarded as it is of no significant use (Jafri et al., 2019).

The first substrate used in this study was the waste shells of the ripened fruits of *Stercutia foetida* which is an unexplored substrate with no economic value and a might be a good source of second-generation biofuel. As of the present time, there exist very few feasible methods for efficient pretreatment of woody materials to produce biofuels. In this work, an attempt has been made to develop an optimal alkaline peroxide pretreatment condition for the effective conversion of lignocellulosic biomass (*Stercutia foetida* fruit shells). In addition, comparative study has been made between the performance of traditional alkaline peroxide pretreatment and liquid hot water assisted alkaline peroxide pretreatment in an autoclave. The mechanism by which this pretreatment method affects the rate and extent to which cellulose present in the substrate undergoes enzymatic saccharification has also been investigated as well as the effect that the pretreatment conditions had on the extracted lignin structure was studied. The mechanism by which this pretreatment method affects the rate and extent to which cellulose present in the substrate undergoes enzymatic saccharification has also been investigated as well as the effect that the pretreatment conditions had on the extracted lignin structure was studied.

The second substrate used in this study was dried bark of *Eucalyptus grandis* which is an unexplored substrate and could be a potent source of second-generation biofuel.

Currently, research is going on to formulate viable techniques to pretreat woody materials to generate biofuel (Ge et al., 2020; Z. Wang et al., 2018). In this study, an optimal condition was developed to effectively convert lignocellulosic biomass (dried bark of *Eucalyptus grandis*). A comparative study has also been made between the performance of traditional alkaline peroxide pretreatment and autoclave assisted alkaline peroxide pretreatment to enhance reducing sugar production.

Third substrate used in this study was mixed garden waste consists of a mix of dried leaves and twigs (80% dried leaves and 20% twigs of plants). The garden waste and/or forest residue in general used as a firewood for domestic purposes and also known as invisible polluter. Also contrary to popular belief, biomass burning contributes to air pollution more than crop burning as the temperature drops in India.

3.1 Material & methods

3.1.1 Biomass

Three types of biomasses were examined in the whole study based on percentage of lignin content. First biomass was ripened and dried fruit shells of *Sterculia foetida*. The outer shells of the fruits of *Sterculia foetida* were collected. The shells were kept in a clean, dry place until they were used. The shells were dried in a hot air oven at $80^{\circ}C$ until a constant weight was achieved. The fibres from within the shells were separated manually and were cut into smaller pieces of length 0.5 cm to 1cm using a locally assembled hand driven cutting mill so as to increase the available surface area for the pretreatment method. Further reduction in size may lead to loss in yield during washing, filtration and subsequent processes.

The second biomass was dried outer skin of *Eucalyptus grandis* bark. Dried bark of *Eucalyptus grandis* was used for all the experiment. The barks were collected locally and washed thoroughly before drying at 80°C in a hot air oven for overnight and stored in a clean, dry container for further use. Size of the barks was reduced to 50 meshes by Willey mill to increase the surface area. For DES (ChCl/Urea) pretreatment *Eucalyptus grandis* bark were cleaned, dried at 105˚C until constant weight and ground to particle sizes of 60 mesh in a Willey mill. The separated lignin was also dried at 105˚C until constant weight.

Garden waste (80% dried leaves and 20% twigs of plants) was acquired from university garden. The wastes were collected and washed thoroughly and air dried at 80°C inside a hot air oven till constant weight is attained. Dried leaves and twigs are then ground into 10 mesh in Willey mill to increase the surface area and to handle at ease.

3.1.2 Chemicals

The chemicals used for the experiment were of analytical grade which includes Sodium hydroxide (NaOH) (ACS grade reagent, Merck), hydrogen peroxide (H_2O_2) (ACS grade reagent, Merck), cellulase (Plant culture tested, Himedia, 10U/mg), β-glucosidase (G0395, 2U/mg, derived from almonds, Sigma Aldrich), citrate buffer (Sigma Aldrich) sodium azide (Merck), and 3, 5-dinitrosalicylic acid (DNS), potassium sodium tartrate, sodium citrate and sodium hydroxide, choline chloride, urea, ethanol were procured from Sigma-Aldrich. Other chemicals such as sulphuric acid (98%, AR grade) was obtained from Merck. Whatman filter paper no. 1 was used in assays and standard filtration practices. All the chemicals used were of analytical grade and was used without further purification.

3.2 Pretreatment Protocols

3.2.1 Alkaline pretreatment

Sodium hydroxide (NaOH) was used as the alkali agent for this pretreatment. Weighed amounts of ground waste was taken in a 500 ml Erlenmeyer flask and slurried in (w/v) of sodium hydroxide (NaOH) (ACS grade reagent, Merck). Solution of different concentrations in a solid to liquid ratio of 1:8. Finally, these flasks were maintained at a temperature of 60°C and/or 80ºC for 3 hours and stirred by means of a magnetic stirrer (REMI 2MLH, average speed~500 rpm). The solid residue was collected by filtration using Whatmann Filter paper (Grade 1, retention 2.5 µm), washed thoroughly with distilled water until pH of the filtrate was found to be neutral and the collected biomass was air dried in an oven.

3.2.2 Alkaline peroxide treatment

Weighed amounts of the fibre was taken in a 500 ml Erlenmeyer flask and slurried in 5% (w/v) of sodium hydroxide (NaOH) (ACS grade reagent, Merck) solution as well as hydrogen peroxide (H_2O_2) (ACS grade reagent, Merck) solutions of different concentrations in a solid to solvent ratio of 1:8. Finally, these flasks were maintained at a temperature of 60°C and/or 80°C for 3 hours using a magnetic stirrer (REMI 2MLH), the average speed of which was maintained~500 rpm. The solid residue was collected by filtration using Whatmann Filter paper having (Grade 1, retention 2.5 µm), washed thoroughly with distilled water until pH of the filtrate was found to be neutral followed by drying of the collected biomass in a hot air oven overnight. For H_2O_2 treatment, same

procedure as described above was followed except that weighed amounts of fibres were dispersed in 100 ml 2% hydrogen peroxide solution with varying NaOH concentration to investigate the efficacy of sodium hydroxide concentration on the pretreatment efficiency. Control experiments were carried out in absence of hydrogen peroxide where the biomass was subjected to the alkaline pretreatment. The combination of treatment is depicted in table 8.

Table 8: Pretreatment combination for both alkaline and alkaline peroxide treatment for *E. Grandis* ,*S. foetida* and garden waste

Substrate	**Pre-treatment 1**	**Pre-treatment 2**	**Pre-treatment 3**	**Concentration of NaOH (%)**	**Concentration of H_2O_2 (%)**
E. Grandis	Alkaline Peroxide treatment at 60°C	Alkaline Peroxide treatment at 80°C	Autoclave assisted Alkaline Peroxide treatment at 121°C in an autoclave	2	**0**
					1
					2
				5	0
					1
					2
				8	0
					1
					2
S. foetida		N. A		5	0
					1
					2
					3
					5
					11
				1	2
				2	
				3	
				1	0

				2	
				3	
Garden waste				1	0
				3	
				5	
				1	5
				3	
				5	

3.2.3 Autoclave assisted pretreatment

In this method of pretreatment, Erlenmeyer flasks containing the slurry were cotton plugged and placed inside a Secor India made laboratory scale Autoclave and was pressurized up to 15 lb/in^2. The time for pretreatment was kept at 15 minutes from the instant the pressure inside the autoclave reached the value of 15 lb/in^2 for the first time. The temperature inside the autoclave was maintained at 121$^{\circ}C$. Since this is a kind of "Steam Explosion" type of pretreatment hence cotton plugging the flasks is of utmost importance to prevent loss of substrate via overflow. The steam generated inside the autoclave was from distilled water. Figure 17 shows a schematic and visual representation of pre-treatments steps starting from raw material to the end product after pretreatment.

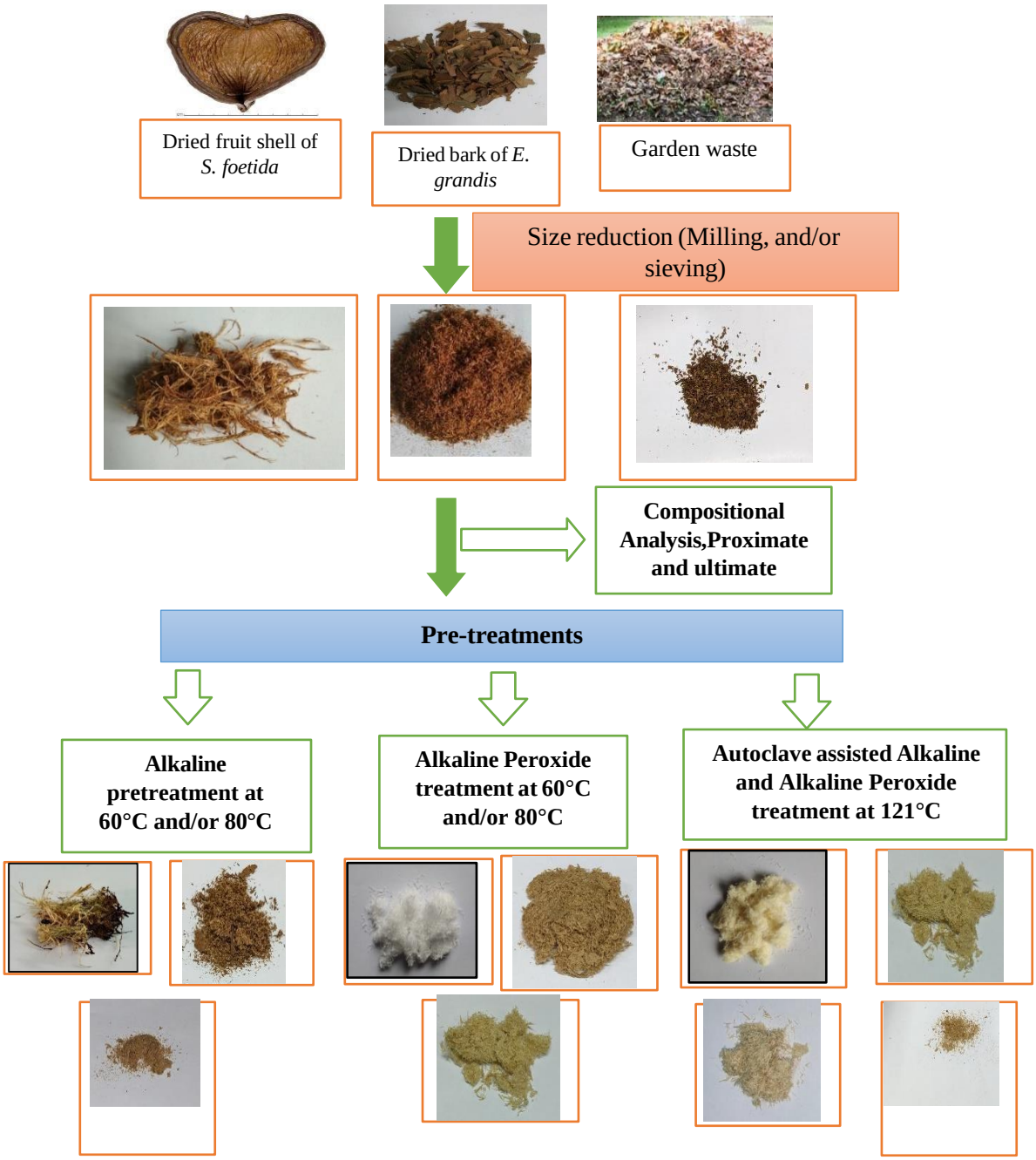


Figure 17: Schematic and visual representation of pre-treatments steps starting from raw material to the end product after pretreatment

3.2.4 Deep eutectic solvent pretreatment:

Preparation of DES:

DES of ChCl/urea mixtures was synthesized by following procedures previously reported (Lian et al., 2015). Before preparation of the solution, ChCl and urea was dried at 60 °C until constant weight is achieved. The eutectic mixtures was produced by mixing freshly dried ChCl and urea at 1:2 molar ratio at 80°C placing it in an oil bath with a magnetic stirrer arrangement at 500 rpm until a clear homogeneous, colorless, odorless and viscous solution was obtained.

Pretreatment:

About 10 g of the oven-dried *Eucalyptus grandis* was added in a solvent to solid ratio of 16:1and were put into 500 mL Erlenmeyer flasks. It was further stirred at 80˚C for 4h, 6h, and 8 h, respectively using an oil bath. Afterward, the reaction mixture was washed with an anti-solvent (water, ethanol) to remove the DES completely, and the residues were separated by vacuum filtration, and then dried at 105˚C to a constant weight. Solubilization rate (SR) of ChCl/urea on samples is calculated according to following equation:

$$SR(\%) = \frac{(m_0 - m_1)}{m_0} * 100$$

where, m_0 is the oven-dried weight of samples, and m_1 is the oven-dried weight of the sample residues after pretreatment.

Recovery of DES:

The pretreated biomass was washed and DES was recovered using deionised water or ethanol as an antisolvent. The anti-solvent was added to the lignocellulsic materials and

DES mixture was separated via centrifugation. The process was repeated until the second phase was no longer observed. Figure 18 represents the schematic of DES pretreatment and recovery steps.

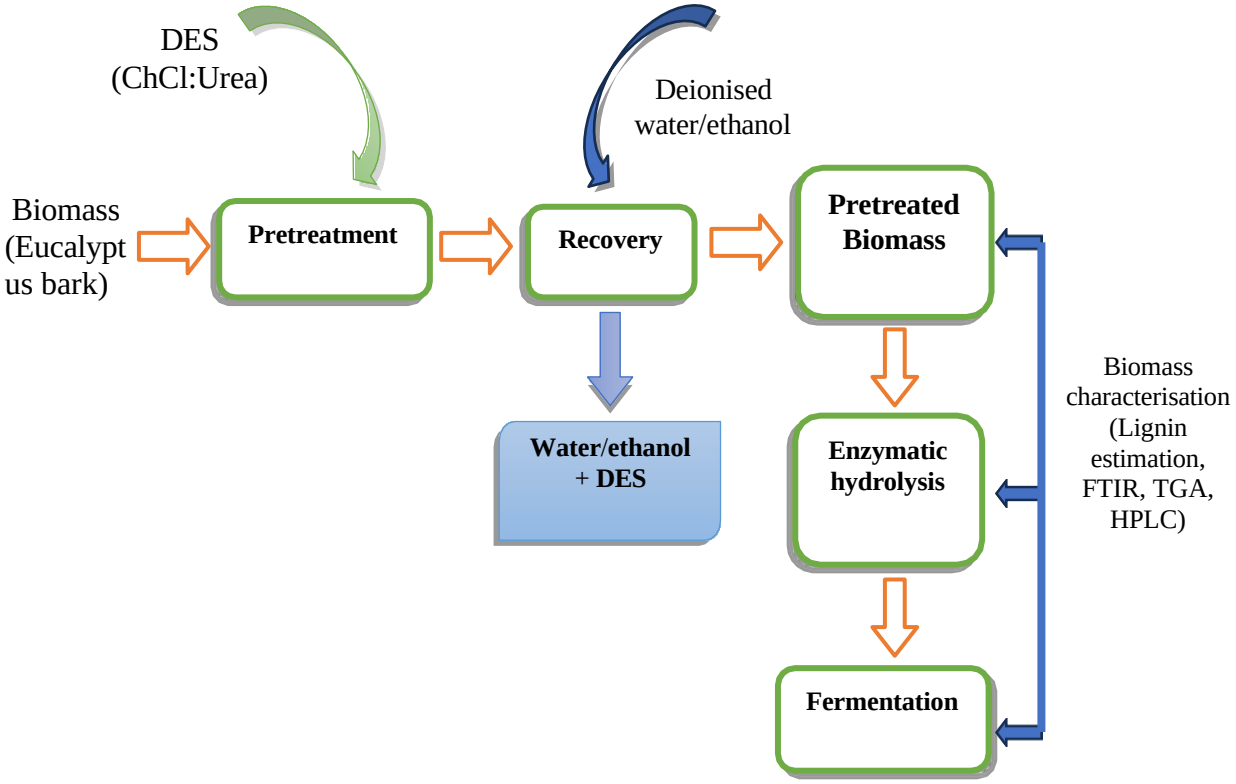


Figure 18: Schematic of DES preteatment on Eucalyptus grandis.

3.3 Measurements and analysis

3.3.1 Determination of composition of biomass

The composition of lignocellulosic materials was determined using standard National Renewable Energy Laboratory (NREL) protocols to govern the moisture content, ash content and fixed carbon and volatile matter. All the experiments were carried out in triplicates and standard deviation was measured and reported accordingly along with the

mean value. Proximate analysis i.e., moisture content, volatile matter and ash content of the biomass was evaluated using standard method (ASTM, 1989; Pazó et al., 2010; Sluiter et al., 2005, 2008) while ultimate analysis for C, H, N was performed in a Perkin-Elmer 2400 Series-II CHN elemental analyser using helium as carrier gas.

To determine moisture content, the glass petri dishes pre-dried at 105 ± 3 °C for minimum 4h was weighed to the nearest 0.1 mg after cooling in a desiccator for avoiding moisture absorption. The samples were mixed thoroughly and 1 g of sample was weighed in the pre-weighed petri dish. It was further heated to 105 ± 3 °C for a minimum of 4h followed by cooling in a desiccator and then weighed to the nearest 0.1 mg. The samples were placed back into the convection oven for an hour and weight was determined again until constant weight is achieved. Constant weight is defined as ±0.1% change in the weight percentage of solids upon one hour of re-heating the sample. The moisture content is the difference in weight divided by the initial weight of sample.

For ash content, the crucibles were weighed to the nearest 0.1 mg by placing it in the muffle furnace at 575 ±25 °C for a minimum of 4h followed by transfer the same directly into a desiccator to avoid absorption of moisture. 1 g of the dried sample was weighed in the crucible and was placed into the muffle furnace at 575 ± 25 °C for complete combustion till the constant weight is achieved. Constant weight is defined as less than ± 0.3 mg change in the weight upon 1h of re-heating the crucible. The ash content of the sample was obtained using the following equation.

$$Ash\ (\%) = \frac{(Weight\ of\ crucible\ with\ ash) - (Weight\ of\ empty\ crucible)}{Weight\ of\ sample} \times 100$$

For volatile matter analysis the 1g of powdered sample was placed in a pre-dried silica crucible with lid in a muffle furnace at 925˚C for 7minutes. The crucible is removed from the furnace and cooled before weighing. The volatile matter can be calculated by determining weight loss during the process divided by the initial weight of the sample and subtracting the moisture content.

$$Volatile\ Matter\ (\%) = \frac{(Weight\ of\ crucible\ with\ residue\ after\ 7min) - (Weight\ of\ crucible\ with\ sample\ intitially)}{Weight\ of\ sample} \times 100 - \%\ Moisture$$

The fixed carbon can be calculated by subtracting moisture content, volatile matter, and ash from 100.

3.3.2 Analytical methods

X-Ray Diffraction (XRD) and crystallinity analysis:

Ultima III X-Ray Diffractometer of Rigaku make was used to quantify the crystallinity of the samples. The scan was set for a rate of 2° per minute with a range of 10° to 70°. The changes in the crystalline nature of the cellulosic structure of the pretreated and untreated biomass were observed in this study. Crystallinity indices were evaluated based on Segal equation from the intensities of the amorphous and crystalline regions (Segal et al., 1959) as follows:

$$CI = \frac{I_{002} - I_{am}}{I_{002}} * 100 \quad (1)$$

Where, I_{002} is the maximum intensity (in arbitrary units) of the 002-lattice diffraction (at $2\theta=22.5°$) observed for the crystalline area and I_{am} is the intensity (at $2\theta=18°$) observed for amorphous structure in same units.

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy:

ATR-FTIR spectroscopy was performed on samples before and after pretreatment for in order to monitor the structural changes that took place due to pretreatment. The organic groups available in the lignocellulosic biomass were detected from FTIR analyses. The FTIR spectra revealed the structural changes occurred during various pretreatment techniques with regard to hemicellulose and lignin content. For that a Simadzu made IRPrestige-21 Fourier Transform Infrared spectrophotometer was used in the range of 400 to 4500 cm^{-1}. The spectra in transmittance mode were used to determine lateral order index (LOI) and total crystallinity index of the (TCI). The LOI and TCI were calculated using the ratio of absorbance at 1430 cm^{-1} to 897 cm^{-1} i.e. (1430cm^{-1}/897 cm^{-1}); and 1375 cm^{-1} and 2900 cm^{-1} i.e. (1375 cm^{-1}/2900 cm^{-1}) respectively.

The lignin content of both raw and pretreated samples was measured based on standard TAPPI Method (T 222 om-02) NREL,(Tappi, 2002) for softwoods. The lignin removal efficacy of a certain pretreatment technique was defined as follows:

$$Lignin\ \mathrm{Re}\ moval(\%) = \frac{InitialLigninContent - FinalLigninContent}{InitialLigninContent} \times 100 \quad (2)$$

3, 5-dinitrosalicylic acid (DNS) assay:

The extent of reducing sugars present in the sample before and after pretreatment were estimated using standard 3, 5 Dinitro salicylic acid (DNS) assay method(Miller, 1959). The reaction was carried out by adding 2.5 mL of DNS to 0.5 mL of supernatant placed in vigorously boiling water bath for 10 min. According to the method, the sample to be evaluated, was added to 3 ml of DNS reagent, and then boiled in a water bath for 10 min. Resulting mixture was then left to cool and the reducing sugar concentration was estimated from the absorbance of the solution at 540 nm by a UV spectrophotometer.

Brunauer-Emmett-Teller (BET) Porosity Measurement:

The total pore volume, average pore diameter and pore size distribution can be calculated using Brunauer-Emmett-Teller equation (BET). The pore volume can be defined as the volume of liquid nitrogen adsorbed at a relative pressure of P/P^0 = 0.99 and the average pore diameter, D_p, was calculated using the relation 4 V_T/S_{BET}, and the pore size distribution, by the BJH method (Basta et al., 2009).

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis was done in a Perkin-Elmer made analyzer for a temperature range of 30-700˚C, in a nitrogen atmosphere.

3.4 Results and discussion

In this section the results obtained during pretreatment of *S. foetida, E. grandis* and garden waste are presented. An optimization study of various process parameters (temperature, time, concentration of salt and peroxide) of pretreatment was carried out to understand the effect of these parameters leading to mechanism of pretreatment. Since

three different lignocellulosic materials was used during this study, the results have been reported based on each lignocellulosic material for better understanding of effect of different process variables on different lignocellulosic materials.

3.4.1 Proximate and Ultimate Analysis Results:

Table 9 denotes proximate and ultimate analysis results of various lignocellulosic materials that have been valorized during the course of this study for bioethanol production. The proximate analysis results revealed highest fixed carbon content for E. *grandis* whereas lowest in garden waste as expected among the three lignocellulosic materials considered during present study. In addition, highest ash content was also observed for garden waste due to presence of various leaf components in it. Volatile matter was highest in *Sterculia foetida* fruit shells as compared to other two materials. The ultimate analysis results support the proximate analysis results as highest carbon content was observed for *E. grandis* whereas highest oxygen content was quantified for Garden waste. Proximate and ultimate values are given in table 9.

Table 9: Proximate and ultimate analysis of various lignocellulosic materials

		Content (wt %)		
	Component	***E. grandis***	***S. foetida***	**Garden waste**
Ultimate Analysis	Carbon	43.90	42.91	38.51
	Hydrogen	5.40	6.07	3.16
	Nitrogen	0.99	0.36	0.31
	Oxygen	49.71	50.66	58.02
Proximate Analysis	Fixed carbon	37.90	30.63	28.15
	Moisture Content	12.6	12.3	9.53
	Volatile Matter	51.2	54.7	49.45
	Ash Content	3.4	2.37	12.87

3.4.2 Pretreatment Results of *Sterculia foetida:*

3.4.2.1 FTIR analysis of Untreated and Pretreated Biomass

The structural formation of untreated *S. foetida* sample was compared with that of pretreated sample under different process conditions to understand the structural modifications occurred during pretreatment using FTIR analysis and presented in Figure 19-26 for *S. foetida*.

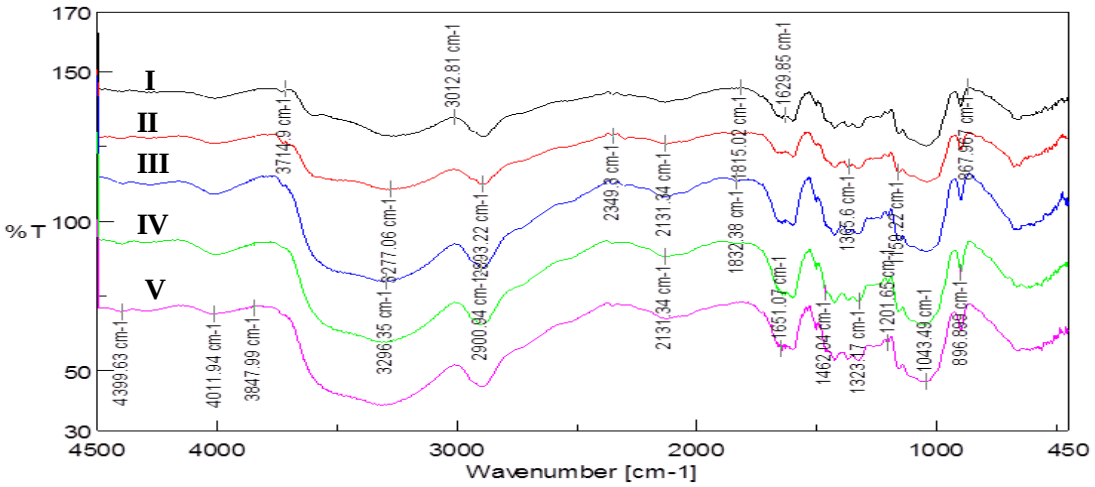


Figure 19: (FTIR spectra of autoclave assisted pretreatment using different concentrations of NaOH): Raw Sample (I), 1% NaOH (II), 2% NaOH (III), 3% NaOH (IV), 5% NaOH (V)

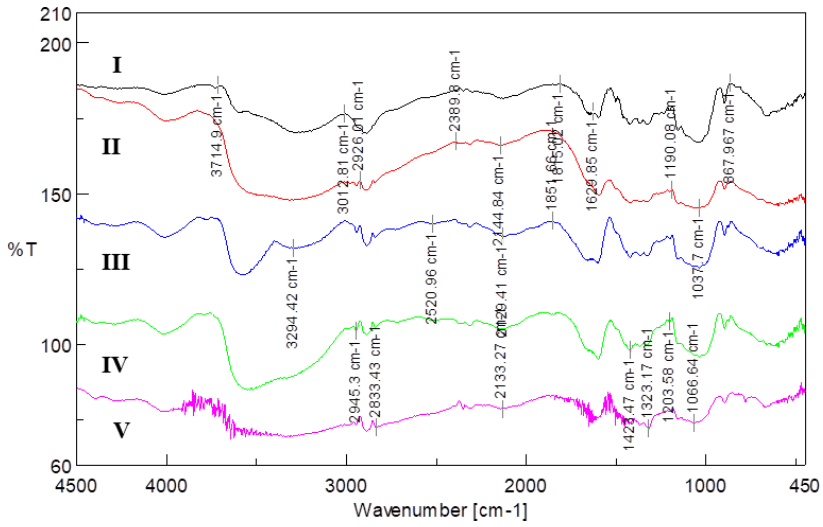


Figure 20: (FTIR spectra of different concentration of NaOH treated by low temperature heating): Raw Sample (I), 1% NaOH (II), 2% NaOH (III), 3% NaOH (IV), 5% NaOH (V)

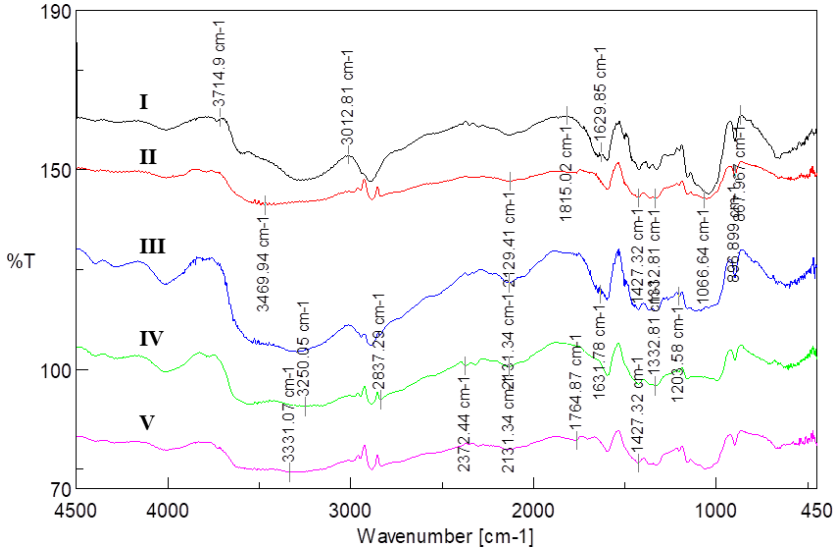


Figure 21: (FTIR spectra of different concentration of NaOH treated in autoclave at constant H_2O_2 (2%) concentration) raw material (I) -1% NaOH (II)-2%NaOH (III)-3%NaOH (IV)-5%NaOH (V)

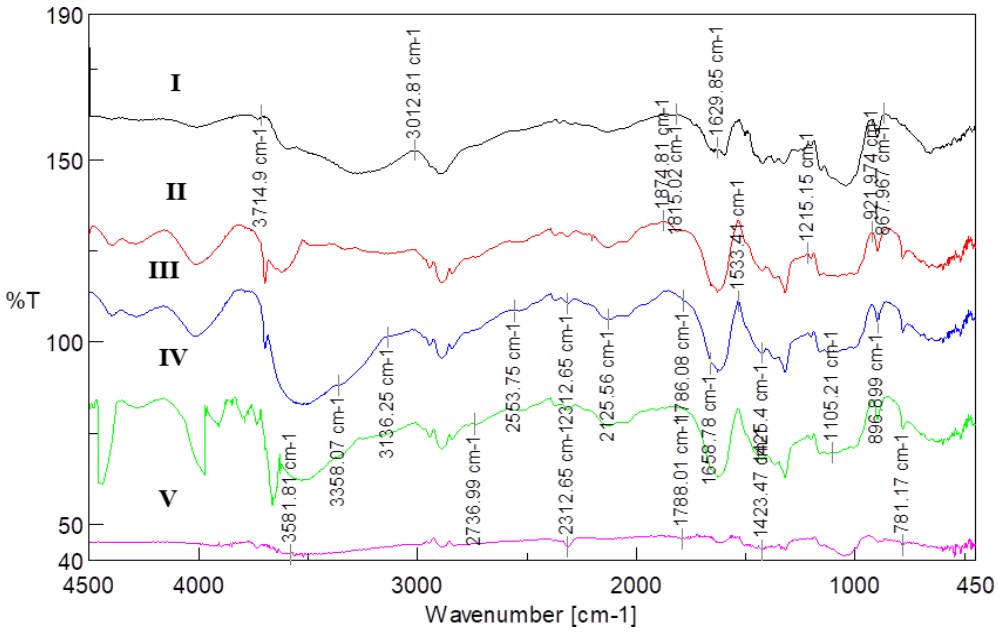


Figure 22: (FTIR spectra of different concentration of NaOH treated in low temperature heating (60°C) at constant H_2O_2 (2%) concentration) Raw Material (I)-1% NaOH (II)-2%NaOH (III)-3%NaOH (IV)-5%NaOH (V)

The untreated biomass sample exhibited a peak at ~1700 cm^{-1} which emanated due to the presence of abundant acid groups in the raw material (Mishima et al., 2006). The band found in the region of 3300-3500 cm^{-1} was attributed to the hydroxyl bond (O-H) stretching. In presence of autoclave assisted alkaline pretreatment the O-H band stretching was found to be more pronounced with the increment in NaOH concentration (Fig 21). However, the extent of O-H band stretching was comparatively less in case of low temperature alkaline pretreated samples (Fig. 22) The appearance of double bands in the region of 2950 cm^{-1} was due to the asymmetric stretching and symmetric vibrations of methylene (-CH_2, -CH_3) groups which are the characteristic peaks of cellulose (Sukarni et al., 2014; Xu et al., 2006). The peak at 2850 cm^{-1} was assigned to the methoxy (O-CH_3) bonds (Y. Chen et al., 2013). The hydrolysis of hemicellulose during pretreatment resulted

in the disappearance of the 1725 cm^{-1} peak which was present in the untreated raw material. It was found upon pretreatment the peaks ascribed to the carbonyl (C=O) groups at ~1600 cm^{-1} became more prominent since during alkaline peroxide pretreatment the carboxylic acid groups got reduced to the carbonyl groups (Fig 23-26).

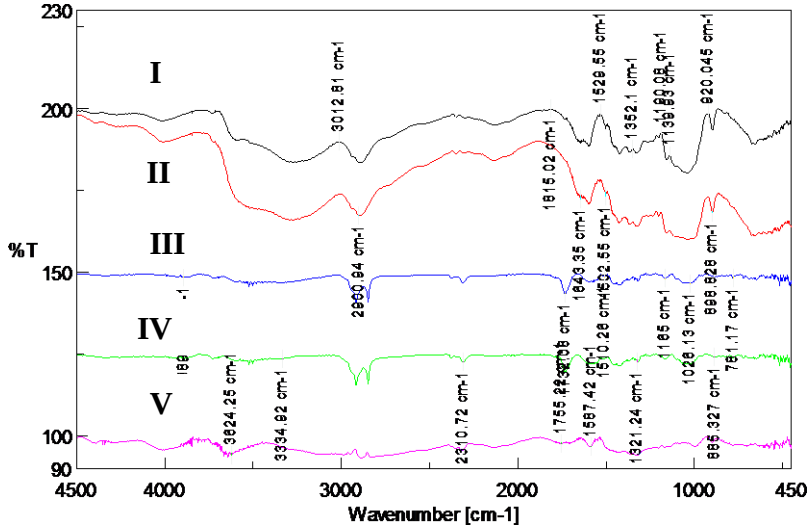


Figure 23: (FTIR spectra of different concentration of NaOH treated in low temperature heating at constant H_2O_2 (11%) concentration) raw material (I)-1%NaOH (II) -2%NaOH (III) -3%NaOH (IV)-5%NaOH (V)

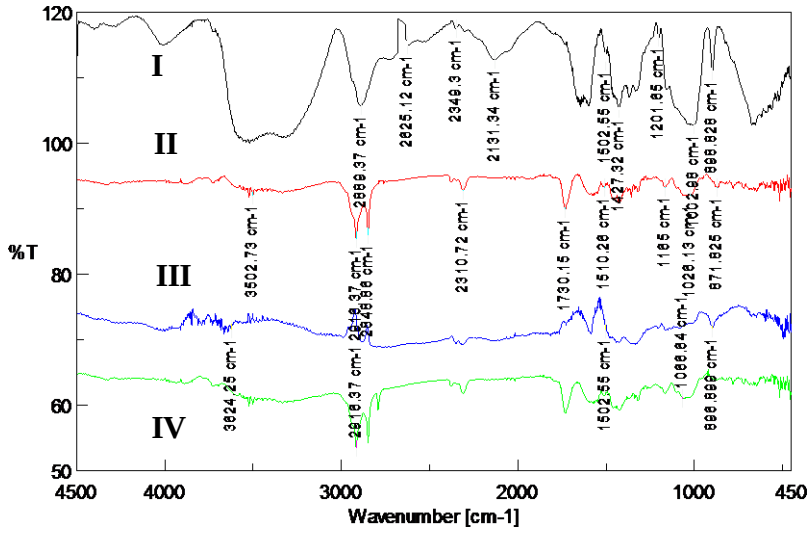


Figure 24: (FTIR spectra of different concentration of NaOH treated in autoclave at constant H_2O_2 (11%) concentration) 1%NaOH (I) -2%NaOH (II)-3%NaOH (III)-5%NaOH (IV)

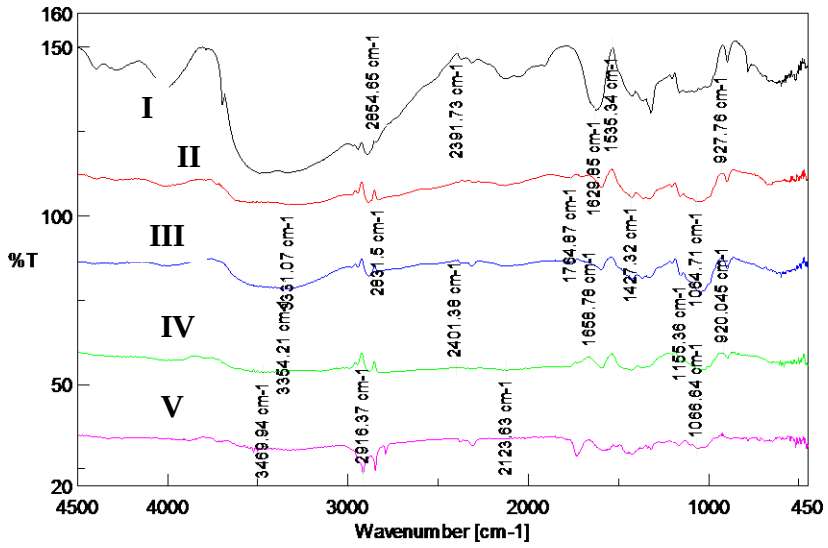


Figure 25: (FTIR spectra of different concentration of H_2O_2 treated in autoclave at constant NaOH (5%) concentration) 1%H_2O_2 (I)-2%H_2O_2 (II)-3%H_2O_2 (III)-5%H_2O_2 (IV)-11%H_2O_2 (V)

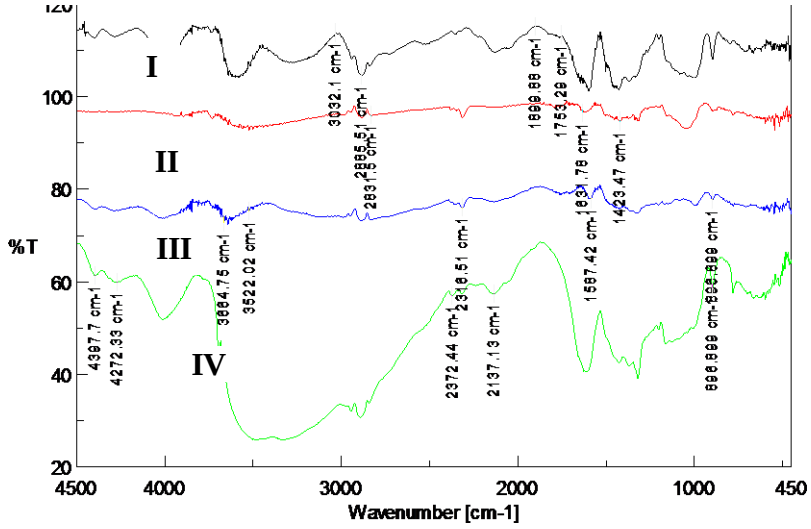


Figure 26: (FTIR spectra of different concentration of H_2O_2 treated in low temperature heating at constant NaOH (5%) concentration) 1%H_2O_2 (I)-2%H_2O_2 (II)-3% H_2O_2 (III)-5%H_2O_2 (IV)

The bands at 850-900 cm^{-1} appeared due to the β-glucosidic bonds between the sugar molecules present (Nelson & O'Connor, 1964). During the pretreatment, α and β-aryl ether glycosidic bonds of polysaccharides were hydrolysed and the acetyl group was removed from the inherent biomass structure (O'Connor et al., 1958). The introduction of ester and ether linkages (band between 1100-1000 cm^{-1}) in the pretreated biomass was a

direct result of the alkaline peroxide treatment since these were less marked in the untreated and alkaline pretreated biomass samples (Qiu et al., 2012; Sukarni et al., 2014). From the FTIR analyses it is clearly evident that both the alkaline and alkaline peroxide pretreatment altered the surface functionalities and structure of the biomass. It could be concluded that the alkaline hydroxide pretreatment was responsible for the increased ester, hydroxyl and ether linkages which was not observed in case of untreated samples while their presence was observed in the alkaline pretreated samples (Fig. 19-20) but to a lesser extent.

3.4.2.2 LOI and TCI of the Pretreated Samples

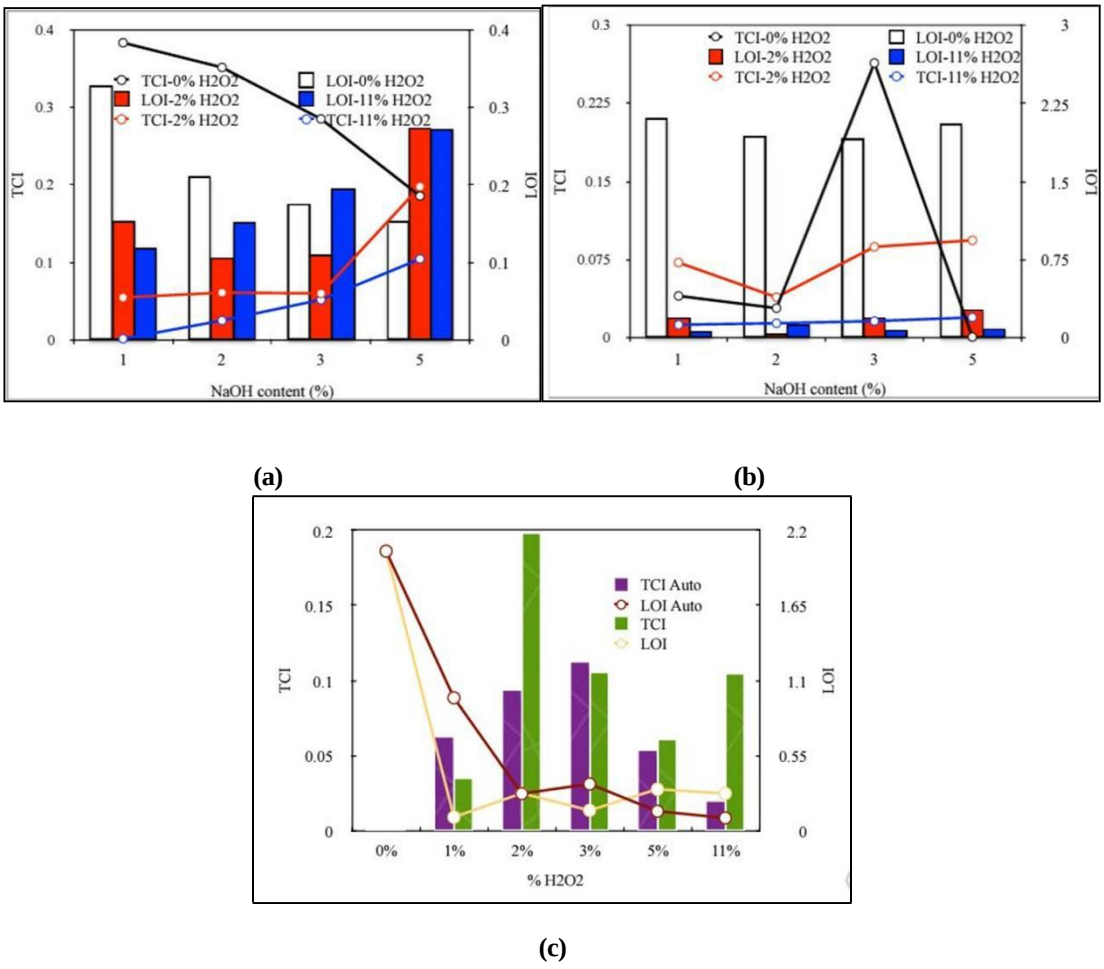


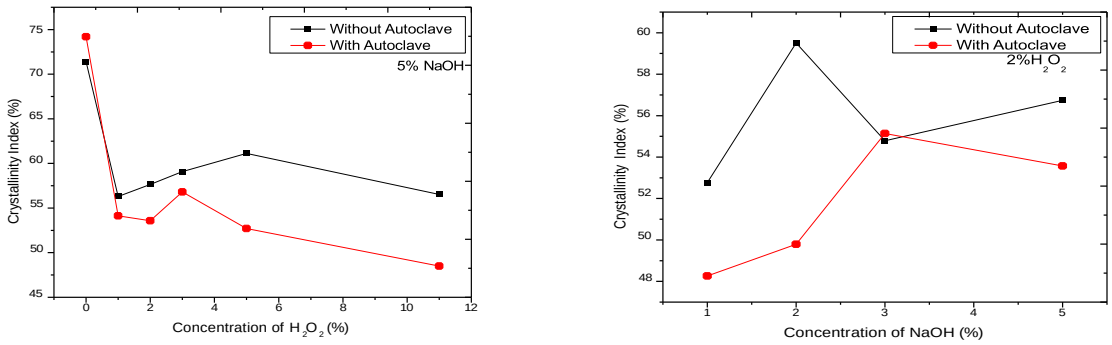
Figure 27: Variation of TCI and LOI for different NaOH and H_2O_2 concentration (a) with autoclave; (b) without autoclave and (c) Variation of TCI and LOI at 5% NaOH concentration with varying H_2O_2 concentration.

From the FTIR spectra of the pretreated biomass, certain parameters can be defined in connection to the structural changes rendered by the pretreatment method. The absorption band between 1420 and 1430 cm^{-1} (A_{1430}) has been attributed to the symmetric $–CH_2$ vibrations and is generally referred to as the "crystallinity band" while the band between 893 and 898 cm^{-1} (A_{898}) is the "amorphous band" appearing due to the C-O-C stretching at the β-glucosidic bonds [47]. The ratio of the area under these two bands (A_{1430}/A_{898}) is defined as the Lateral Order Index (LOI) which physically signifies the amount of crystalline to amorphous regions present in the cellulose and high LOI values are indicative of an ordered structure (Karimi & Taherzadeh, 2016). Another important parameter that can be obtained from the FTIR analysis is the Total Crystallinity Index (TCI) (A_{1373}/A_{2917}) which is defined as ratio of the area under absorption bands corresponding to 1373 cm^{-1} (C–O vibration in the syringyl ring) and 2917 cm^{-1} (asymmetric $–CH_2$ stretching) (Poletto et al., 2011). However, it is well documented that results based upon TCI values were rarely reported in available literature (Isogai & Atalla, 1991; R. Kumar & Wyman, 2013).

Figure 27 illustrates the variation of LOI and TCI values of the pretreated samples at different pretreatment conditions. In the autoclave assisted alkaline peroxide pretreatment, it was noted that the LOI values underwent a steady decline while the TCI values increased up to 3% H_2O_2 concentration and then decreased significantly. The result was indicative of the disruption of the lateral order structure of the biomass while the decrement in TCI values was in accordance with the lignin removal data. The increase in lignin removal suggested that the amount of cellulosic fibres in the biomass became more pronounced resulting in an increase in the TCI values. For the low temperature heating

pretreatment, the TCI & LOI followed a similar trend attaining the minimum value at the pretreatment condition where the lignin removal percentage was maximum while at the other conditions their values increased which corroborated the recalcitrance nature of the cellulose exposed. The effect of H_2O_2 concentration on the TCI and LOI values were also investigated. In absence of peroxide the LOI values of the pretreated samples were significantly higher than that for the peroxide treated samples suggesting that only alkaline pretreatment was unable to disrupt the inherent lignocellulosic structure to facilitate the accessibility of cellulose. The crystallinity of cellulose has a direct role on the thermal stability (Karimi et al., 2013) and hence samples with high values of TCI and LOI can be expected to show higher thermal stability. It is worth mentioning that cellulose contains both amorphous and crystalline regions as well as a small amount of mixed region; however, the hydrolysis of cellulose is facilitated by the presence of the amorphous fraction (Terinte, Ibbett, et al., 2011). There are physical and chemical treatments which can convert crystalline cellulose to its amorphous counterpart but it has been reported that in presence of moisture amorphous cellulose is thermodynamically unstable and is prone to the partial conversion to the ordered crystalline structure (Filho et al., 2007). Therefore, an optimum pretreatment should be characterized by decent lignin removal as well as a moderate value of TCI necessary for the faster and easier conversion of the pretreated biomass to biofuels.

3.4.2.3 XRD analysis of Pretreated Biomass



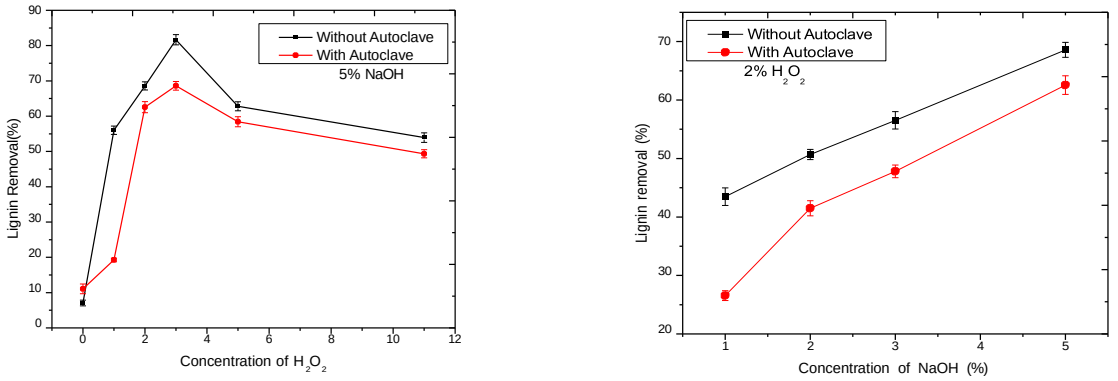
(a) (b)

Figure 28: Variation of Crystallinity Index of the pretreated samples with and without autoclave treatment for different (a) H_2O_2 and (b) NaOH concentrations

Crystallinity is one of the most important parameters that are often correlated with the conversion of lignocellulose (A. K. Kumar, Parikh, & Pravakar, 2016). The crystallinity of the biomass samples is dependent on the inherent framework of the cellulosic fibres and is a function of the procedure of measurement (XU et al., 2007). Cellulose crystallinity is believed to play an important role in its biological conversion. The biomass samples exhibited the characteristic basal reflection peak (002) which illustrated the crystalline cellulose zone (S. Singh et al., 2014) however the intensity of this peak varied with the kind of pretreatment employed for lignin removal. From the XRD plots, it was seen that the amount of free cellulose increased which may be attributed to the changes in biomass structure (Sindhu et al., 2012). The raw and pretreated biomass samples exhibited the characteristic 2θ peaks at ~22° and ~18° indicative of the crystalline and amorphous regions respectively. Figure 28 showed the variation in CI values with the pretreatment conditions for both the low temperature heating and autoclave assisted methods. The

cellulosic fraction of the biomass imparts crystallinity to the samples while the hemicellulose and lignin contributes to its amorphous nature. Hence, the CI of the raw biomass was found to be the lowest due to presence of relatively higher amounts of lignin and hemicellulose and this is in agreement with previous works (Kshirsagar et al., 2015). The increment in the CI values after pretreatment was indicative of the pretreatment affected the amorphous region of the biomass to a greater extent than the crystalline section(Hsu et al., 2010). The change in cellulose crystallinity after pretreatment dictates the enzymatic hydrolysis performance of the lignocellulosic biomass(Rahnama et al., 2013). It was observed that the CI values initially increased after pretreatment which may be attributed to the hydrolysis of glycosidic bonds in the cellulose accessible regions(Martel & Gould, 1990). Similar increase in the CI values after pretreatment have been reported for rice straw treated with dilute alkali and acid (Argyropoulos & Menachem, 1997; G. Shen et al., 2011) while increase in the crystallinity after alkaline peroxide pretreatment has also been reported (Ralph et al., 2007). But as the concentration of the peroxide solutions was increased the CI values decreased as the lignocellulosic matrix expanded and the cellulosic regions present were gradually deformed (B. Y. Yang & Montgomery, 1996) while the amorphous regions of the biomass matrix also got partially disrupted. All these structural changes play a vital role in efficient enzymatic hydrolysis for bioethanol production. It is worth mentioning, however, that the exact mechanism by which the alkaline peroxide treatment affects the CI of the pretreated samples is yet to be fully comprehended.

3.4.2.4 Lignin Removal



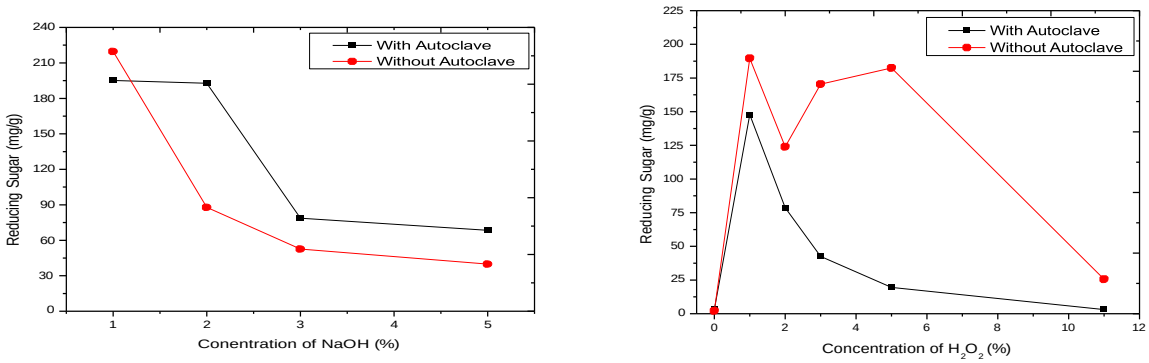
(a) **(b)**

Figure 29: Effect of (a) H_2O_2 and (b) NaOH concentration on lignin removal

The presence of carbon-carbon bonds in softwoods like *S. foetida* is more pronounced compared to hardwoods and is often held responsible for the added difficulty in pretreating softwoods (Knill & Kennedy, 2003). The structure of softwood lignin has been reported to be somewhat more complex than hardwood lignin which affects the solubility of the lignin during extraction (Novotný et al., 2008). The removal of lignin from the biomass improves the accessibility of cellulose and overall surface area for downstream enzymatic hydrolysis which is facilitated by a porous and expanded structure of the substrate biomass. Figure 29 shows the variation in lignin removal with the change in pretreatment conditions. It was observed that the low temperature heating method (81.66%) yielded better results with respect to lignin removal than the autoclave assisted method (68.61%) for the combination of 3% H_2O_2 and 5% NaOH pretreatment solution. The better performance of the low temperature heating (60 ℃) compared to the autoclave assisted method (121 ℃) was

probably due to the instantaneous decomposition of H_2O_2 at higher temperatures. The lignin removal was facilitated by the gradual increase in NaOH concentration under both autoclave assisted and low temperature condition mainly due to the production of OH^- ions which attacked the lignin structure. The peroxide concentration improved the lignin removal and showed an optimum condition at 3% after which it decreased probably due to the formation of recalcitrant products. The optimum condition coincided with the condition at which the pretreated biomass structure showed increased crystallinity and expansion as evidenced by the TCI & LOI data.

3.4.2.5 Reducing Sugars Estimation by DNS Assay



(a) (b)

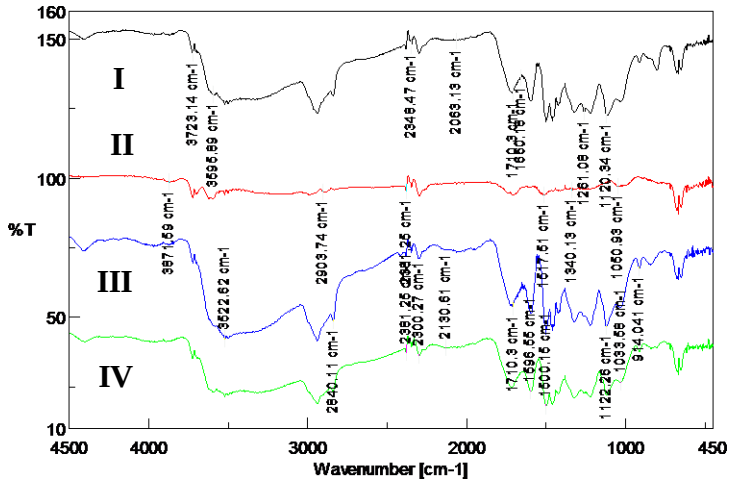
Figure 30: Effect of (a) sodium hydroxide (NaOH) and (b) hydrogen peroxide (H_2O_2) concentration on reducing sugar yield with or without autoclave

The maximum reducing sugar content obtained during the study was 220 mg/g of dried biomass and the sugar content was found to be varied significantly upon changing the pretreatment conditions. After the removal of lignin, the structure of the biomass was supposed to be rearranged so that the monomeric sugars become easily accessible. The

highest reducing sugar yield for *Sterculia foetida* could be obtained when the pretreatment was carried out employing 2% H_2O_2 and 1% NaOH at 60$^{\circ}C$ for 3h while the same combination yielded the maximum reducing sugars (195 mg/g of dried biomass) during the autoclave assisted pretreatment method (121$^{\circ}C$ for 15 min). Again, the pretreatment severity of the autoclave assisted method has been held responsible for the 11.36% reduction in the sugar concentration as compared to the pretreatment carried out at 60$^{\circ}C$. The reducing sugar yield was found to decrease to an appreciable extent with the progressive increase in the concentration of the alkaline peroxide solutions. As evident from the plots, the pretreatment carried out at below and beyond the optimum condition yielded lower reducing sugar content. Figure 30 illustrates the interaction plots of the variables involved in the pretreatment process where the effect of two independent variables was gauged while keeping the other variable constant. The variables used in this study for the reducing sugar concentration were the alkaline concentration, peroxide concentration and reaction temperature. From the results it was clear that both the alkaline concentration and temperature played an important role in determining the reducing sugar content of the biomass and increment in either of these parameters led to a marked decrease in the sugar yield. It can also be inferred from the interaction plots that pretreatment temperature and time had an appreciable effect on the reducing sugar yield. High temperature pretreatment was found to be disadvantageous from the perspective of reducing sugar content. The high alkali concentration coupled with the high temperature may be detrimental to the reducing sugar yield since a portion of the sugar may be lost via oxidation to multiple carboxylic acids as well as sugar molecule degradation under the said conditions (Hendriks & Zeeman, 2009; Naumann et al., 1991; Nie et al., 2013). Moreover, there

remains a possibility of formation of refractory compounds and organic matter decomposition (Faix, 1991). It is probable that the high alkaline concentration (5% NaOH and 3% H_2O_2) yielding best lignin removal efficiency interfered with the amount of reducing sugars available due to the degradation of carbohydrates(R. Gupta & Lee, 2010a). Although, the transfer of sugar to the pretreatment liquor diminishes the reducing sugar of the samples, the liquor can find its use as a potential feedstock for biofuels and other value-added chemicals. However, the production of biofuels or chemicals employing the liquor having high sugar content was not investigated in the present study.

3.4.2.5 ***FTIR analysis of the Extracted Lignin***



(a)

Figure 31: FTIR spectra of extracted lignin (a) from 1% NaOH (I)- 2% NaOH (II) -3% NaOH (III)- 5% NaOH (IV) at constant H_2O_2 concentration (2%)

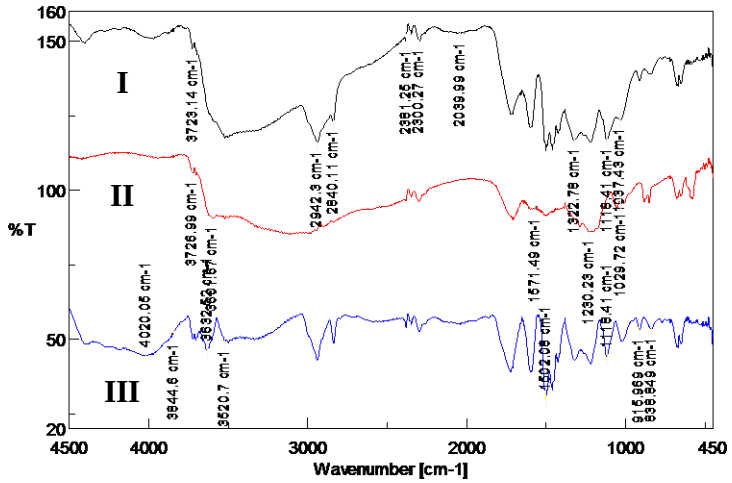
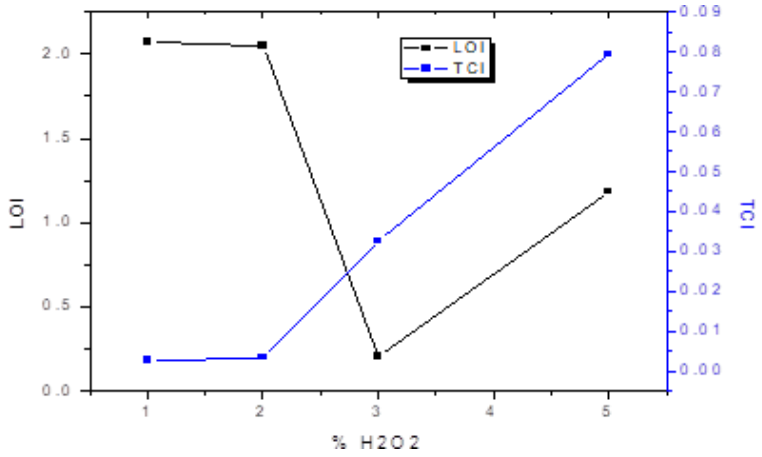


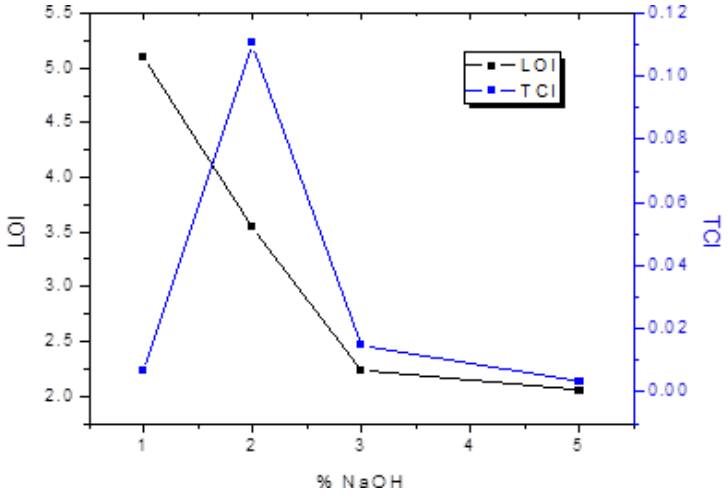
Figure 32: FTIR spectra of extracted lignin from 1% H_2O_2 (I) - 3% H_2O_2 (II) - 5% H_2O_2 (III) at constant NaOH concentration (5%)

The lignin extracted from the pretreated biomass was analyzed using FTIR to investigate the structural changes caused due to the pretreatment. The FTIR spectra of the lignin extracted from the various pre-treatment combinations shows that the band at 900-1200 cm^{-1} was of much diminished intensity compared to the raw material as expected due to the removal of polysaccharides from the biomass matrix(G.-L. Guo et al., 2009). The peak at 1300 cm^{-1} was due to the C-O bond stretching while the aromatic skeleton vibration was marked in 1500 cm^{-1} region(Selig et al., 2009). The band at 2850 cm^{-1} became prominent due to the presence of sufficient methoxy groups in the lignin structure. From the figure 31, it was observed with the increase in alkaline concentration, most of the peaks associated with oxygenated functional groups increased in their intensity, a fact supported by the formation of more and more hydroxyl ions at higher NaOH loading which led to the increment in the surface functionalities. Figure 32 showed the changes in lignin structure upon varying the H_2O_2 concentration at constant NaOH. The peaks at ~1800 cm^{-1} was found to be more intense at higher peroxide concentrations suggesting the presence of acid moieties in the structure. Figure 33 shows the TCI and LOI of the lignin samples extracted after pretreatment. It can be said from the LOI values that the structure of lignin was

progressively disrupted as the concentration of the peroxide increased in the pretreatment solution presumably due to the presence of more and more highly reactive hydroxyl radical. The TCI values also suffered a decrease indicating the amorphous nature of the lignin extracted. However, the presence of NaOH affected the TCI values in a different fashion although the trend of LOI values did not change. The sudden decrease in TCI value at 5% NaOH concentration may be due to the presence of carbohydrates in the solid phase at that particular condition (Sardar et al., 2021).



(a)



(b)

Figure 33: TCI and LOI of lignin samples extracted after pretreatment using (a) H_2O_2 and (b) NaOH

3.4.2.7 Mechanism of Alkaline & Alkaline Peroxide Pretreatment

Alkaline peroxide pretreatment of biomass is an oxidative pretreatment method which employs H_2O_2 as an oxidizing agent. The presence of H_2O_2 in the reaction medium assisted the solubilization of lignin and hemicellulose present in the raw biomass sample(Martel & Gould, 1990). In an alkaline medium, H_2O_2 promptly dissociates into hydrogen cation and hydroperoxyl anion which then undergoes in-situ reaction with the unreacted H_2O_2 to form hydroxyl radicals which are responsible for attacking the lignin framework. The efficacy of the pretreatment is higher since the produced hydroxyl radical is a stronger oxidizing agent than H_2O_2 itself. The reaction of lignin with the hydroxyl radical results in the formation water soluble by-products while reducing the robustness of the cell wall at the same time (Selig et al., 2009) thereby leading to an expanded lignocellulosic structure. The oxidative pretreatment rendered the cellulosic fraction of the biomass more accessible to the subsequent enzymatic hydrolysis. The produced hydroxyl radicals attack the ether linkages of lignin thereby separating the sugars and solubilising the hemicellulose fraction.

$$H_2O_2 \leftrightarrows H^+ + HOO^-$$

$$H_2O_2 + HOO^- \leftrightarrows H_2O + OH^{\cdot} + O_2^{-}$$

3.4.3 Pretreatment (Alkaline peroxide) Results for *Eucalyptus grandis*:

3.4.3.1 FTIR analysis of treated and untreated biomass

In order to understand the structural changes transpired during pretreatment under different pre-treatment conditions, the data obtained from FTIR analysis of untreated and pretreated samples were compared for various pretreatment conditions.

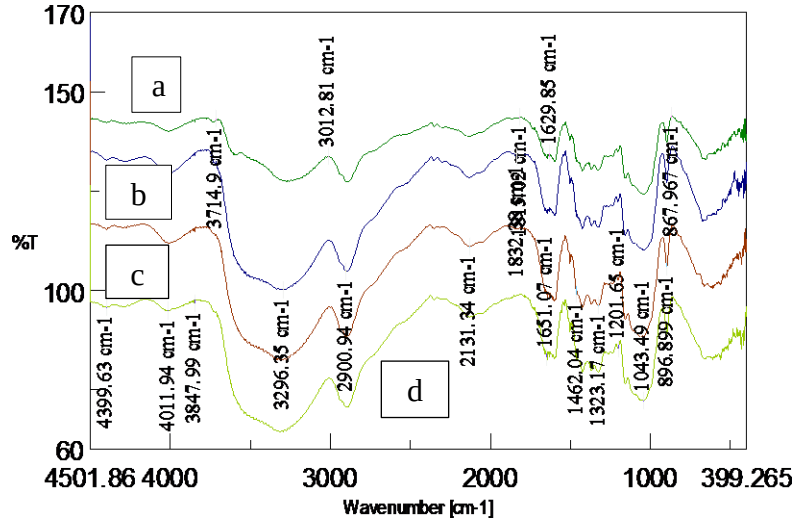


Figure 34: Comparison of FTIR spectra of *E. Grandis* treated at 121°C (autoclave assisted) with 0% (Raw Sample) (a), 2% (b), 5% (c), 8% (d) NaOH.

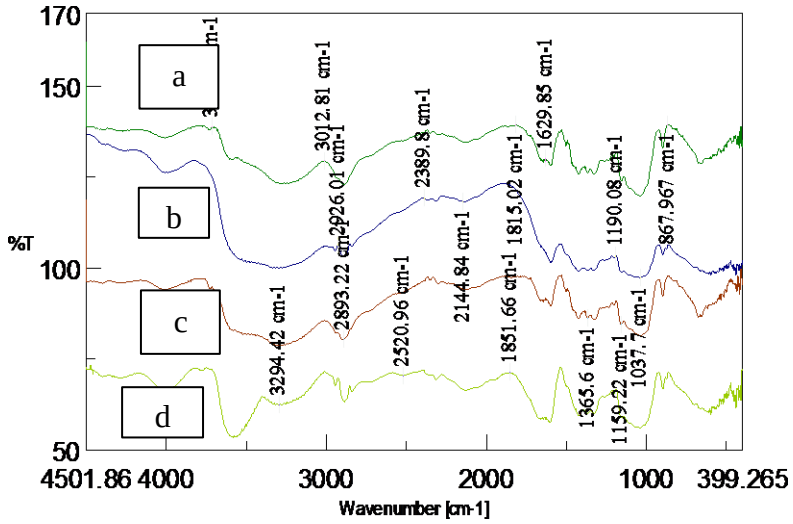


Figure 35: FTIR spectra of *E. Grandis* treated at 60°C with 0% (Raw Sample) (a), 2% (b), 5% (c), 8% (d) NaOH.

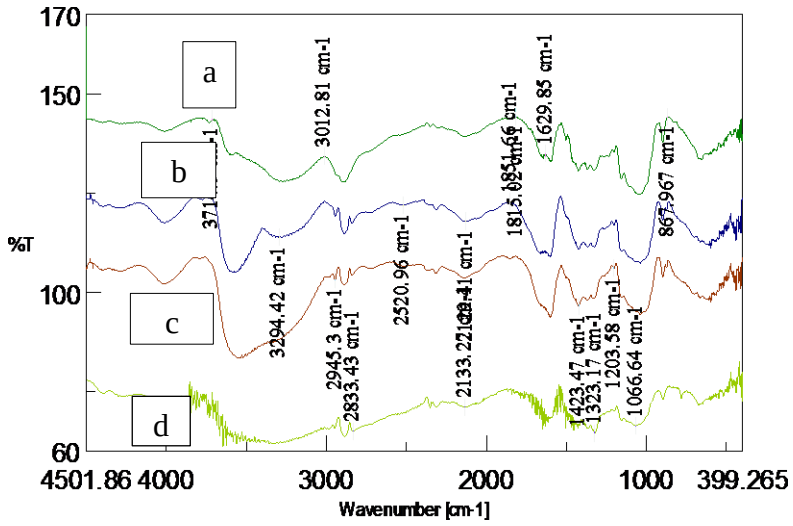


Figure 36: FTIR spectra of *E. Grandis* treated at 80°C 0% (Raw Sample) (a), 2% (b), 5% (c), 8% (d) NaOH.

The untreated dried bark of EG biomass exhibited a broad and strong absorption band in the region of 3300-3500 cm^{-1} attributed to hydroxyl group (O-H). For AAHP (autoclave assisted alkaline hydrogen peroxide treatment) this hydroxyl band stretching was observed to be more distinct as the NaOH concentration increases (Fig 34). However, the magnitude of the hydroxyl band stretched was comparatively less for AHP 60 (alkaline hydrogen peroxide pretreatment at 60˚C) and AHP 80 (Fig 35-36). The band observed at 2900cm^{-1} was allocated to the C-H vibration of methoxy (O-CH_3) bonds. The peak at~1725 cm^{-1} corresponds to the carbonyl (C=O) stretch present in uronic ester groups of hemicellulose or hemicellulose-lignin heterostructure complexes, disappeared during the course of pretreatment. This confirms solubilization and removal of large portions of hemicellulose during AHP treatment (Fig 37-42). The peaks at 1600cm^{-1}, 1506 cm^{-1}, corresponds to aromatic skeleton vibration of lignin while peak at1460cm^{-1} attributed to aromatic ring vibration of lignin. Decrease in these peak after AHP was in accordance with the lignin removal data. Decrease of 1326cm^{-1} peak corresponding to syringyl and condensed

guaiacyl structure if lignin was due to the demothoxylation reaction during AHP treatment. The peak around 1246cm^{-1} appeared due to ester linkages between hemicellulose and lignin (stretching and deformation of (C-OH) of syringyl ring and (C-O) of acetyl group in xylan) also reduced during AHP indicates depolymerization of lignin and hemicellulose during AHP.

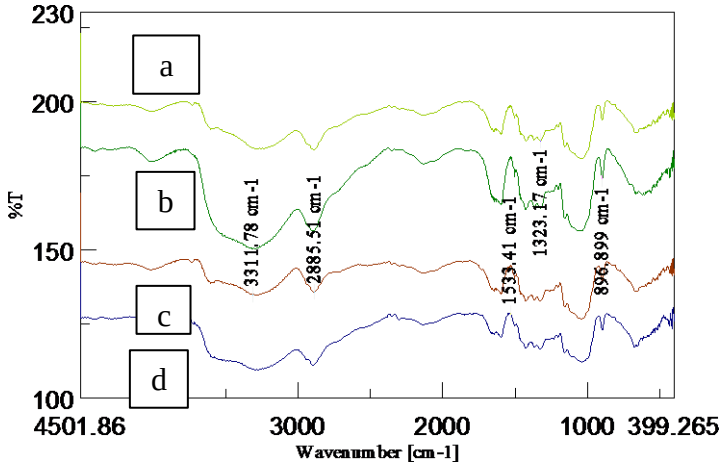


Figure 37: FTIR spectra of *E. Grandis* treated at 2% H_2O_2 under autoclave assisted condition (121°C) with (a)0% NaOH, (b) 2% NaOH, (c)5% NaOH, (d)8% NaOH.

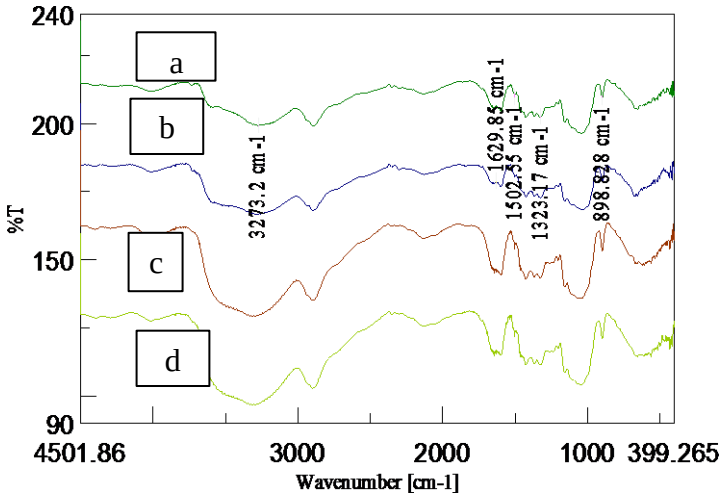


Figure 38: FTIR spectra of *E. Grandis* treated at 1% H_2O_2 under autoclave assisted condition (121°C) with (a)0% NaOH (b)2% NaOH, (c)5% NaOH, (d)8% NaOH.

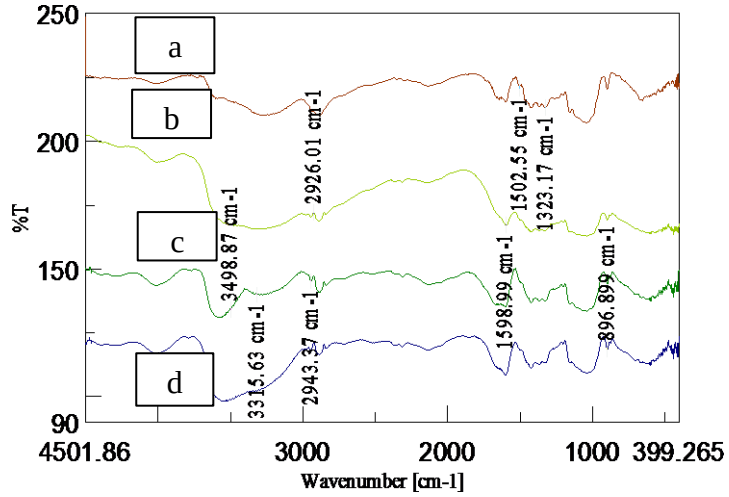


Figure 39: FTIR spectra of *E. Grandis* treated at 1% H_2O_2 under low temperature (60°C) along with (a)0% NaOH (b) 2% NaOH, (c) 5% NaOH and (d) 8% NaOH.

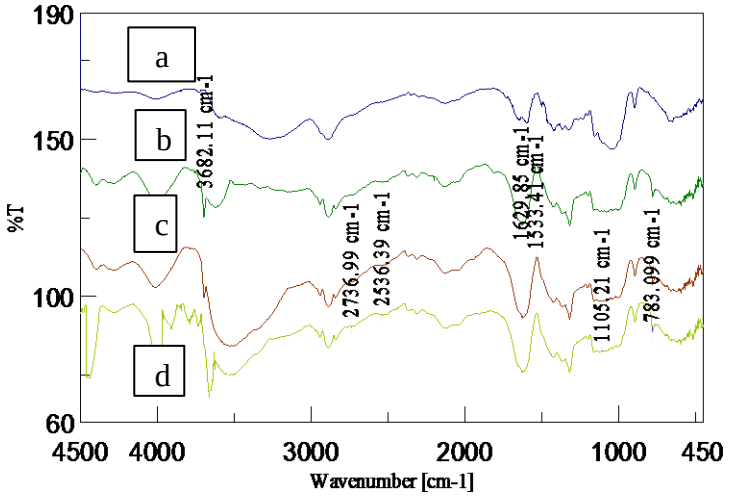


Figure 40: FTIR spectra of *E. Grandis* treated at 2% H_2O_2 under low temperature condition (60°C) along with (a)0% (b) 2% (c) 5% and (d) 8% NaOH.

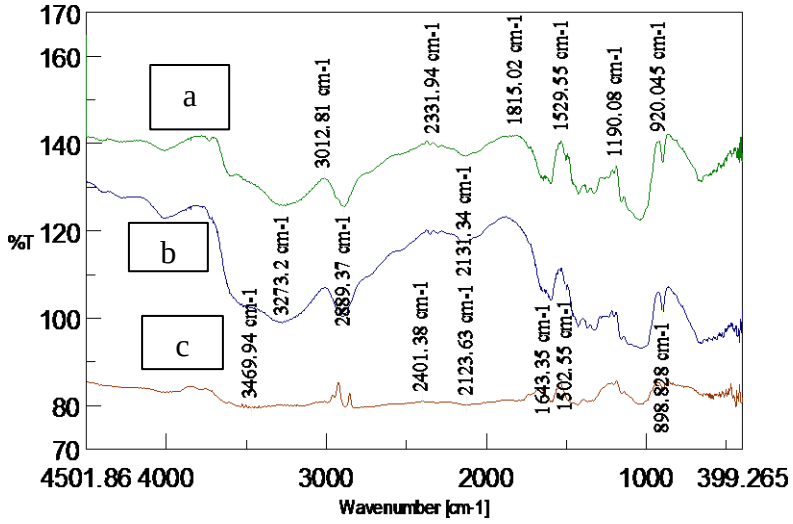


Figure 41: FTIR spectra of *E. Grandis* treated at 1% H_2O_2 under 80°C along with: (a) 2% (b) 5% and (c) 8% NaOH.

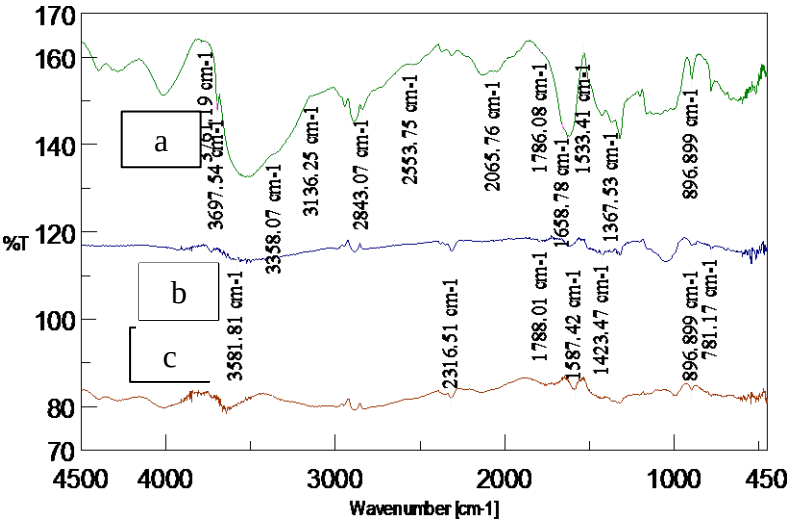


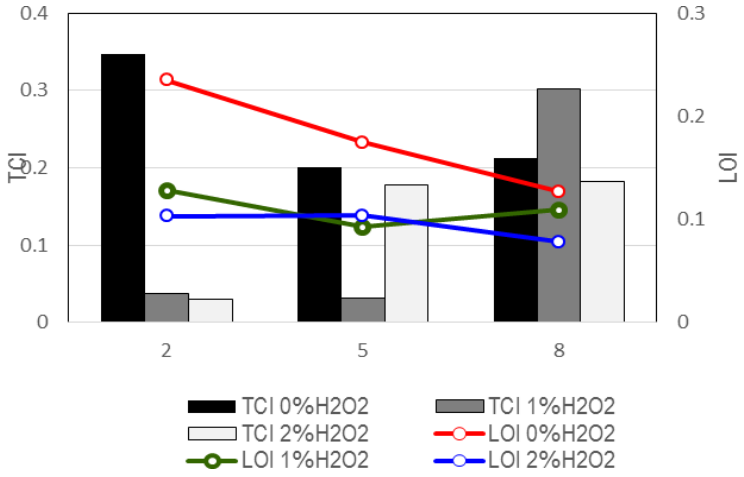
Figure 42: FTIR spectra of *E. Grandis* treated at 2% H_2O_2 and 80°C along with: (a)2% (b)5% and (c)8% NaOH.

The bands at 896 cm^{-1} attributed to the β-glucosidic bonds from the sugar molecules present in cellulose structure becomes intense confirming abundance of cellulose after AHP treatment. The absorbance at 834cm^{-1} represents the C-H out of plane bending of syringyl lignin, also reduced during pretreatment was direct result of delignification of EG biomass during pretreatement. At the time of pretreatment, the removal of acetyl group from the integral biomass structure and α and β-aryl ether glycosidic bonds of polysaccharides were hydrolyzed. Introduction of ester and ether linkages (band between 1100-1000 cm^{-1}) in the pretreated biomass was a direct result of the AHP treatment since these peaks were less intense in the untreated and alkaline pretreated biomass samples. The FTIR data evidently indicates that surface functionalities and inherent structure were altered during alkaline and alkaline peroxide treatment. It could also be concluded that the increase in ester, hydroxyl and ether linkages were due to the presence of alkaline hydroxide in pretreatment solution. The details of the spectra available is summarized in table 10.

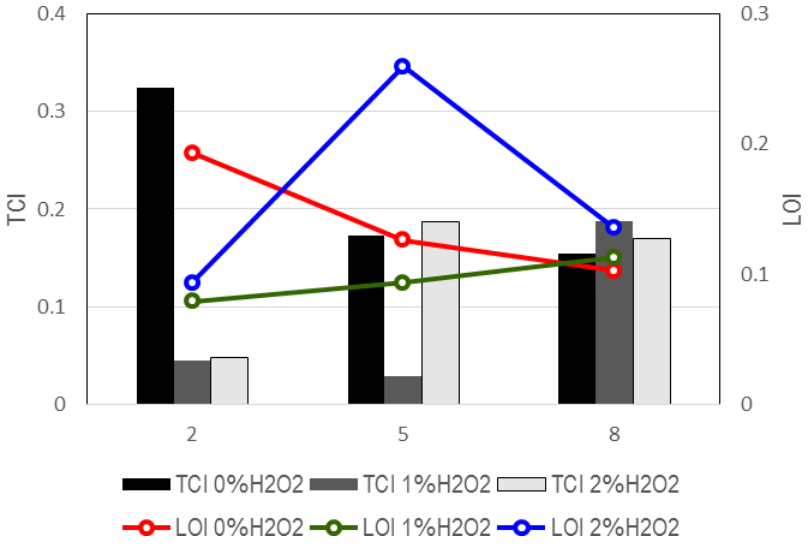
Table 10: FTIR spectra and the source of the spectrum (Bodîrlău & Teacă, 2009; Pandey, 1999; Schultz et al., 1985)

Wave number of the FTIR Spectra (cm^{-1})	Significance	Source
3300-3500	Hydroxyl bond (O-H) stretching	Water and general organic matter
2950	asymmetric stretching of methylene ($-CH_2$, $-CH_3$) groups	Cellulose
1725	Stretching of (C=O) of carbonyl and ester groups	Pectin and xylan
1500-1600	Stretching of (C=C) of aromatic ring	Lignin
1373	C–O vibration in the syringyl ring	Lignin and hemicellulose
1240	Stretching and deformation of (C-OH) of syringyl ring and (C-O) of acetyl group in xylan	Lignin and hemicelluloses (xylan)
1050	(C-OH) of secondary (2°) alcohol	Cellulose and hemicellulose
896	β-glycosidic bond	Cellulose

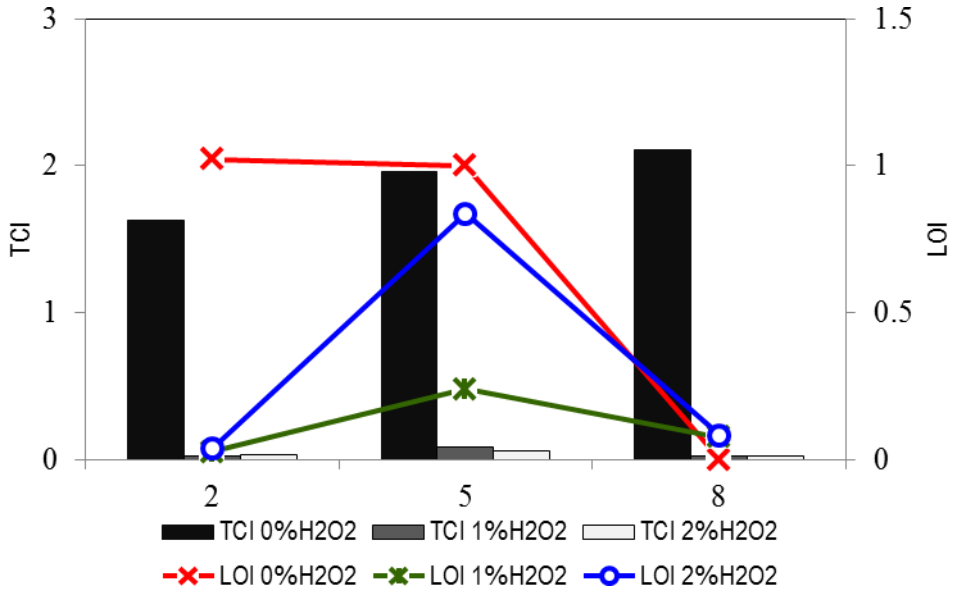
3.4.3.2 LOI and TCI of the pretreated sample



(a)



(b)



(c)

Figure 43: Changes in TCI and LOI at different temperature and different concentration of NaOH and H_2O_2 (a) at 60°C; (b) at 80°C and (c) at 121°C in an autoclave

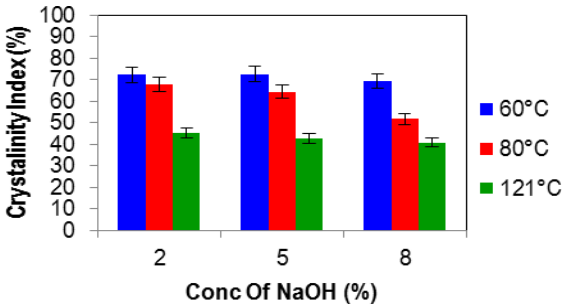
Total Crystallinity Index (TCI) and Lateral Order Index (LOI) among all other parameters can be defined from the FTIR spectra available, in connection to the structural changes resulted from the pretreatment method. The absorption band observed between 1420 and 1430 cm^{-1} (A_{1430}) has been recognized to be that of symmetric $–CH_2$ vibrations and is generally known as "crystallinity band". Likewise, the band between 893 and 898 cm^{-1} (A_{896}), known as "amorphous band" attributed to be that of the C-O-C stretching at the β-glycosidic bonds (Fan et al., 2012). Lateral Order Index (LOI) can be defined as the ratio of the area under these two bands (A_{1430}/A_{898}). LOI indicates the extent of crystalline to amorphous regions there in the cellulose. High LOI values point toward an ordered structure (Karimi & Taherzadeh, 2016). Total Crystallinity Index (TCI) on the other hand is the ratio between absorption bands 1373 cm^{-1}, resulted from C–O vibration in syringyl

ring, and 2917 cm^{-1}, resulted from asymmetric $–CH_2$ stretching, i.e. (A_{1373}/A_{2917}) (Poletto et al., 2011).

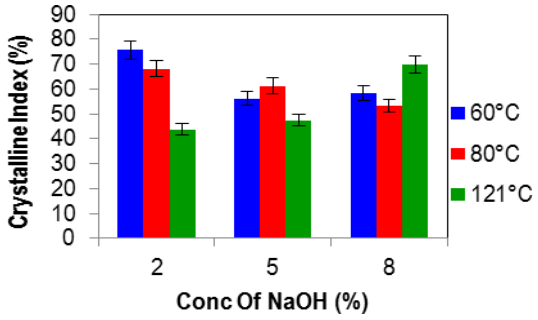
Figure 43 represents TCI and LOI variation during AHP pretreatment. A steady decline of LOI and an increment followed by a significant decrease in TCI values were observed for AAHP pre-treatment using 0% H_2O_2 on EG biomass. This indicates that the lateral order structure of the biomass was disrupted and the decrement in TCI values was compliant with the lignin removal data. The increase in lignin removal suggested that the amount of cellulosic fibers in the biomass became more accessible resulting in an increase in the TCI values. For AHP 60, the TCI & LOI followed a similar trend attaining the minimum value at the pretreatment condition where the lignin removal percentage was maximum while at the other conditions their values increased which corroborated the recalcitrance nature of the cellulose-hemicellulose -lignin hetero structure. The effect of H_2O_2 concentration on the TCI and LOI values were explored as well. LOI values of the sample treated with peroxide were found to be lower than that of the sample treated in absence of peroxide. The result suggests the incompetency of only alkaline pretreatment to disrupt the intrinsic lignocellulosic structure to aid the accessibility of cellulose. The crystallinity of cellulose can have an impact on thermal stability and therefore higher thermal stability can be observed on samples with elevated TCI and LOI values. Cellulose contains both amorphous and crystalline regions as well as a small amount of mixed region. The hydrolysis of cellulose is facilitated by the presence of the amorphous fraction(Terinte, Schuster, et al., 2011). Since, in presence of moisture amorphous cellulose is thermodynamically unstable and is prone to the partial conversion to the ordered crystalline structure, an optimum pretreatment should be characterized by significant lignin removal

as well as a moderate value of TCI necessary for the faster and easier conversion of the pretreated biomass to biofuels (Filho et al., 2007).

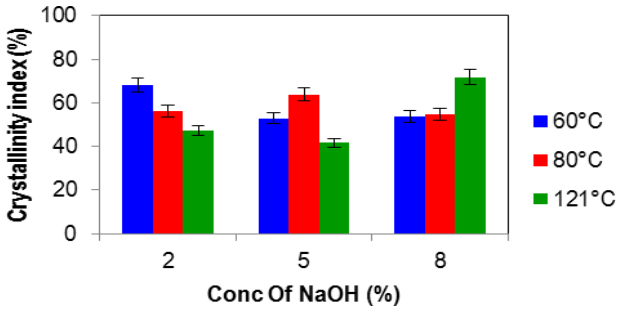
3.4.3.3 XRD analysis of pretreated sample



(a)



(b)



(c)

Figure 44: Variation of Crystallinity Index of the pretreated samples at 60°C, 80°C and 121°C for (a) 0% H_2O_2 (b) 1% H_2O_2 (c) 2% H_2O_2 concentrations.

XRD was used in this study to investigate the variation in the crystallinity at different pretreatment conditions based on the amount of the crystalline cellulose got exposed on the surface due to the pretreatment (L. Liu et al., 2009). Cellulose crystallinity is assumed to play an important part in its biological conversion for the production of monomeric sugars. Crystallinity index of the raw material was 42.6% as calculated from XRD data. The *Eucalyptus grandis* exhibited the characteristic basal reflection peak (002) which illustrated the crystalline cellulose zone however the intensity of this peak varied with the kind of pretreatment employed for lignin removal (Liao et al., 2011). The raw and pretreated biomass samples exhibited the characteristic 2θ peaks at~ 22° and ~ 18° indicative of the crystalline and amorphous regions respectively. Figure 44 represents the variation in CI values at different pretreatment conditions. The cellulosic fraction of the biomass imparts crystallinity to the samples while the hemicellulose and lignin contributes to its amorphous nature. Hence, the CI of the raw biomass was found to be the lowest due to presence of relatively higher amounts of lignin and hemicellulose which is in agreement with previous works (Kshirsagar et al., 2015). The fact that the untreated biomass had lower value of CI than its pretreated counterparts suggested that the modification in the composition of the pretreated material dictated the nature of the CI values. The increment in the CI values after pretreatment was indicative of the pretreatment affected the amorphous region of the biomass to a greater extent than the crystalline section (Ge et al., 2020).

From Figure 44 it can be observed alkali pretreatment in absence of H_2O_2, facilitated decrease in the crystallinity index with increase in temperature and NaOH concentration. For instance, at 2% NaOH concentration the crystallinity indices were 72.1, 67.9 and 45.29 for AHP-60, AHP 80 and AAHP respectively. Similarly at 80˚C, the values

of the same index were 67.9, 64.4 and 51.8 for 2%, 5% and 8% NaOH concentrations respectively, which indicated that pretreatment at higher NaOH concentrations and temperature is more effective. An opposite trend that is increase in crystallinity index was observed for samples treated with higher concentration of H_2O_2 and NaOH at higher temperature. At higher temperature, water and the pretreating reagent enters into the para-crystalline and amorphous cellulose at the time of pretreatment. As a result the para-crystalline cellulose recrystallizes and water molecules were released because of high temperature conditions (Biswas et al., 2011) resulting increase in crystallinity index. Similar increase in crystallinity has been reported in previous works as well (Z. Wang et al., 2018). But as the concentration of the peroxide solutions was increased the CI values decreased, for instance at 60˚C and 5% NaOH concentrations, the crystallinity index values are 72.6, 56.2 and 52.9 for 0%, 1% and 2% H_2O_2 concentrations respectively, as the lignocellulosic matrix expanded and the cellulosic regions present were gradually deformed (B. Y. Yang & Montgomery, 1996) while the amorphous regions of the biomass matrix also got partially disrupted. All these structural changes play a vital role in efficient enzymatic hydrolysis for bioethanol production. It is worth mentioning, however, that the exact mechanism by which the alkaline peroxide treatment affects the CI of the pretreated samples is yet to be fully comprehended.

3.4.3.4 Yield and lignin removal

The main physical hindrance for enzyme accessibility of cellulose for production of monosugars from lignocellulosic biomass is lignin due to its inherent recalcitrant nature. Alkaline peroxide pre-treatment found to be effective in removal of lignin from lignocellulosic biomass (Karimi & Taherzadeh, 2016). Various process parameters like NaOH and H_2O_2 concentrations, pretreatment time and temperature play important role in

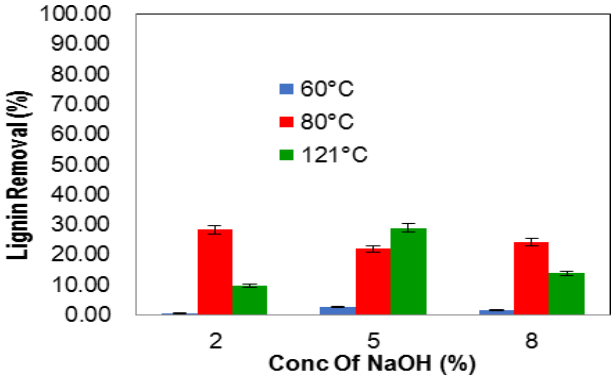
effectiveness of pretreatment on easy accessibility of cellulose for enzymatic hydrolysis and are examined in this study for valorization of EG dried bark, a waste material mostly generated in pulp and paper, plywood industry across India. The compositional analysis of EG dried bark used during this study is evaluated by proximate and ultimate analysis and results are presented in Table 9 with 18.7±0.16% lignin content.

Figure 45 depicts effectiveness of delignification with varied pretreatment intensities of different process parameters. It is evident from figure 45 that the percentage of lignin removal increased as temperature and H_2O_2 concentration increases. During the control experiments (while no H_2O_2 was added to the reaction mixture) temperature rather than NaOH concentration seemed to be the key factor for lignin removal (Fig 45a). With increase in temperature from 60˚C to 80˚C lignin removal percentage escalated from 0.53% to 28.34% during treatment with 2% NaOH concentration, 2.67% to 21.93% while treated with 5% NaOH and 1.60% to 24.06 % when treated with 8% NaOH. Whereas in case of AAHP there was a decrease in lignin removal percentage to 9.63% when treated with 2% NaOH and 13.90% at 8% NaOH concentration while an increase was observed for 5% NaOH to 28.88%. Nevertheless, the delignification was not effective without the addition of H_2O_2. Addition of H_2O_2 significantly increased delignification percentage while an increase in delignification was observed with increase in H_2O_2 concentration (1% to 2%). The highest lignin removal percentage (73.21%) was detected when AAHP was used with 8% NaOH and 2% H_2O_2. Higher lignin removal percentage was strongly dependent on temperature due to the fact that higher temperature facilitate generation of more active hydroxyl radicals (HO*) which directly contributes in degradation reaction of lignin (R. C. Sun et al., 2000). The hydroxyl radicals and the anion radicals (O_2*) which generated on decomposition of peroxide probably also aided in dissolution of lignin and to some

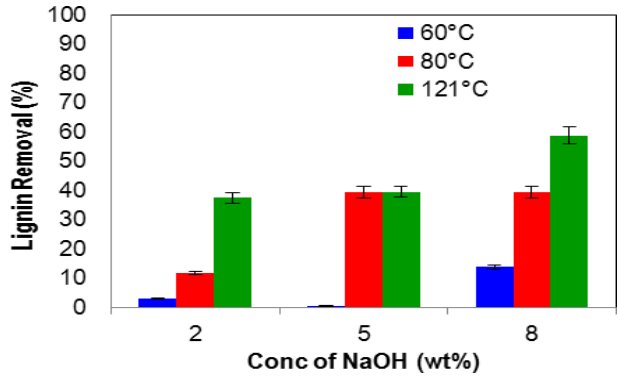
extent(W., 1996). However, due to its high cost the percentage of H_2O_2 to be used in the experiment should be minimized. It was also observed that the AAHP effectively removed a large percentage of lignin (73.21%) compared AHP 60 AND AHP 80 (9.63% and 50.27%) for 2% H_2O_2 and 8% NaOH. The lignin removal was facilitated by the gradual increase in NaOH concentration for AHP 60, AHP 80 and AAHP mainly due to the production of OH^- ions which directly help to dissociate various bonds present in lignin hetero-polymer structure. Alkaline peroxide pretreatment of biomass is an oxidative pretreatment method and utilizes H_2O_2 as an oxidizing agent. The presence of H_2O_2 in the reaction medium assisted in solubilizing lignin and hemicellulose present in the raw biomass sample (Bianco et al., 2021). In an alkaline medium, H_2O_2 promptly dissociates into hydrogen cation and hydroperoxyl anion which then undergoes in-situ reaction with the unreacted H_2O_2 to form hydroxyl radicals which are responsible for attacking the lignin framework. The efficacy of the pretreatment is higher since the produced hydroxyl radical is a stronger oxidizing agent than H_2O_2 itself. The reaction of lignin with the hydroxyl radical results in the formation water soluble by-products while reducing the robustness of the cell wall at the same time (R. C. Sun et al., 2000) thereby leading to an expanded lignocellulosic structure. The oxidative pretreatment rendered the cellulosic fraction of the biomass more accessible to the subsequent enzymatic hydrolysis. The produced hydroxyl radicals attack the ether linkages of lignin thereby separating the sugars and solubilizing the hemicellulose fraction (Baksi, Saha, et al., 2019).

$$H_2O_2 \leftrightarrows H+ + HOO–$$

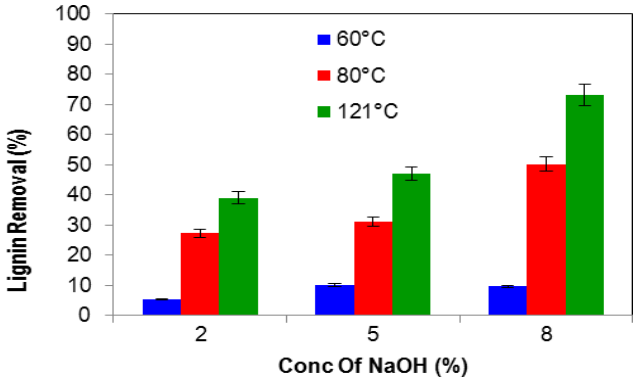
$$H_2O_2 + HOO– \leftrightarrows H_2O + {}^{\cdot}OH + O_2^-$$



(a)



(b)



(c)

Figure 45: Effect of NaOH and H_2O_2 concentration on lignin removal (a) 0% H_2O_2 (b) 1% H_2O_2 and (c) 2% H_2O_2 with change in temperature

The surface area, pore size, and pore volume of pretreated Eucalyptus grandis were increased (Table 11) due to lignin removal leading to fragmentation of the lignin-cellulose-hemicellulose hetero matrix during pre-treatment. The lignin removal from the biomass improves the accessibility of cellulose and overall surface area for downstream enzymatic hydrolysis which is facilitated by a porous and expanded structure of the substrate biomass. It was further correlated to kinetics of enzymatic hydrolysis as reported by various researchers (Arantes & Saddler, 2010; S. W. Kim et al., 2015; Y.-H. P. Zhang & Lynd, 2004).

Table 11: Surface area, pore size, and pore volume of untreated and pre-treated *Eucalyptus grandis* bark

Sample	Surface area m^2/g	Pore size[a] Å	Pore volume[b] $\times 10^{-2}$
Untreated	12.49±0.14	19.22±0.38	1.967±0.076
Pre-treated	20.47±0.18	36.07±0.41	2.253±0.57

[a.] Average pore radius.

[b.] Total pore volume for pores with radius less than 1636.54 Å at P/P0 = 0.99.

Yield of the pretreated biomass decreased with increase in temperature as well as H_2O_2 and NaOH concentration. From figure 46 it can be observed that the percentage yield of biomass after pre-treatment was greater when treated in absence of H_2O_2 at lower temperature. From lignin removal data and the visual appearance of the treated sample it can be implied that the inefficient lignin removal in these pre-treatment conditions was responsible for higher yield. Increase in temperature and concentrations of NaOH and H_2O_2 directly help to disrupt the lignin-hemicellulose-cellulose hetero-matrix facilitating removal of higher amount of lignin along with hemicellulose solubilization resulting lower

yield. The yield percentage significantly decreased for AAHP which is in accordance with the lignin removal data For AHP 60 lignin removal percentage ranges from 0.53% to 14% and for AHP 80 it ranges from 20% to 50.27%, whereas for AAHP the lignin removal percent ranges mostly varied between 30-73.2%. Comparing both yield and lignin removal data 8% NaOH and 2% H_2O_2 provides significantly high yield percentage for both AHP 80 and AAHP with autoclave assisted one offering the highest (73.2%) lignin removal and significant (74.8%) yield.

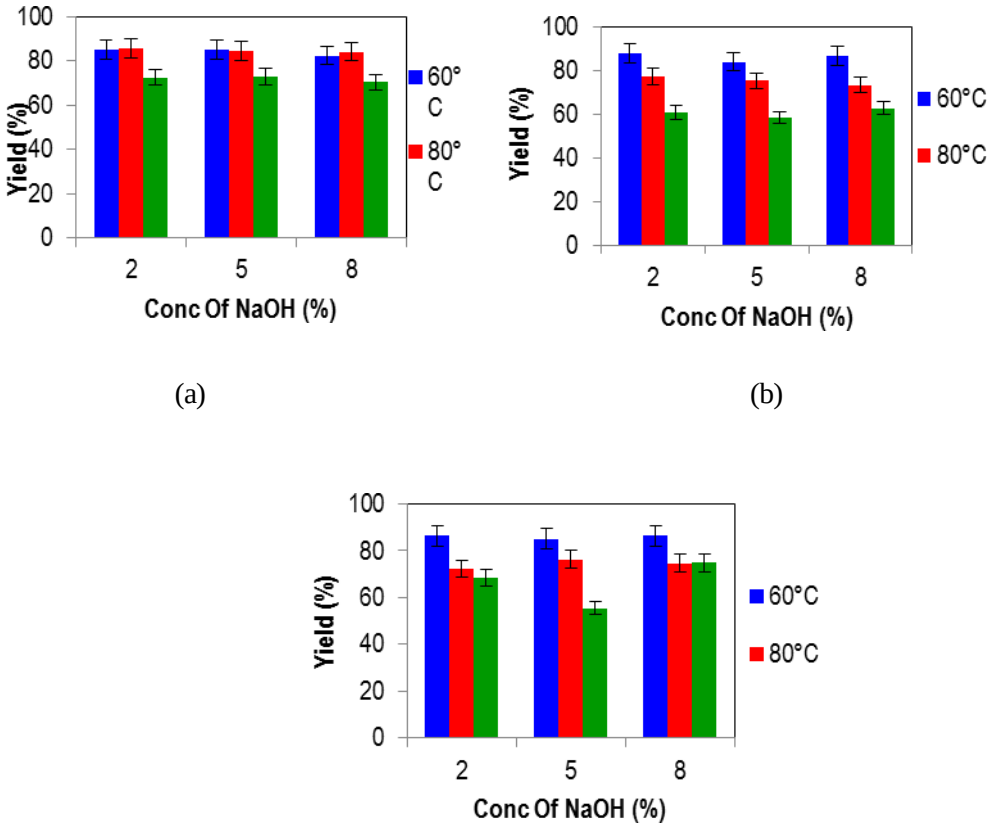
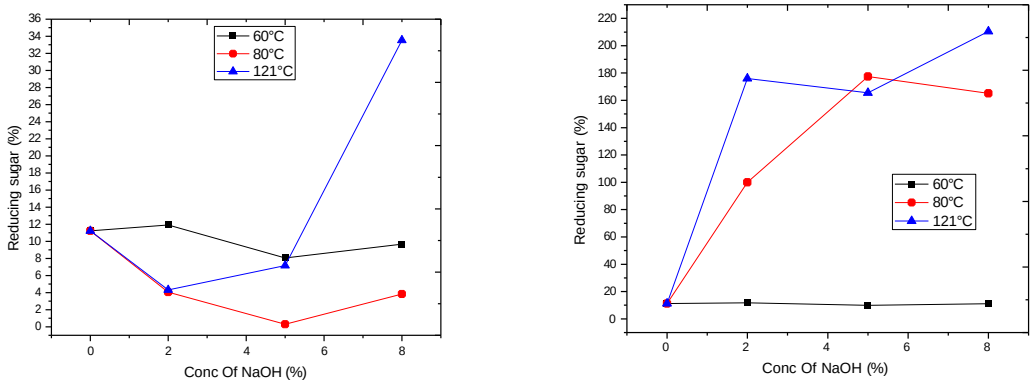
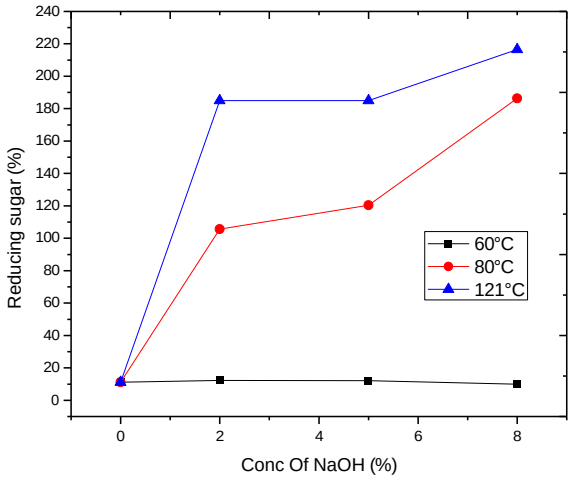


Figure 46: Effect of NaOH and H_2O_2 concentration on yield at different temperature conditions (a) 0% H_2O_2 (b) 1% H_2O_2 and (c) 2% H_2O_2

3.4.3.5 Reducing sugar estimation by DNS Assay



(a) (b)



(c)

Figure 47: Effect of sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2) concentration (a) 0% H_2O_2, (b) 1% H_2O_2 and (c) 2% H_2O_2 on reducing sugar yield at different reaction temperature

The reducing sugar content of the raw material was 11.21 mg/g. From figure 47 it can be seen that in the absence of H_2O_2 the reducing sugar obtained during DNS reaction is very low and ranges between 0.3mg/g to 11.9mg/g. Although at elevated temperature i.e., at 121˚C in assistance with autoclave (AAHP), the reducing sugar content increased significantly with increasing NaOH concentration from 4.31mg/g to 33.54 mg/g. On the other hand, when treated with H_2O_2, with increasing H_2O_2 concentration and increasing temperature there were noticeable increase in reducing sugar content. For example, at 60˚C it was 11.81mg/g for the combination of 2% NaOH and 1% H_2O_2 and increased to 12.30mg/g when H_2O_2 percentage was further increased to 2% H_2O_2. Similarly, as temperature increases i.e., at 80˚C the reducing sugar content for the same combination was 99.92 mg/g and 105.69 mg/g respectively. This same combination when treated under autoclave assisted environment yielded 175.99mg/g and 185.00mg/g reducing sugar. The reducing sugar content was observed to be varied with varying treatment intensities and reached its maximum at 216.5 mg/g of dried biomass. Maximum reducing sugar of 216.5 mg/g of dry biomass was obtained when treated under autoclaved assisted pretreatment with 8% NaOH and 2% H_2O_2. This could be due to the fact that after the removal of lignin, the structure of the biomass was rearranged for easy accessibility of cellulose resulting higher monomeric sugar formation. The highest reducing sugar yield for *Eucalyptus grandis* was found to be in the pretreatment condition employing 2% H_2O_2 and 8% NaOH at 80°C as well as at 121°C in case of autoclave assisted pre- treatment. However, at 60°C the reducing sugar content dropped significantly which could be attributed to the fact that at higher temperature water and pretreating reagents able to penetrate the para-crystalline cellulose and para-crystalline cellulose recrystallizes and become accessible to the

pretreating reagents (Biswas et al., 2011). The same combination (2% H_2O_2 and 8% NaOH) at 121°C treated in an autoclave also yielded the maximum lignin removal (73%). From the plots it can be observed that the pretreatment performed at below and ahead of the optimum condition yielded lower reducing sugar content. Autoclave assisted treatment proved to be highly effective although the pre-treatment was carried out for a very small time. It can also be inferred from the plots that pretreatment temperature and time had an appreciable effect on the reducing sugar yield. High temperature pretreatment was established to be beneficial from the perception of reducing sugar content. Although, It is probable that the high alkaline concentration coupled with high temperature may lead to the degradation of carbohydrates in some cases but in this study effect of higher alkaline concentration was not investigated (R. Gupta & Lee, 2010b) due to the corrosive nature of high alkaline condition. The effect of lignin content and crystallinity index (CI) on reducing sugar production is also investigated (Figure 48 and 49). The results directly indicate presence of higher amount of lignin inhibit reducing sugar formation due to recalcitrant nature of lignin while Crystallinity index did not show any direct correlation on reducing sugar formation.

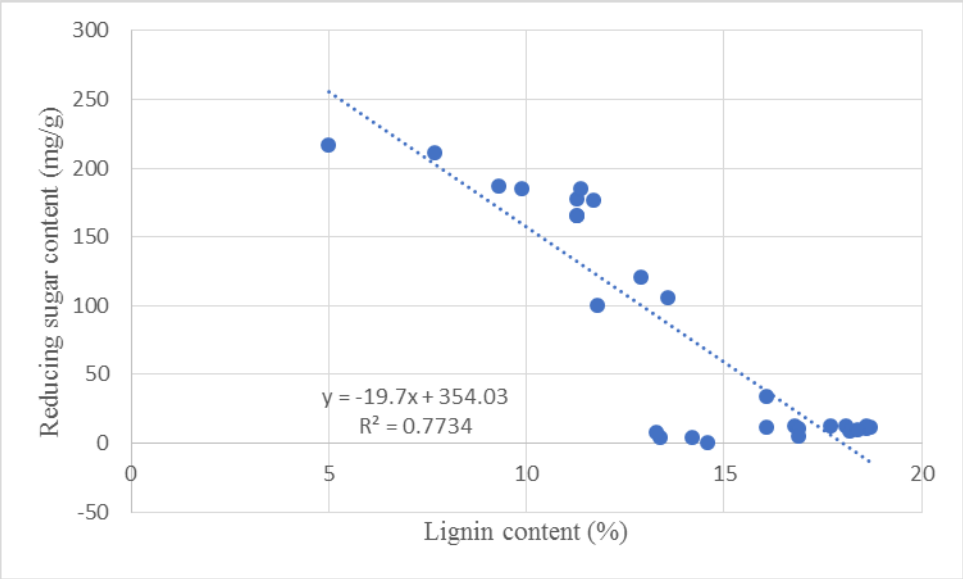


Figure 48: Variation of reducing sugar production with lignin content in pre-treated EG

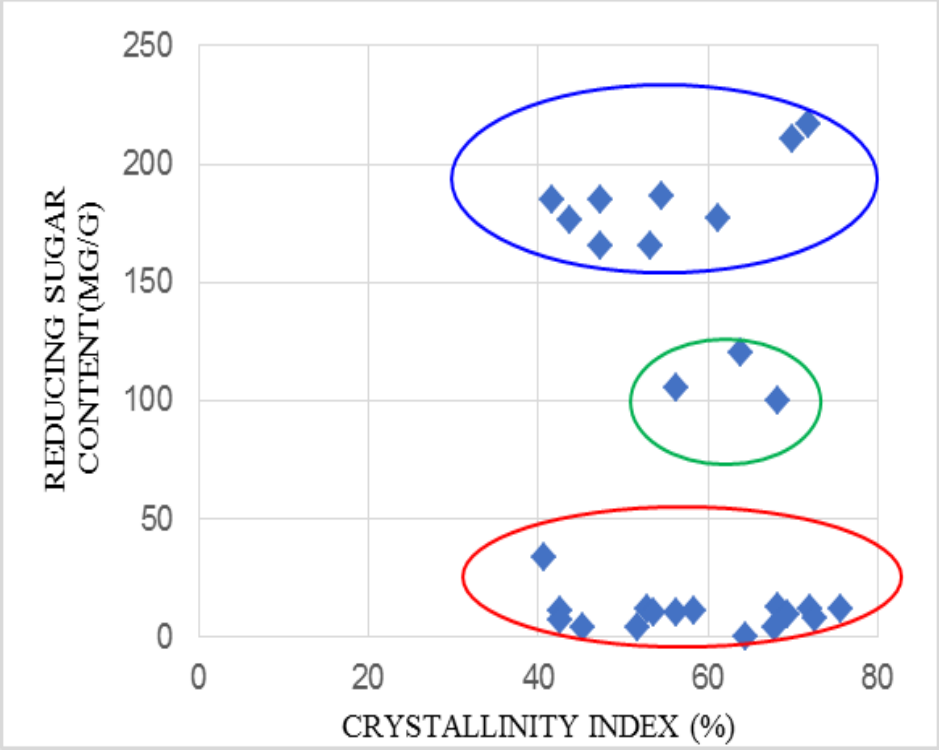


Figure 49: Variation of reducing sugar with crystallinity index

3.4.4 Pretreatment (Deep Eutectic Solvent) Results for *Eucalyptus grandis*

Other than alkaline peroxide preptreatment efficacy of DES was also investigated to pretreat E. *grandis*. The efficacy of DES pretreatment on ethanol production from E. *grandis* was also evaluated and compared in next chapter.

3.4.4.1 Effect of DES on morphology

The effect of ChCl/urea pretreatment on morphological changes happened can be observed with the help of SEM images of the pretreated *E grandis* and the acid insoluble lignin extracted from the sample. With ChCl/urea pretreatment, the surface structure of Eucalyptus residues and the acid insoluble lignin changed significantly. Figure 50 represents the SEM of DES treated sample and extracted lignin.

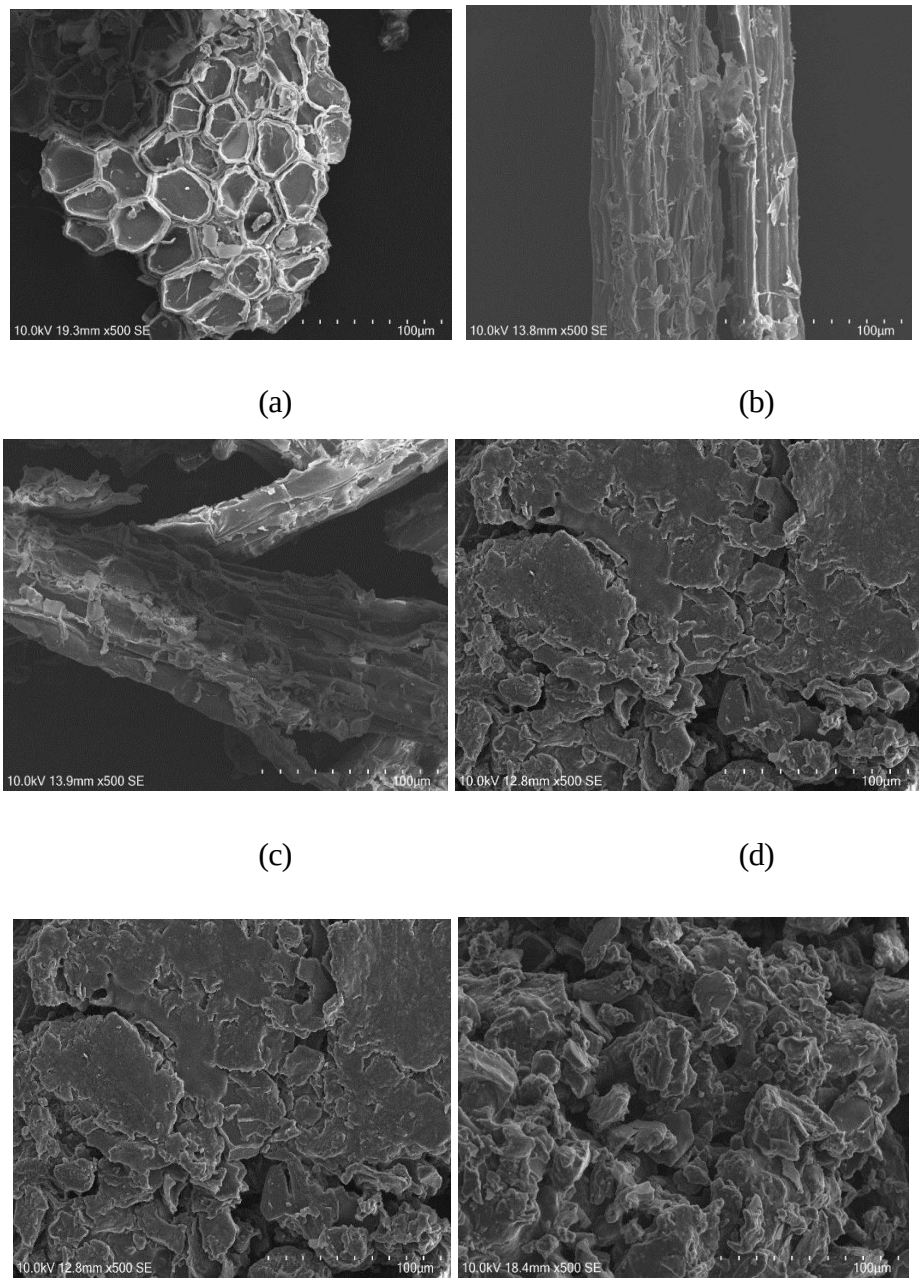


Figure 50: SEM images of DES treated *E. grandis* treated at 80˚C for (a)4 hrs (b) 6 hrs and (c) 8 hrs and the acid insoluble lignin extracted from sample treated for (d)4 hrs (e) 6 hrs and (f) 8 hrs.

E. grandis internal structure was ruptured by ChCl/urea pretreatment and visible cracks and pores can be observed at the surface as presented in Fig 50 (a, b and c). The solvation action of ChCl/urea might be responsible for the disruption of the surface resulting in swelling of the lignocellulosic matrix. The visible cracks and pores in the surface of the residues from ChCl/urea pretreatment can be advantageous for the subsequent enzymatic hydrolysis. Fibrillar structure of the residue was visible after 8h pretreatment with ChCl/urea. It is may be due to the removal of a portion of hemicellulose and amorphous cellulose.

3.4.4.2 FTIR analysis of treated and untreated biomass:

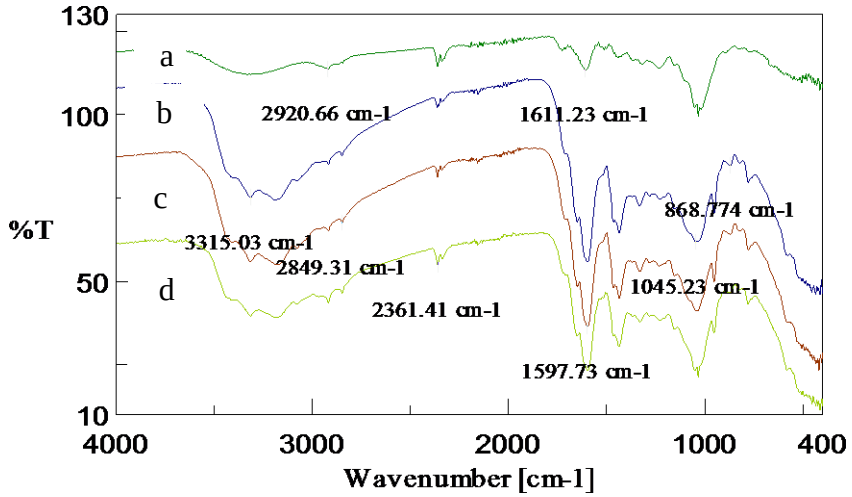


Figure 51: Comparison of FTIR spectra of *E. Grandis* treated with DES at 80°C for 0 (Raw Sample) (a),4 (b), 6(c), 8(d) hours.

Fig 51 shows the FTIR spectra of *E. grandis* under ChCl/urea pretreatment. The shift of peaks from 3415 to 3436 cm^{-1} can be observed which attributes to the formation of the intramolecular hydrogen bonds in cellulose and can also leads to deconstruction of intramolecular hydrogen bonds network. The peaks at 2900 cm^{-1}, attributed to C-H

stretching in the methylene of cellulose and hemicellulose, also changed after pretreatment. The slight decrease in peaks at 1515 cm^{-1} is possibly due to the removal of lignin. The absorption band at 894 cm^{-1} indicates the presence of cellulose in the pretreated sample.

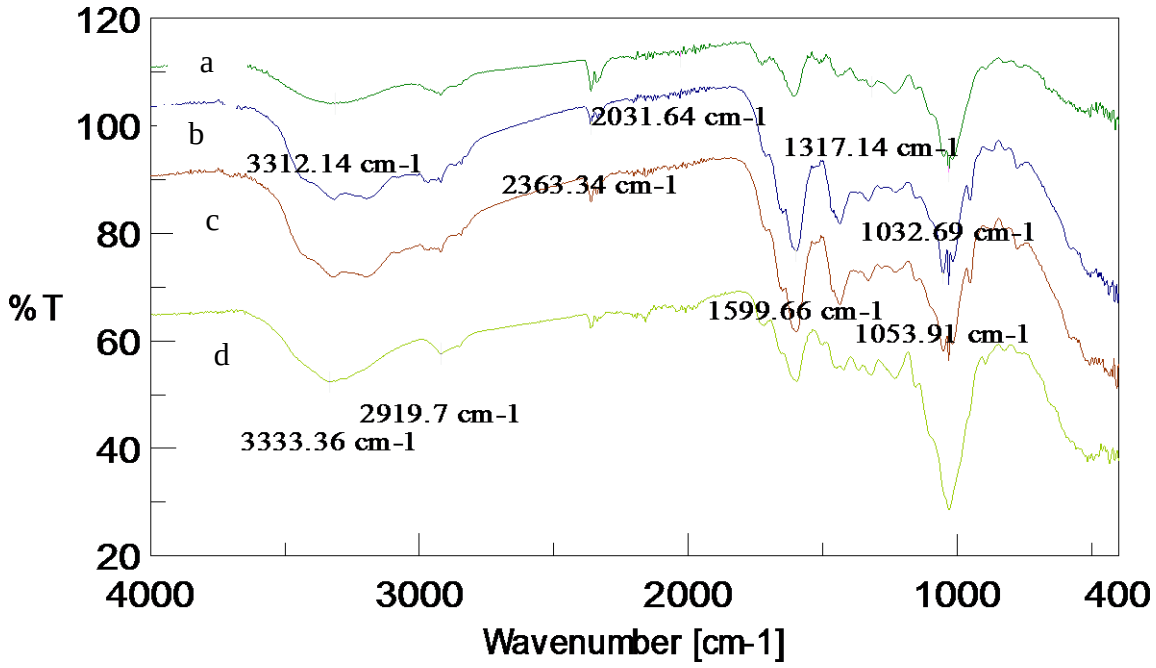


Figure 52: Comparison of FTIR spectra lignin extracted from *E. Grandis* treated with DES at 80°C for 0 (Raw Sample) (a),4 (b), 6(c), 8(d) hours.

Fig 52 represents the FTIR spectra of the lignin extracted from the pretreated sample, the disappearance of absorption band at 894 cm^{-1}, indicates the absence of hemicellulose and cellulose in the sample. The peaks at 3426, 1656 and 1513 cm^{-1} were also observed which also contributes to the presence of lignin. The disappearance at 894 cm^{-1} also indicates amorphous nature of the lignin sample extracted and it is devoid of cellulose.

3.4.4.3 Solubility and lignin removal:

Solubization rate (SR) is determined to assess the effect of ChCl/urea pretreatment on the separation capacity of *E. grandis* and its chemical fractions. It can be defined as the

rate at which a solvent solubilizes and in a medium and remains active. For sample pretreated by DES (ChCl/urea), SR was improved from 12.24% to 17.31% with an increase in the contact time from 4 h to 6 hr. ChCl/urea had an almost identical SR also from 17.31% to 18.03% with an increase in the rection time from 6 h to 8 h at 80˚C. It is concluded that the reaction time help to solubilize the biomass to a certain extent but it does not have significant effect. The lignin removal rate of the treatment is also not of much significant in this case. Whereas in case of alkaline peroxide treatment at 80 ˚C the highest lignin removal achieved was 50.27%, in DES at the same temperature highest lignin removal of 11.13% was obtained. So depending upon the lignin removal efficiency, the optimum condition for this treatment can be considered as 80˚C and 8h. The sample obtained from this condition can be used for subsequent hydrolysis and fermentation to compare with the sample obtained after alkaline peroxide treatment at the same temperature, although as indicated by previous study that reaction temperature rather than reaction time has significant effect in case of DES pre-treatment, the effect of temperature for this particular biomass is yet to be investigated.

3.4.4.4 Thermogravimetric analysis (TGA) of extracted lignin:

TGA can be used to determine the thermal stability and the decomposition pattern. The plots represent the interaction between weight loss percentage and the temperature. The analysis was done for the extracted lignin also. The aromatic rings present in lignin leads to a comparative wide range of degradation temperature from 0 to 700˚C as expressed by Lyu et al, 2019 (Lyu et al., 2019). At the end due to the presence of highly dense aromatic ring, produced during pyrolysis 30 to 40 wt.% of all lignin samples did not get volatized at 700˚C as seen in Fig 53. The degradation occurs in three stages, at first

evaporation of water occurred at around 30-120˚C. At next stage the carbohydrates degrade and releases volatile gases such as CO, CO_2 at around 180-350˚C. Lastly, above 350˚C, the volatile product such as phenolics, alcohols get removed. The plots also indicate that to initiate the degradation heat energy gets absorbed and the molecular chain degrade.

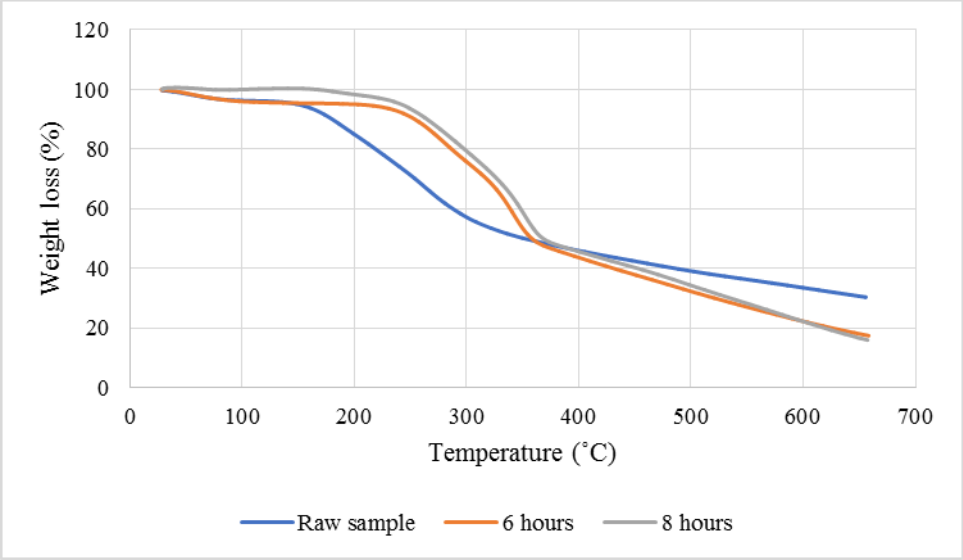
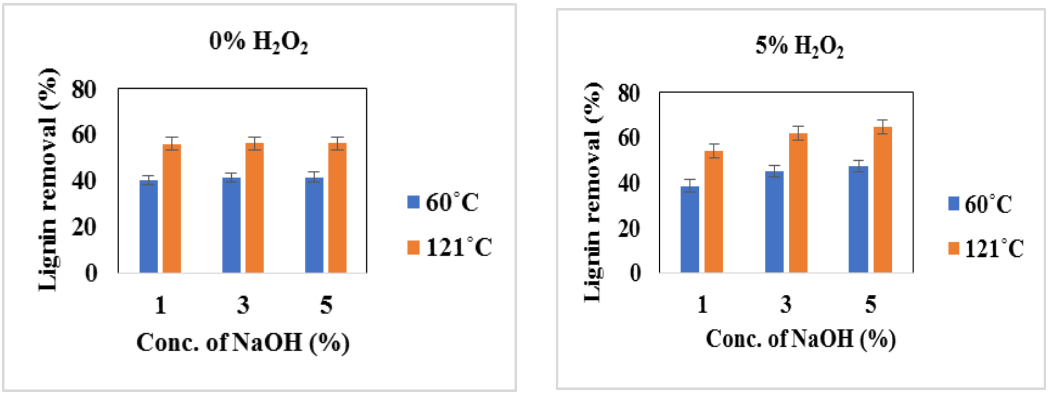


Figure 53: TGA plots of lignin from raw sample and sample treated for 6 hours and 8 hours obtained under nitrogen atmosphere at 10˚C/min.

3.4.5 Pretreatment of Garden Waste

3.4.5.1 Effect of NaOH and H_2O_2 Concentration and Temperature on Lignin Removal



(a) (b)

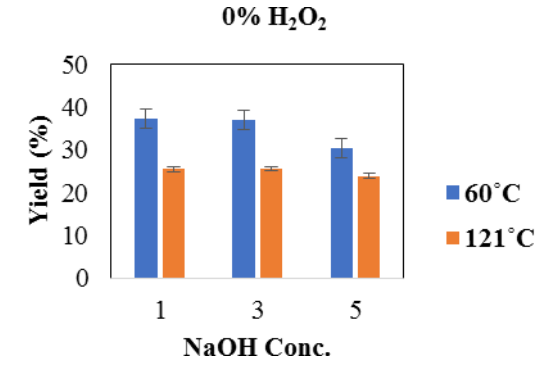
Figure 54: Effect of NaOH and H_2O_2 concentration on lignin removal percentage at different temperature conditions (a) 0% H_2O_2 (b) 5% H_2O_2

Figure 54 represents the effect of NaOH and hydrogen peroxide (H_2O_2) on lignin removal percentage. Figure 54 (a) depicts that for NaOH treated sample, the percentage of lignin removal dose not vary significantly at 60˚C as well as at 121˚C for different concentration of NaOH. But for a particular concentration of NaOH, as temperature increases from 60˚C to 121˚C the lignin removal percentage increases from significantly. This is due to the fact that at 121˚C the sample was treated in an autoclave under elevated temperature and pressure and the lignocellulosic matrix got disrupted in the process. As a result, lignin got removed from the sample. So, during control experiment where no hydrogen peroxide was added, temperature is an important factor rather than concentration of NaOH. From figure 54 (b), in presence of H_2O_2 in the reaction mixture it can be observed that as temperature increases lignin removal also increases significantly at 60˚C as well as for autoclave assisted treatment at 121˚C. As concentration of NaOH is concerned, with increase in

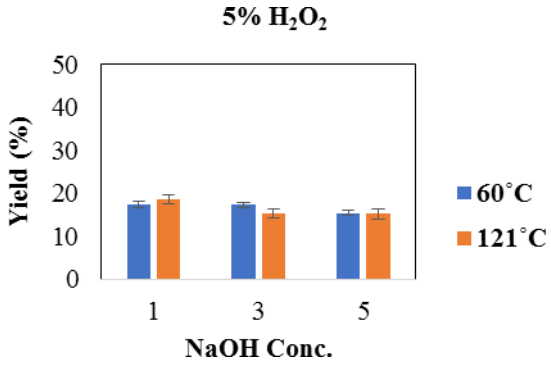
concentration the lignin removal percentage also increases for both the operating conditions and highest lignin removal percentage was obtained at a combination of 5% NaOH and 5% H_2O_2 under autoclave assisted treatment at 121˚C. The results showed that alkaline peroxide treatment under autoclave condition as an effective delignification technique for garden waste. It also suggests that the procedure is highly temperature dependent. As at high temperature active hydroxyl radicals (HO*) were formed which helps in degradation of lignin (Sardar et al., 2021). The hydroxyl radicals and the anion radicals (O_2*) which generated on decomposition of peroxide probably also assisted in disbanding of lignin and to some extent, in bleaching (Dence, 1996).Under autoclave assisted condition due to the production of OH- ions the bonds present in lignin hetero polymer structure gets dissociated. In alkaline peroxide pretreatment H_2O_2 acts as an oxidizing agent, thereby making the process oxidative. The presence of H_2O_2 in the reaction medium assisted in solubilizing lignin and hemicellulose present in the raw biomass sample (G. Shen et al., 2011). Figure 55 represents the visual of lignin extracted and isolated with the help of Gooch crucible by following the TAPPI protocol mentioned earlier for acid soluble and insoluble lignin.

Figure 55: Extracted lignin and the Gooch crucible used to extract the lignin.

3.4.5.2 Effect of NaOH and H_2O_2 Concentration and Temperature on Yield



(a)



(b)

Figure 56: Effect of NaOH and H_2O_2 concentration on yield at different temperature conditions (a) 0% H_2O_2 (b) 5% H_2O_2

Figure 56 represents the change in percentage of yield at different pretreatment conditions and at different concentrations of H_2O_2 and NaOH. As evident from the graph, as temperature increases from 60˚C to 121˚C i.e., from normal heating to autoclave assisted heating, the yield gradually decreases and it continued to decrease as the percentage of H_2O_2 increases. This could be due to the hard to avoid material loss during the pretreatment process and subsequent washing and filtration process. Lower lignin content (7.92%) can

also play a significant role. Moreover, the overall yield from the pretreatment obtained is also not sufficient enough to proceed for further treatment from a techno economic stand point.

3.4.5.3 Valorization of Solid Residue as Fertilizer

The solid residue obtained after the pretreatment was endeavored to valorize as a fertilizer. The carbon-to-nitrogen ratio of organic matter means the amount of carbon relative to the amount of nitrogen present. The carbon-to-nitrogen ratio (C: N) and is usually a single number (Akratos et al., 2017).The carbon to nitrogen (C/N) ratio is a substantial factor in composting as a well-balanced carbon and nitrogen ratio, ranging between 25 to 35, is required for microorganisms to continue on being active and thrive. High values of carbon to nitrogen ratios can affect the duration of composting by making it longer than usual and low values of carbon to nitrogen ratios can augment the nitrogen loss (Brust, 2019). As the C:N ratio decreases, release of nitrogen into the soil will increase for the crops to use immediately (Watson et al., 2002). whereas a carbon to nitrogen ratio less than 35 indicates a possibility of immobilization of the microbes. In this study the carbon to nitrogen ratio of all the samples obtained after treated with different concentration of NaOH, H_2O_2 and under different operating conditions, are found to be in the range of 30 to 35. The highest and the lowest C:N ratio was obtained respectively at 1%NaOH under 60˚C and with the combination of 5%NaOH, 5% H_2O_2 in an autoclave under 121˚C. Therefore, it can be concluded that the solid residue obtained after pretreatment can be used as fertilizer. The total nitrogen (N), phosphorus (P), and potassium (K) content as obtained from the product has been tabulated in table 12 which shows the suitability to be used as an organic fertilizer.

Table 12: N, P and K ratio of the product obtained after pretreatment.

Method	Component	%
Phytate Phosphorus	phosphorus (P)	0.84
Kjeldahl method	Total nitrogen (N)	1.01
Potassium	Potassium (K)	0.40

3.5 Conclusion

In this study, the potential of *Sterculia foetida* fruit shells as feedstock for bioethanol production have been demonstrated by employing effective pretreatment methods. Alkaline pretreatment was found to be ineffective with respect to lignin removal compared to alkaline peroxide pretreatment. The alkaline peroxide pretreatment of the biomass was carried out with two different sets of conditions: low temperature heating (60℃) and in an autoclave (121℃) while the former being more effective leading to a conclusion that time rather than temperature was more predominant factor in alkaline peroxide pretreatment. The biomass structure before and after pretreatment was analysed using FTIR and XRD techniques. The highest lignin removal was achieved by using the combination of 3% H_2O_2 and 5% NaOH solution. It was also observed that upon increasing the pretreatment severity there was an appreciable decrease in the reducing sugar yield. Moreover, the effect of the pretreatment conditions on the lignin chemistry was investigated. Although there is a need for further research regarding scale-up, it can be said from the above results that optimized alkaline peroxide pretreatment has immense potential as a suitable pretreatment technique for the production of biofuels from lignocellulosic

biomass. It is hoped that the results of this study will provide valuable insight into the low-cost alkaline peroxide pretreatment method for lignocellulosic biomass.

In the present study, the prospect of *Eucalyptus grandis* bark as feedstock for bioethanol production have been investigated by using different pretreatment conditions. Alkaline pretreatment at low temperature (60°C) as well as at high temperature was observed to be not effective with regard to lignin removal and improving reducing sugar content compared to that of alkaline peroxide pretreatment. Out of the three temperature conditions (60℃, 80°C and 121℃, in an autoclave) the alkaline peroxide pretreatment was executed at, former was more inefficient and lead to a fact that reaction temperature rather than reaction time was more major factor in alkaline peroxide pretreatment. Lignin removal was obtained to be highest for the combination of 2% H_2O_2 and 8% NaOH solution, especially in high temperature conditions. Although additional study is needed concerning scale-up, it can be concluded based on the data obtained that improved alkaline peroxide pretreatment has enormous prospect as a pretreatment technique suitable for utilizing the biomass. It is hoped that the results of this study will provide valuable insight into the low-cost alkaline peroxide pretreatment method for lignocellulosic biomass.

As from a techno economic stand point, for garden waste the overall yield obtained from the pretreatment is not sufficient to proceed for hydrolysis and subsequent fermentation to produce bioethanol, pretreated garden waste was investigated to be used as biofertilizer.

CHAPTER 4

ENZYMATIC HYDROLYSIS AND FERMENTATION

CHAPTER 4

ENZYMATIC HYDROLYSIS AND FERMENTATION

The percentage of sugar yield is highly reliant on the type of pretreatment and effectiveness of enzymes. Lack of proper pretreatment may require higher enzyme loading which in turn jeopardizes the techno economic scenario and increases the cost of production (Piccolo & Bezzo, 2009). The activity of enzymes influences the product release, as well as enzyme substrate interaction (Chen et al., 2008). The products of enzymatic hydrolysis include hexose or pentose sugars such as glucose, cellobiose, xylose, arabinose, glucan. Cellulase and and β-glucosidase is the primary enzymes required for the enzymatic hydrolysis of lignocellulose (Juturu & Wu, 2014). Along with cellulase, usage of hemicellulase, xylanase, and lignolytic enzymes are important to hydrolysation of the lignocellulosic biomass to produce fermentable sugar (Beg et al., 2001). The enzymes can be used as a cocktail or even separately also. Enzyme hydrolysis generally carried under mild conditions with pH-4.8 and temperature 45-50 °C and could take several days to form product. This provides advantages over comparatively harsh conditions acidic hydrolysis (Binod et al., 2019). Number of active sites controls the rate of reaction and more active sites yields higher activity. As a result, the reaction is initiated at a faster pace and forms the product. Main source of cellulases are fungi such as *Trichoderma reesei* and *Aspergillus niger*, and both are used to produce commercial enzyme cocktail (Kalyani et al., 2013). There are also instances where use of enzymes isolated from soil, tree bark and termites to hydrolyse the biomass was observed to yield reducing sugars. Although the process is a slow one, the usage of microbes derived enzymes are frequently reported in the literature (L. Chen et al., 2013a; Sukumaran et al., 2009).

Depending on the type of enzyme used and the source of the biomass, composition of the product or sugar hydrolysate varies (Skoulou et al., 2011).

For fermentation of hydrolyzed biomass, microorganisms such as *Saccharomyces sp., Zymomonas sp.,* are widely used commercially to produce ethanol industrially in a large scale (A. Gupta & Verma, 2015). These organisms often genetically modified and the modified strains are used to obtain high productivity and a wide range of tolerance towards varying reaction condition (T. Guo et al., 2013; Ndimba et al., 2013).High-performance liquid chromatography (HPLC) can be used for quantification of the product.

The aim of this study was to obtain optimal enzyme concentrations and enzyme mixtures for hydrolysis of pretreated biomass of *Eucalyptus* and *Sterculia*. The study was divided into two stages: first, thorough cleaning of the biomass after pretreatment to wash out any inhibitors formed during pretreatment, and secondly, treating the biomass with different enzyme to substrate ratio. *Saccharomyces cerevisiae* was used to ferment the sugar hydrolysate obtained after the enzymatic saccharification of alkaline peroxide pre-treated *Sterculia foetida* and *Eucalyptus grandis* for the production of biofuel.

4.1. Material & methods

4.1.1. Materials

Enzymatic hydrolysis of the pretreated sample was carried out on two samples which during pretreatment showed significantly better results in respect to reducing sugar production and lignin removal. Hydrolysis was done in a round bottom flask using a cocktail of enzyme prepared in combination with cellulase (Plant culture tested, Himedia, 10U/mg) and β-glucosidase (G0395, 2U/mg, derived from almonds) in a ratio of 1:2 (unit basis). 50 ml of citrate buffer (pH 4.8) along with 0.1% (w/v) of sodium azide to prevent any microbial contamination.

The reaction mixture, i.e., substrate, sodium azide and citrate buffer, was placed in a conical flask and agitated at 110 rpm. As the reaction temperature reached 50˚C, the enzyme cocktail is added to start hydrolysis. Sampling was done at different time intervals. Enzyme cocktail concentration of 1.5 g/L and 10.5 g/L was used for the study. After each sampling, the samples collected from enzymatic hydrolysis required to be kept in a boiling water bath for a minimum of 10 minutes in order to deactivate the enzyme and to terminate the reaction (L. Chen et al., 2013b).

For enzymatic hydrolysis of DES treated *E. grandis* sample, 1g of pretreated biomass was hydrolyzed with the enzyme cocktail used previously along with 10 mL citric acid-sodium hydroxide buffer solution (pH 4.8) and were taken into a flask and incubated at 50˚C for 48 h.For this case also collected samples were boiled for a minimum of 10 minute to quench the reaction and centrifuged to further use the sample for sugar analysis.

Following table 13 demonstrates the experimental design:

Table 13: Experimental design of enzymatic hydrolysis

Substrate	Pretreatment combination		Enzyme conc. (g/l)	Substrate Conc. (g/l)
S. foetida	1 NaOH and 2 H_2O_2 under low temperature condition (60˚C)	5 NaOH and 3 H_2O_2 treated under low temperature condition	1.5 g/L	10
				30
				50
			10.5 g/L	10
				30
				50
E. grandis	8 NaOH and 2 H_2O_2 under autoclave assisted condition	8 NaOH and 2 H_2O_2 under high temperature condition (80˚C)	1.5 g/L	10
				30
				50
			10.5 g/L	10
				30
				50
	DES (ChCl/urea) (molar ratio 1:2) at 80 ˚C		1.5 g/L	10
				30
				50
			10.5 g/L	10
				30
				50

The sugar hydrolysate used for the fermentation study was obtained from Eucalyptus and *Sterculia foetida* sample which had been pretreated with different concentrations of alkaline peroxide at different reaction conditions and then enzymatically hydrolysed.

4.1.2. Microorganism and media:

Saccharomyces cerevisiae strain was procured from MTCC, Chandigarh and was grown at 30±5˚C and maintained at 4˚C on YPD agar both in liquid and solid media. YPD is also known as YEPD for its composition of yeast extract, bacteriological peptone, glucose, and agar. Media was made by suspending 65 g in 1 L of distilled water followed by boiling while stirring to dissolve all ingredients completely. Finally, autoclaving was done for 15 minutes at 121˚C.

4.1.3. Inoculum preparation:

Volumes of 50ml media was autoclaved in 250 ml cotton plugged Erlenmeyer flasks followed by incubation for 30h at 35±0.5˚C in an incubator shaker with a shaker speed of 130 rpm. After the incubation period is over the content of the flasks were centrifuged and used for fermentation. It resulted in 0.98±0.3g biomass of *S. cerevisae* in 100ml working volume in all fermentation experiments.

The fermentation was done in cotton plugged Erlenmayer flasks kept at 30±5˚C at initial pH of 4.5 and 150 rpm for 42 hours. The sample has been collected periodically after a certain time gap.

4.1.4. Analytical Procedure:

Waters made HPLC with RI detector (Waters, model 2414) was used to quantify sugars, and ethanol obtained after enzymatic hydrolyzation and fermentation of the biomass with the help of a waters made amino column. HPLC grade acetonitrile was used as mobile phase at ambient temperature with HPLC grade water [80:20 (v/v)]. Sugars are quantified with the help of standard curve of glucose. Glucose is one of the most important substrates for fermentation process in order to produce ethanol.

4.1.5. Kinetics of enzymatic hydrolysis:

According to literature (Andrić et al., 2010), the enzyme activity often gets inhibited by the glucose produced during enzymatic hydrolysis. The literature also indicates that competitive inhibition can be observed in a system involving cellulase, glucose and β- glucosidase (Baksi, Sarkar, et al., 2019).

A model based on pseudo-homogenous Michaelis-Menten mechanism, from where the production of sugar from initial concentration of a soluble substrate to the final concentration of the product is predicted (Teoh & Mat Don, 2011):

$$\frac{dC}{dt} = \frac{K[E_0](C_{ult} - C)}{K_M\left[1+\left(\frac{1}{K_1}\right)C\right]+0.9(C_{ult} - C)} \quad (2a)$$

In case of no inhibition involved,

$$\frac{dC}{dT} = \frac{k[E_0]S}{K_M + S} \quad (2b)$$

Here, C_{ult} indicates the ultimate concentration of the glucose concentration. 0.9 (c_{ult}-C) indicates the hypothetical substrate concentration where the ratio of the molecular weight of glucose in cellulose to that of glucose, 0.9 is constant. K is the apparent rate constant indicating binding frequency between cellulose and cellulase, K_M is apparent Michaelis constant which indicates the affinity between cellulose and cellulase. K_1 is proposed to be the apparent competitive inhibition constant between total sugar and cellulases.

To examine the kinetic model in Eq. (2a), parameters of the processes are determined as:

First, total sugar produced at initial stage ($t \rightarrow 0$) can be neglected ($C \rightarrow 0$), therefore,

$$\left(\frac{dC}{dt}\right)_{t\to 0} = \frac{K[E_0]C_{ult}}{K_M + 0.9C_{ult}}$$

Or

$$\frac{1}{\left({}^{dC}/_{dt}\right)_{t\to 0}} = \frac{K_M}{k[E_0]C_{ult}} + \frac{0.9}{K[E_0]} \qquad (3)$$

Second, integrating Eq. (2b) under the conditions ($C=C_0$ at $t=0$ and $C=C$ at $t=t$) to determine the parameter K_1 the equation is as follows:

$$\frac{t}{0.9(C-C_0)} = \beta \frac{ln\left[{}^{C_{ult}-C_0}/_{C_{ult}-C}\right]}{0.9(C-C_0)} - \gamma \qquad (4)$$

Or $Y = \beta X - \gamma$ Where,

$$\beta = \frac{K_M C_{ult}}{KK_1[E_0]} + \frac{K_M}{K[E_0]} \qquad (5)$$

$$\gamma = \frac{K_M}{0.9KK_1[E_0]} - \frac{1}{K[E_0]} \qquad (6)$$

From the slope and intercept of the plot of Y vs. X, the values of β and γ can be calculated. The scattering nature in the data plotted on Eq. (6) makes the simultaneous determination of accurate values of K_M, K_1 and K values more difficult. The two step determination of obtaining the values has been adopted in order to remedy the problem.

4.1.6. Kinetics of fermentation:

As the growth kinetics of the microorganisms, modified Monod kinetics has been used (D. Wang et al., 2004), as the classical Monod model has comparatively low chances to fit processes of fermentation in many cases. As per the literature, an equation based logistic model for growth associated production of ethanol is followed considering the boundary conditions as:

$$t = 0, X = X_0, S = S_0.$$

The substrate consumption rate for ethanol fermentation process includes the reducing sugar consumption for the growth of the microorganism and the maintenance of the biomass. The consumption rate of sugar is:

$$-\frac{dS}{dt} = \frac{1}{Yx_{/s}} \cdot \frac{dX}{dt} + m \cdot X \qquad (7)$$

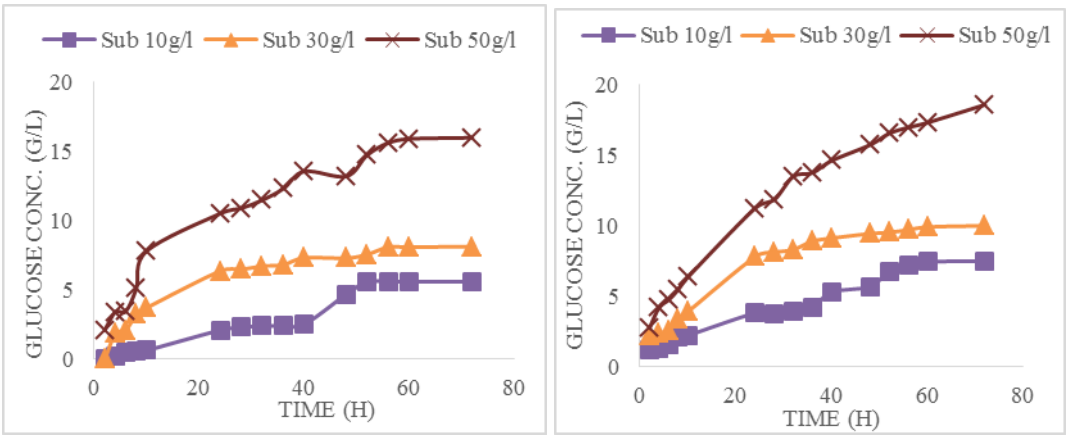
To incorporate the yeast lag growth phase, lag time parameter, Δt is introduced and the ethanol production rate was modified as:

$$\frac{dP}{dt} = Y_{p_{/x}} \frac{dX}{d(t-\Delta t)} \qquad (8)$$

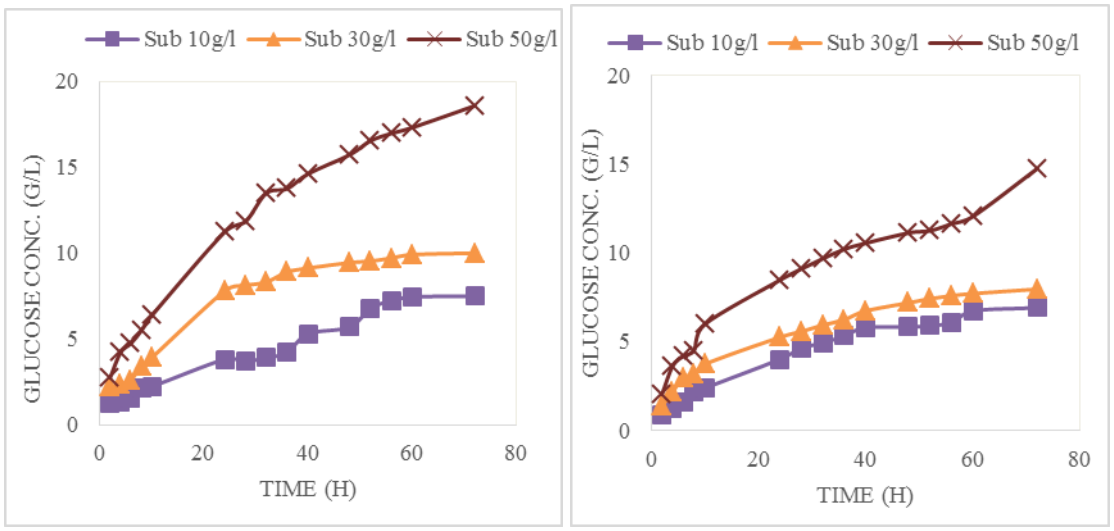
Yield coefficient of ethanol on biomass, $Y_{p_{/x}}$ can also be determined from this equation.

4.2. Results & discussion

4.2.1. Enzymatic hydrolysis (*Sterculia foetida):*



(a1) (a2)



(b1) (b2)

Figure 57: Variation of glucose production with time under different enzyme concentration and different pre-treatment combination of *Sterculia foetida* (a)1%NaOH & 2% H_2O_2 and (b) 5% NaOH & 3% H_2O_2 at (1) 1.5 g/L enzyme conc. and (2) 10.5 g/L enzyme conc.

From Figure 57 it can be observed that the production of glucose increases as substrate concentration increases from 10g/L to 30g/L and 30g/L to 50g/L in case of a certain

enzyme concentration. However, as substrate concentration further increases in case of 5% NaOH and 3% H_2O_2, glucose recovery decreases significantly. That could be due to the inhibitory products formed during the process. It can be explained with the help of table 14 containing the values of kinetic constants. Additionally, higher concentration of enzymes for a certain substrate concentration can be linked with increased glucose generation. In case of 1% NaOH and 2% H_2O_2 as enzyme concentration as well as substrate concentration increases the glucose concentration also increases. The results with 3% NaOH and 5% H_2O_2 also exhibited the same trend but in this case the glucose concentration produced is not that high. This could be due to the severity of the pretreatment conditions which is in accordance with the XRD data.

Table 14: Kinetic constants obtained from the model for product inhibition of enzymes.

		E=1.5 G/L			**E=10.5G/L**		
SAMPLE	kinetic constants	10g/L	30g/L	50g/L	10g/L	30g/L	50g/L
1% NaOH 2%H_2O_2	K		2.64			0.113	
	K_m		26.04			14.16	
	K_1	6.09	12.82	31.13	3.28	5.95	14.13
5%NaOH 3%H_2O_2	K		9.51			1.26	
	K_m		42.05			19.34	
	K_1	8.37	40.36	50.49	16.67	40.62	42.35

It can be observed from the table that for the combination of 1% NaOH and 2% H_2O_2, the K_1 values increases with increase in enzyme concentration from 1.5g/L to 10.5g/L. It showed a similar trend in case of 5% NaOH and 3% H_2O_2. In the context of substrate concentration also, as the substrate concentration increases from 10g/L to 30g/L as well as 30g/L to 50g/L, the value of K_1, competitive inhibition constant of glucose and cellulase increases. Although, it has been reported that after a certain increment in enzyme concentration, the K_1 values start to reduce due to the decreased ability of the enzymes to

penetrate in to the biomass (Baksi, Sarkar, et al., 2019; Teoh & Mat Don, 2011). From the results obtained, It can be concluded that the enzyme concentration can be increased upto 10.5g/L of substrate and substrate concentration can be increased upto 50g/L without decreasing the accessibility of enzyme particle to the core area of the biomass.

4.2.2. Enzymatic hydrolysis (*Eucalyptus grandis)*

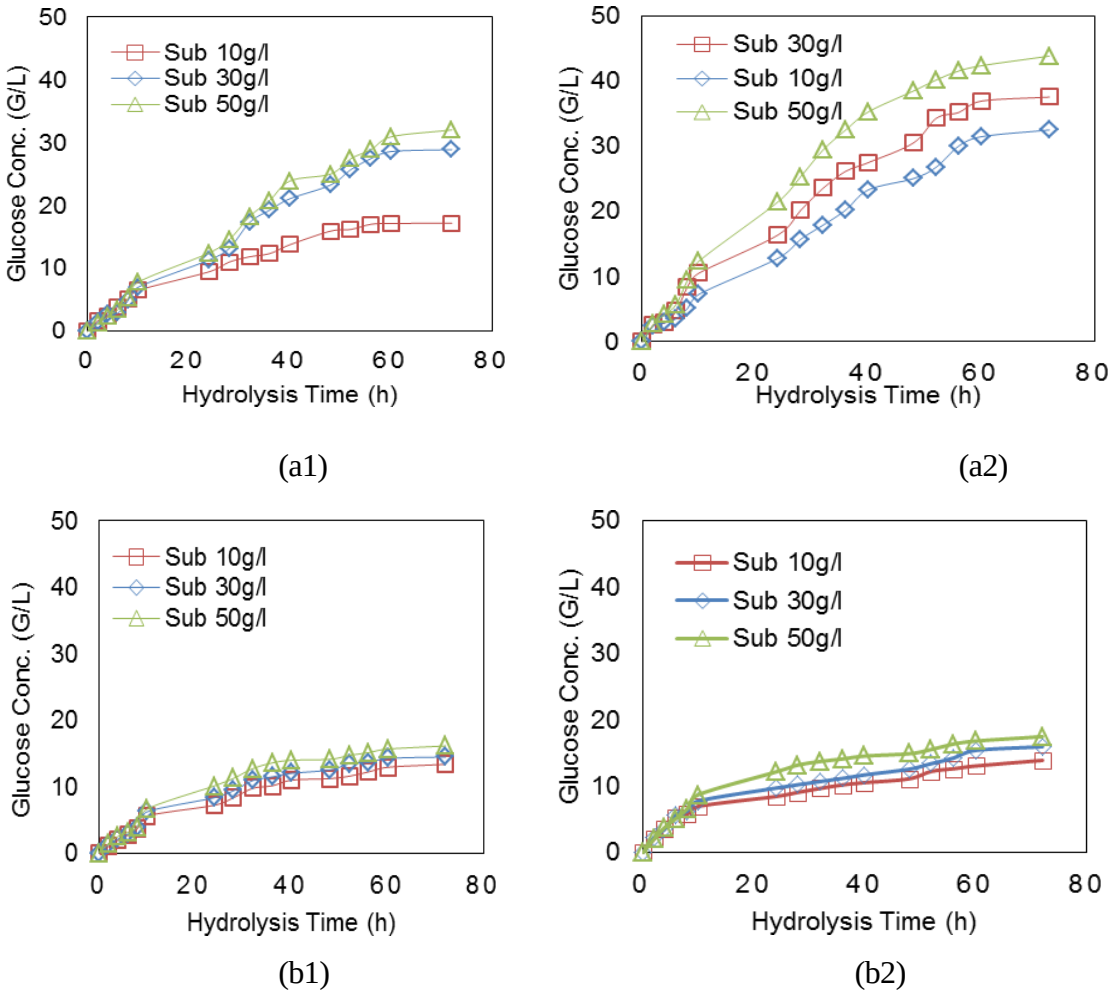


Figure 58: Enzymatic hydrolysis of *E.grandis* as a function of time at different enzyme concentration and different pre-treatment combination (a)8%NaOH & 2% H_2O_2 for AAHP and (b)8%NaOH & 2% H_2O_2 for AHP-80 at (1) 1.5 g/L enzyme conc. And (2) 10.5 g/L enzyme conc.

From Figure 58 shows the glucose concentration measured by HPLC over time during enzymatic hydrolysis of pretreated EG biomass. From Fig 58, it can be observed that glucose generation increased over time with the increase in substrate concentration for a particular enzyme loading. Also, for a certain substrate concentration while reaction condition become severe i.e., reaction temperature increases, glucose recovery increases significantly. It was probably due to the fact that as lignin removal percentage increases the enzyme gets more active sites. It can also be explained with the help of Table 15 containing the values of kinetic constants.

Table 11 also shows that the pretreatment increases the accessible surface area and pore size and increased pore volumes caters to the increase in glucose yield due to hydrolysis. Additionally, higher concentration of enzymes for a certain substrate concentration facilitated increased glucose generation. This was due to availability of higher amount of enzymes which can attack more active sites within a certain period of time generating higher amount of glucose. Table 15 demonstrates the values of the kinetic constants obtained from the kinetic model. For both AAHP and AHP-80, it is observed that the variation in substrate concentration can influence the apparent rate constant k which indicates the binding frequency between substrate and enzyme. As substrate concentration increases the apparent rate constant decreases significantly in case of AAHP. The values of the apparent Michaelis constant, K_M which indicates the affinity of cellulase to cellulose also decreases in case of AAHP as substrate concentration increases. An opposite trend was observed for AHP-80 for which K_M increases with increase in substrate concentration. It signifies that hydrolysis process was highly dependent on substrate concentration. The K_M is at its lowest at AHP-80 for enzyme

concentration 1.5 g/L but the highest concentration of glucose was obtained at AAHP and 10.5 g/L enzyme concentration. This may be due to the low lignin removal percentage at AHP-80 conditions which hinders the accessibility of the cellulose matrix. On the other hand, at AAHP, the pretreatment condition similar to steam explosion leads to more efficient rupture of lignocellulosic matrix, thereby aiding the glucose production.

Table 15: Values of the kinetic constants obtained for the product inhibition of enzymes

Sample		E=1.5 g/L			E=10.5g/L		
	Substrate Conc.	10g/L	30g/L	50g/L	10g/L	30g/L	50g/L
AAHP (8%NaOH 2%H_2O_2)	C_{ult}	13.5	14.8	16.3	13.91	15.96	17.49
	k (h^{-1})	4.95			0.142		
	K_M (g/L)	166.4			16.38		
	MSE	0.372	0.29	0.27	3.49	4.87	2.41
AHP-80 (8%NaOH 2%H_2O_2)	C_{ult}	18.45	34.41	37.59	33.57	40	44.5
	k (h^{-1})	0.41			0.165		
	K_M (g/L)	12.4			16.82		
	MSE	2.71	7.03	11.73	40.33	31.95	12.62

Validation of the kinetic model with respect to the obtained experimental data is essential. Comparison of the data obtained from theoretical and experimental outcome under different conditions are presented in figure 59 and figure 60. It can be observed that the Michaelis Menten kinetics based on no inhibition model fits well with the experimental data with MSE value <5% except for AHP-80 with enzyme conc. 10.5g/L.

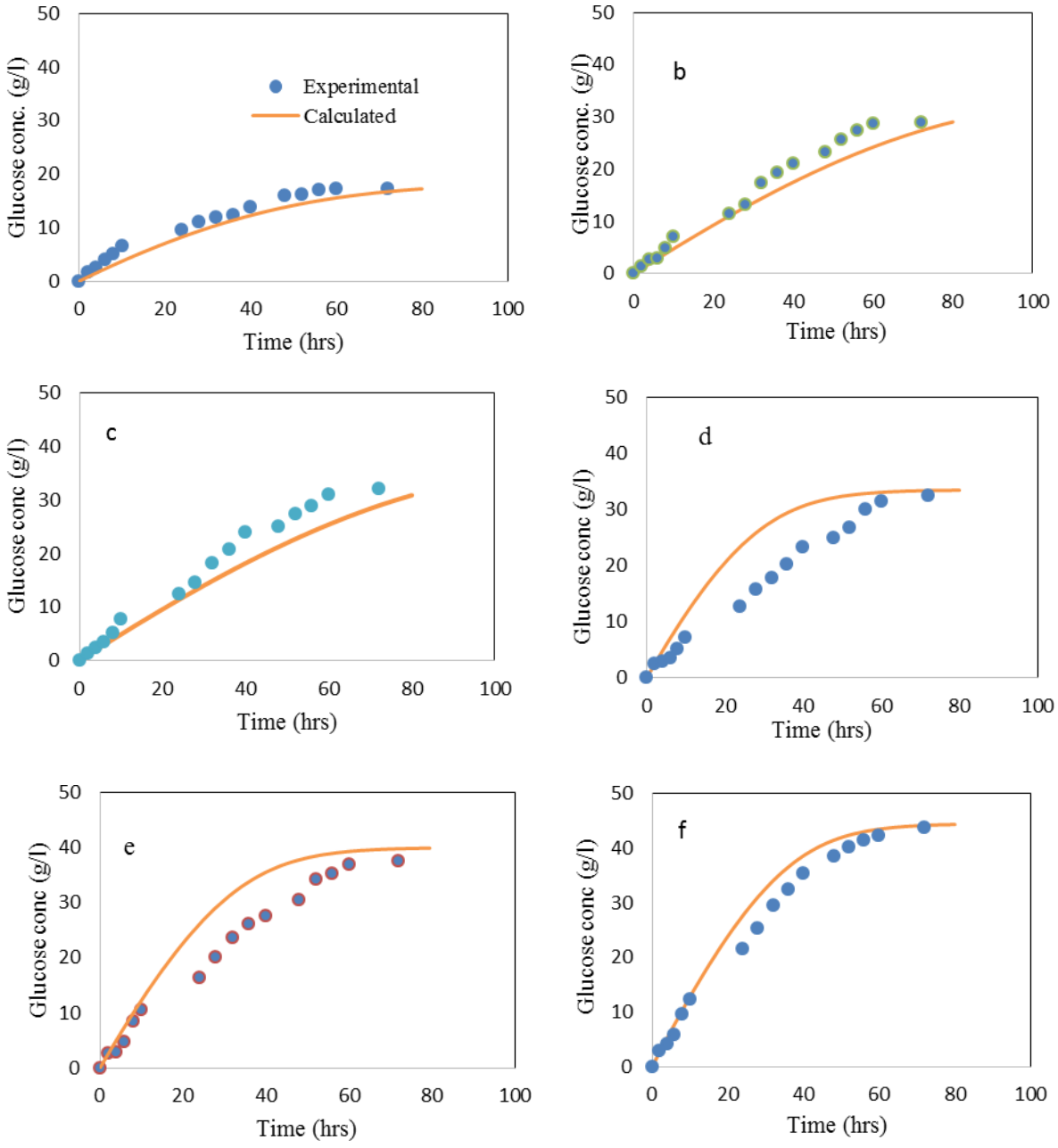


Figure 59:Validation of theoretical and experimental data with various substrate and enzyme loading for AHP-80 [a:(S)=10g/l,(E)=1.5g/L, b: (S)=30g/l,(E)=1.5g/L, c: (S)=50g/l,(E)=1.5g/L, d: (S)=10g/l,(E)=10.5g/L, e: (S)=30g/l,(E)=10.5g/L, f: (S)= 50g/l,(E)=10.5g/L]

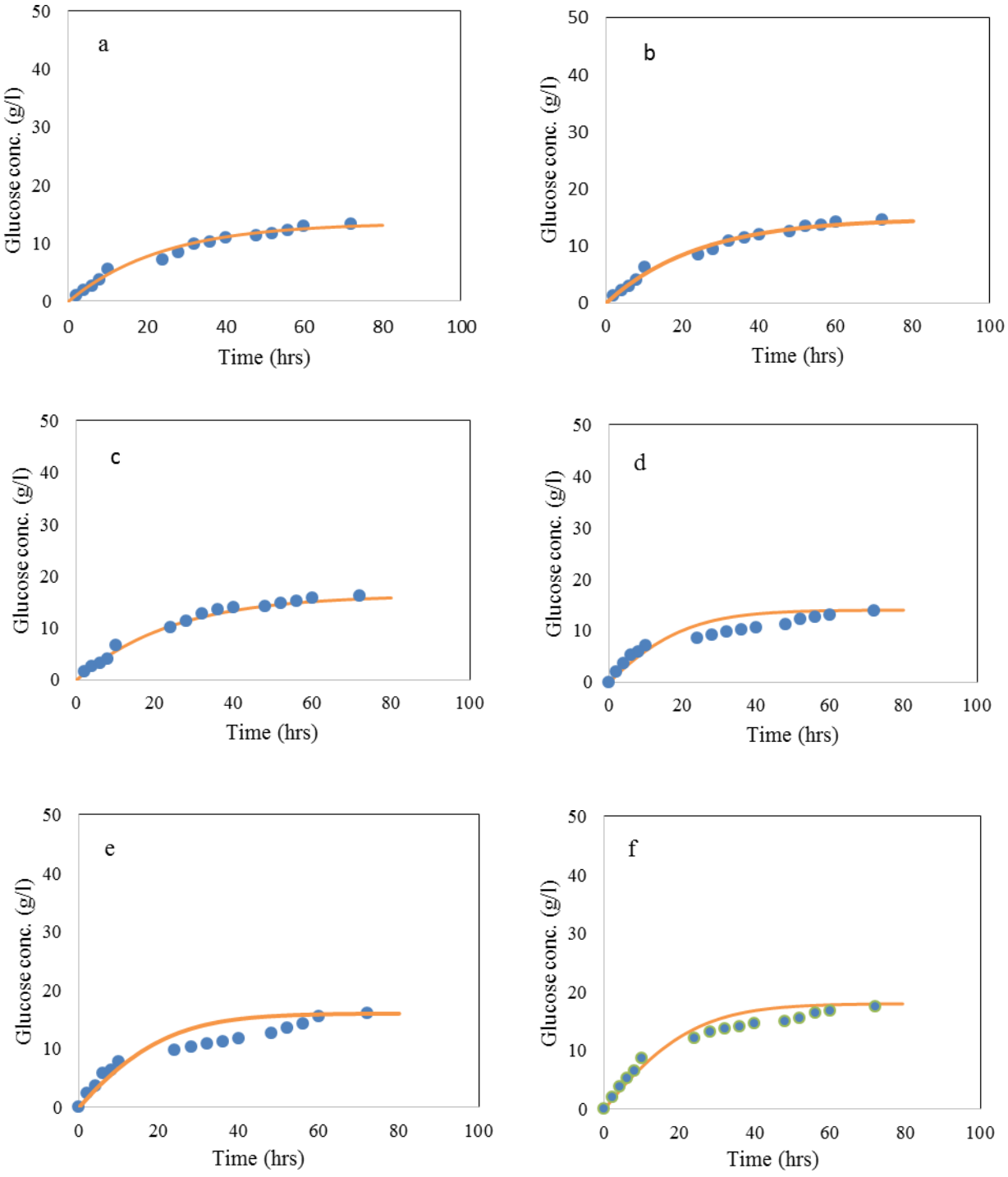


Figure 60:Validation of theoretical and experimental data with various substrate and enzyme loading for AAHP [a:(S)=10g/l,(E)=1.5g/L, b: (S)=30g/l,(E)=1.5g/L, c: (S)=50g/l,(E)=1.5g/L, d: (S)=10g/l,(E)=10.5g/L, e: (S)=30g/l,(E)=10.5g/L, f: (S)=50g/l,(E)=10.5g/L]

4.2.3. Enzymatic hydrolysis of DES pretreated *Eucalyptus grandis*

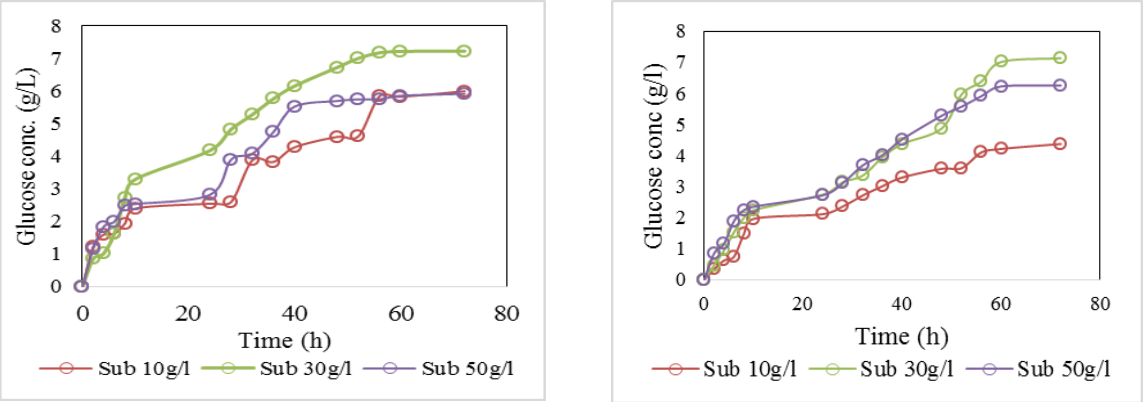


Figure 61: Variation of glucose production with time under different enzyme concentration for E.grandis treated by DES at 80˚C, 8h at (1) 1.5 g/L enzyme conc. And (2) 10.5 g/L enzyme conc.

Figure 61 represents the glucose yield in the enzymatic hydrolysis of *E.grandis* under enzyme concentration of 1.5g/L and 10.5g/L. The substrate concentration was maintained at 10g/L, 30g/L and 50g/L. It was found that the enzymatic hydrolysis did not produce significant sugar concentration as compared to that treated with alkaline peroxide at same temperature.

Table 16: Values of the kinetic constants calculated from the model for DES treated *E.grandis*

		E=1.5 G/L			E=10.5G/L		
SAMPLE	kinetic constants	10g/L	30g/L	50g/L	10g/L	30g/L	50g/L
DES (80˚C, 8H)	K		1.31			0.49	
	K_m		12.63			7.29	
	K_1	7.69	28.83	35.21	3.21	5.37	11.05

Table 16 represents the values of kinetic constants, which are in accordance with the results obtained. It may be due to the inefficiency of ChCl/urea pretreatment at a lower

temperature of 80˚C. The lower saccharification efficiency may be due to the fact DES has influence of cellulase enzyme (A. K. Kumar, Parikh, Shah, et al., 2016). In this study the highest glucose yield of 7.22g/L is observed at a substrate concentration of 30g/L and enzyme concentration of 1.5g/L. It also followed a same trend when hydrolysed with enzyme concentration of 10.5g/L and same substrate concentration. The sample with higher glucose yield will be used for fermentation in order to investigate and compare the results.

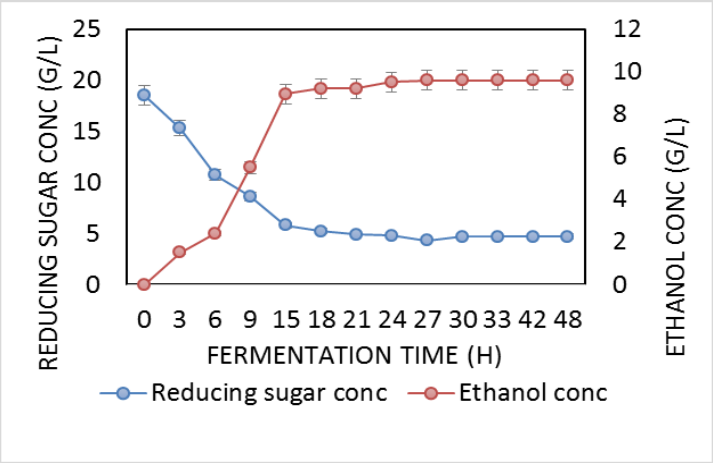
In order to compare the glucose concentration obtained in this study with other works as reported in the literature, from these three types of garden wastes, a tabulated version, table 17 is represented below. As mixed garden waste and fruit shell of *S. foetida* is an unexplored source of biofuel the glucose concentration data obtained after hydrolysis is unavailable for these two.

Table 17: Comparison of glucose concentration data as reported in literature

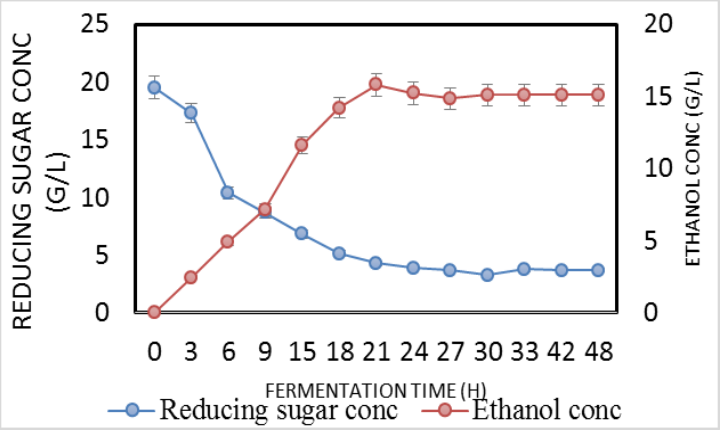
Biomass	Reaction condition	Glucose concentration (g/L)	Reference
Eucalyptus grandis bark	Pretreatment used: AAHP Enzyme: Cellulase and β glucosidase Enzyme conc: 10.5g/L Substrate conc: 50 g/L Time: 72 h	43.82	(Present study)
Eucalyptus grandis sawdust	Pretreatment used: Alkaline pretreatment with NaOH, 155 ˚C and 45 min Enzyme: Novozyme CelliCTec 2 Enzyme loading:16% (w/v) solid load	12.0 ± 0.1 xylo-saccharides, 2.0 ± 0.1 gluco-saccharides, 0.4 ± 0.1 arabino-saccharides	(Guigou et al., 2023)
Eucalyptus globulus bark	Pretreatment used: Hydrothermal (Tmax=228˚C) Enzyme: Cellic Ctec2 Enzyme Loading: 20 FPU/g solid	54 g/100 of raw material	(Gomes et al., 2021)
Eucalyptus globulus bark	Pretreatment used: Kraft pulping Enzyme used: cellulase consortium (Cellic® CTec2), with additives (PEG 4000, Tween 80) Time: 24 h	161	(Amândio et al., 2023b)

4.2.4. Fermentation (*S. Foetida*):

Since sugars are available in a degradable form and because of the ability of yeast cells to metabolize sugar directly, the process requires low cost operation. The reducing sugar consumption and ethanol production were investigated for 48 hours of ethanol fermentation using *S. cerevisiae* yeast in the hydrolysed biomass obtained after enzymatic hydrolysis. Concentration of ethanol produced and consumption of reducing sugar increased significantly till 27h for *Sterculia foetida* sample pretreated under 1%NaOH and 2%H_2O_2 concentration. After 27 h the concentration of ethanol remains unchanged throughout the experiment time (48h), which indicates the stationery growth phase for yeast. The same trend is observed in case of *Sterculia foetida*, hydrolysed at 1.5g/l of enzyme concentration and pretreated under 5%NaOH and 3% H_2O_2 concentration. In this case, the ethanol production and reducing sugar consumption was at its peak at 21h with a production rate of 15.8 g/l and after 21 hour it decrease upto 27 hours and then remains unchanged. For *Sterculia foetida* the combination 5NaOH, 3 H_2O_2 gives better ethanol conversion when fermentation followed by hydrolysis at substrate conc of 50g/L and 1.5g/L of enzyme concentration. The results represented in figure 62 also showed that the hydrolysed *S. foetida* biomass was suitable for *S. cerevisiae* cell to grow, consume reducing sugar and produce ethanol for as long as 27 hours without any detoxication process whatsoever. Between the two hydrolysed sample, the one pretreated under 5%NaOH and 3% H_2O_2 concentration and hydrolysed at 1.5g/l of enzyme concentration provided the highest ethanol yield in a lower time span of 21 hours.



(a)



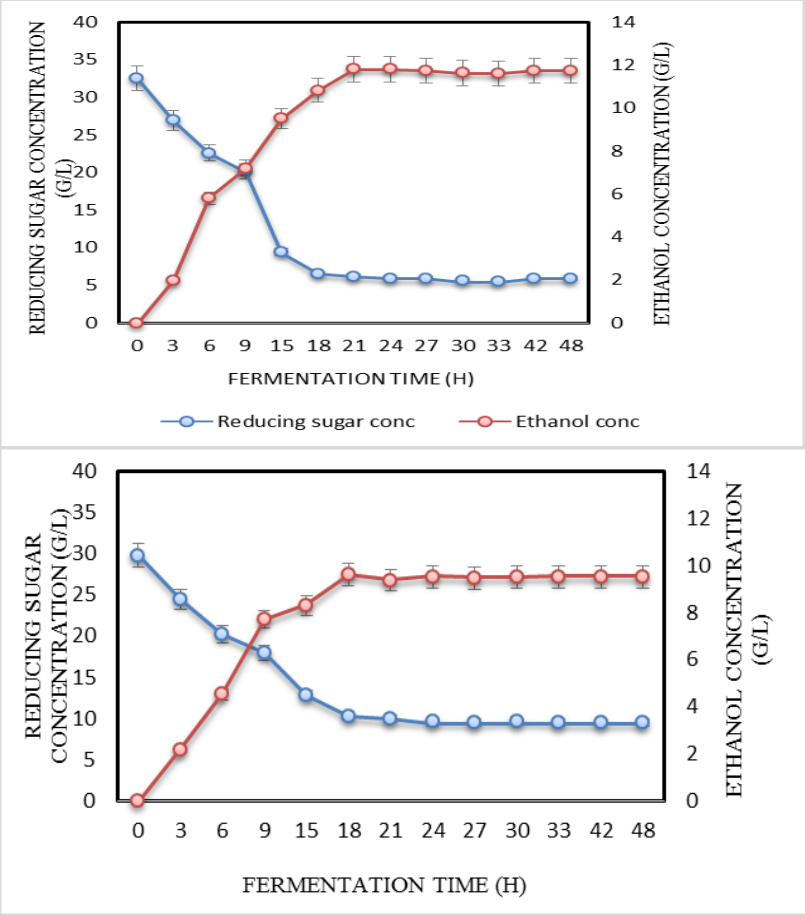
(b)

Figure 62: Ethanol production and reducing sugar consumed during ethanol fermentation with Saccharomyces cerevisiae in the extract obtained after enzymatic hydrolysis. (a)1NaOH $2H_2O_2$(b) 5NaOH $3H_2O_2$

4.2.5. Fermentation (*E.grandis*):

The ethanol yield of the two samples AHP-80 and AAHP was measured using HPLC and transient behavior is presented in Fig 63. It can be inferred from the Fig 63 that the ethanol yield was increased steadily for initial 21h and 18h of fermentation for AAHP and AHP samples respectively. Exponential growth rate of yeast was probably the reason for this steady increase in ethanol concentration. There was a minute decrease in ethanol concentration which remained constant thereafter for both the samples. Ethanol inhibition of yeast may be responsible for this decrease (Lin & Tanaka, 2006; E. Palmqvist et al., 2000a, 2000b) while the constant ethanol yield phase was related to stationery growth

phase of the yeast. EG treated with AAHP, gives better ethanol conversion when fermentation followed by hydrolysis at substrate concentration of 50g/L and 10.5g/L of enzyme concentration compared to AHP-80. The result is in accordance with the reducing sugar production data available as this combination provides the highest reducing sugar production also.



(a)

(b)

Figure 63: Ethanol production and reducing sugar consumed during ethanol fermentation with *Saccharomyces cerevisiae* in the extract obtained after enzymatic hydrolysis. (a)AAHP (b) AHP-80

The kinetic parameters such as, maximum biomass concentration (X_m), the yield coefficient of ethanol on biomass $(Y_{p/x})$, lag time for yeast growth (Δt), the yield coefficient of biomass on sugar $(Y_{x/s})$ and maintenance coefficient (m) obtained from the kinetic model based on the experimental data with the help of Eq.7 and 8 and are tabulated in table 18.

Table 18: Values of the kinetic parameters obtained from experimental data of ethanol production for AAHP and AHP-80

Parameter	AAHP	AHP-80
$Y_{x/s}(g/g)$	0.117	0.16
$Y_{p/x}(^{g}/_{g})$	9.941	8.152
m (h^{-1})	0.114	0.114
X_m (gL^{-1})	73.63	70.68
Δt (h)	5.17	3.64

Biomass concentration i.e the cell concentration at different temperature were plotted along with expected data according to the kinetic model in fig. 64 using the estimated parameters. It can be observed from the model that the data fits the model sufficiently well (correlation coefficient, $r^2 \geq 0.95$) and can be used to predict the cell growth data.

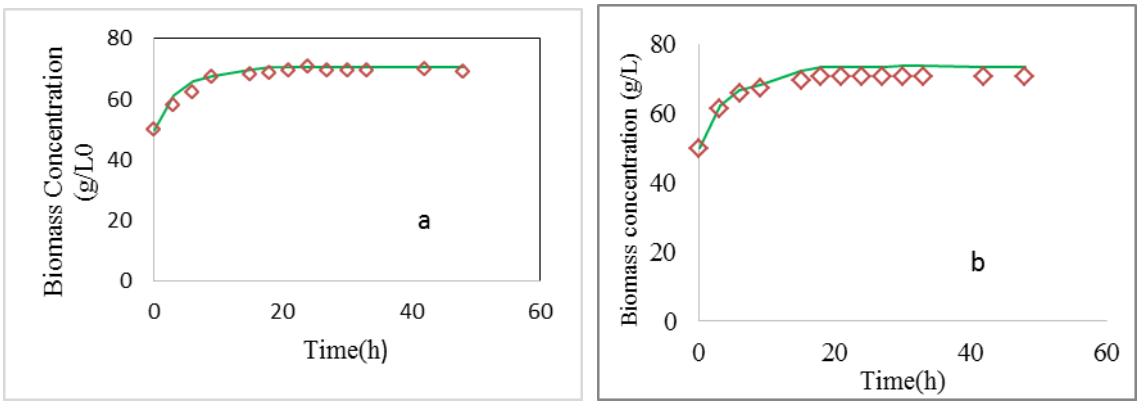


Figure 64: Experimental and kinetic model data for yeast growth for (a) AHP-80 and (b) AAHP

The yield coefficient of ethanol on biomass $(Y_{p/x})$ for AAHP exhibited a higher value than AHP-80, indicating higher ethanol conversion for AAHP compared to AHP-80. This was probably due to the substrate inhibition as enzymatic hydrolysis yields higher amount of glucose for AHP-80 compared to that of AAHP. *S.cerevisiae* is glucophilic in nature and formation of ethanol results from sugar metabolism by the yeast, the fermentable sugar in

the solution aids in the growth of *Saccharomyces cerevisiae* which leads to high biomass concentration and better ethanol yield for AAHP(Bisson, 1999) compared to AHP-80. Presence of higher amount of glucose at the initial phase helps to grow the yeast faster with lower lag time obtained for AHP-80 but due to substrate inhibition effect the yield of ethanol was lower for AHP-80.

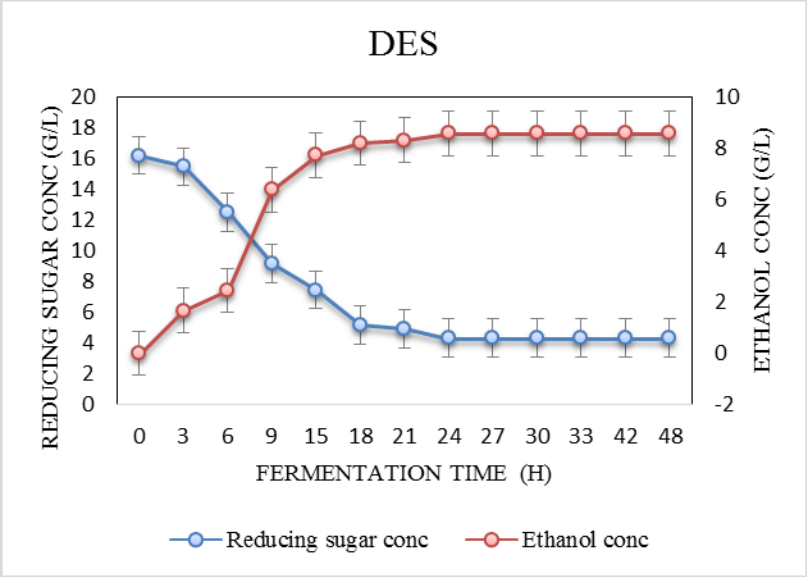


Figure 65: Ethanol production and reducing sugar consumed during ethanol fermentation with *Saccharomyces cerevisiae* in the extract obtained after enzymatic hydrolysis under DES pretreatment at 80˚C for 8h.

E. grandis when pretreated with ChCl/urea and hydrolysed at the same substrate concentration and enzyme concentration mentioned previously, does not produce significant concentration of ethanol. Figure 65 represents the ethanol production and reducing sugar consumption during ethanol fermentation using *Saccharomyces cerevisiae* from the hydrolysate obtained after enzymatic hydrolysis under DES pretreatment at 80˚C for 8h. The highest ethanol yield is observed at 24h with a ethanol concentration of 8.57g/l. This may be due to the inefficiency of ChCl/urea residues treated at a comparatively low reaction temperature of 80˚C to biodegradation.

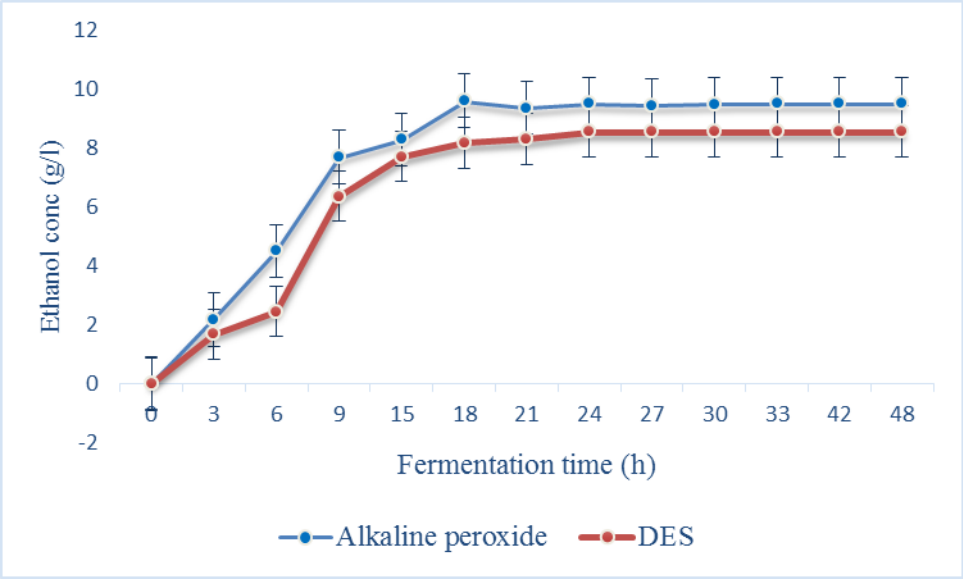


Figure 66: Ethanol production during ethanol fermentation with *Saccharomyces cerevisiae* in the extract obtained after enzymatic hydrolysis under DES pretreatment at 80˚C and alkaline peroxide pretreatment at 80˚C

The comparison between alkaline peroxide pretreated sample and DES pretreated sample at 80˚C as shown in figure 66 also showed competency of ethanol production significantly better than that of DES pretreated sample. This may be due to the fact that DES performed better at an elevated temperature and reaction temperature rather than reaction time is a defining factor for Choline based DES to work efficiently.

4.3. Conclusion:

For hydrolysis, highest substrate concentration of 50g/L with 10.5g/L of enzyme concentration found to be more effective for production of fermentable sugar for both *Sterculia foetida* and *Eucalyptus grandis*. The kinetics of enzymatic hydrolysis of delignified biomass shows product inhibition to be lower with higher substrate concentration under a particular enzyme loading. Optimum fermentation time is found to be 21hrs for around 83% of reducing sugar conversion to ethanol except for DES treated biomass where production of ethanol was hampered due to insufficient removal of lignin and inability to produce reducing sugar.

CHAPTER 5

CONCLUSION AND FUTURE WORK

CHAPTER 5

CONCLUSION

Efficient low-cost pre-treatment methods (alkaline, alkaline peroxide, DES) are applied for valorization of three novel lignocellulosic materials (*S. foetida*, *E. grandis* and *mixed garden waste*) for improved lignin removal for easy accessibility of cellulose and hemicellulose for subsequent enzymatic hydrolysis and fermentation for production of ethanol. The residues are further treated with cellulase and β-glucosidase at various substrate and enzyme loading conditions for conversion of C6 and C5 sugars to monomeric sugars which undergo fermentation with *Saccharomyces cerevisiae* for production of ethanol. Efficient lignin removal is found to be the key factor for maximizing the ethanol yield among all the lignocellulosic materials with various pretreatment. The kinetics of enzymatic hydrolysis is also evaluated to understand the effect of various inhibitors formed in the process.

For *Sterculia foetida* fruit shells, in terms of pretreatment, alkaline peroxide pretreatment is observed to be effective in lignin removal as well as for producing reducing sugar than alkaline peroxide pretreatment. An increase in crystallinity of the pre-treated samples was observed which indicated the removal of lignin as well as easy accessibility of cellulose. At higher chemical concentrations, the yield decreased due to the in-situ degradation of the released sugars in presence of NaOH and H_2O_2. The optimum lignin removal of 81.66% was achieved by heating the sample at 60° for 3 hours using 3% H_2O_2 and 5% NaOH aqueous solution. A maximum reducing sugar yield of 220 mg/g was obtained at 1% NaOH and 2% H_2O_2 treated at low temperature condition of 60˚C. It can also be concluded that 60˚C temperature is sufficient in terms of lignin removal and reducing sugar production in alkaline peroxide tratment. In terms of hydrolysis and

fermentation for *S. foetida* combination of 1% NaOH and 2 % H_2O_2 gives best results at substrate conc of 50g/L and 10.5g/L of enzyme concentration. Whereas, for the combination of 5% NaOH and 3% H_2O_2, shows comparatively better results at substrate conc of 50g/L and 1.5g/L of enzyme concentration but not as much as that of the combination of 1% NaOH and 2 % H_2O_2.

Enzyme conc of 1.5g/L gives the best result with the same substrate concentration. Although, when compared between these two combinations, 5% NaOH and 3 % H_2O_2 gives best results with glucose concentration of 18.59g/L and continues to provide better results with 97% ethanol yield in 21 hours of fermentation time and with reducing sugar conversion rate of 87% thereby optimizing the reaction conditions for this biomass for an efficient bioethanol generation.

For *Eucalyptus grandis*, in terms of pretreatment, Alkaline pretreatment at low temperature of 60°C as well as at high temperature of 80˚C was ineffective with regard to lignin removal and improving reducing sugar content compared to that of alkaline peroxide pretreatment. Autoclave assisted alkaline peroxide pretreatment produces best results both in terms of lignin removal and reducing sugar content when treated in combination with 2% H_2O_2 and 8% NaOH solution and even after fermentation continues to provide best ethanol yield when hydrolyzed at high substrate concentration of 50g/L with 10.5g/L of enzyme concentration and fermented by *Saccharomyces cerevisiae* strain for 21 hours of fermentation time. This is the optimized condition to obtain 11.8 g/L of ethanol concentration for this particular biomass. For DES pretreatment, 80˚C is not sufficient to produce significant amount of ethanol. Moreover, at similar temperature alkaline peroxide pretreated sample provides better ethanol yield than DES treated sample. Therefore, higher temperatures need to be applied to optimize the conditions for DES.

As for garden waste, from a techno economic stand point, the overall yield obtained from the pretreatment is not sufficient to proceed for hydrolysis and subsequent fermentation to produce bioethanol. Alternatively, pretreated garden waste can be used as biofertilizer.

Future work:

Garden waste as well as the huge waste generated from the pulp and paper industry could be used as a potent source of energy as well as different value-added products. The scope of the work includes extraction, purification as well as valorization of lignin and the inhibitory products may have produced during pretreatment such as furan and furfural. A lot of research has already been going on in this area. Since *S. foetida* and the bark of *Eucalyptus grandis* is newly explored sources of bioethanol, life cycle assessment as well as economic analysis could be done to evaluate the potential impacts of the products and the process and to gauge the feasibility of the same for large scale application.

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