SHORT-TERM LIME PRETREATMENT AND ENZYMATIC CONVERSION OF SAWDUST INTO ETHANOL

By

Augustine Omoniyi AYENI
(CUGP050132)

A Thesis Submitted in the Department of Chemical Engineering to the
School of Post-graduate Studies

As part of the requirements for the award of the degree of Doctor of Philosophy (Ph.D.)
in Chemical Engineering of Covenant University, Ota, Ogun State, Nigeria

June, 2013

CERTIFICATION

I hereby certify that this Thesis is an original research work carried out independently by Augustine Omoniyi AYENI under our supervision in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy (Ph.D.) in Chemical Engineering of Covenant University, Ota, Ogun State, Nigeria.

Mr. A.O. Ayeni
(Author of Thesis)
Department of Chemical Engineering
Covenant University, Ota, Nigeria.

Dr. R.A. Pandey
(Co-Supervisor)
Scientist "G" & Head
Environmental Biotechnology Division,
National Environmental Engineering Research Institute, Nehru Marg, Nagpur, India

Prof. F.K. Hymore
(Co-Supervisor)
Professor & Head
Department of Chemical Engineering,
Covenant University, Ota, Nigeria.

Prof. J.A. Omoleye
(Supervisor)
Professor of Chemical Engineering
Department of Chemical Engineering,
Covenant University, Ota, Nigeria.

DEDICATION

To God Almighty from whom all blessings flow.

To my beloved wife Afolajaye Omoyeni and

our lovely children Oluwasemilore, Ifeoluwa, Toluwalase, and Boluwatife.

To my parents Pa.(Late) P.A. Ayeni and Mrs. V.E. Ayeni

ACKNOWLEDGEMENTS

Many people have contributed in one way or another to the success of this work. Without them it would not have been possible.

I thank the Nigerian Conservation Foundation, Lekki, Lagos and Chevron Nigeria Limited for the award of Chief S.L. Edu Ph.D. research grant 2008. I thank the Academy of Sciences for the Developing Countries (TWAS), Italy and Council for Scientific and Industrial Research, New Delhi, India for the award of CSIR-TWAS fellowship for research and advance training 2009 tenable at Environmental Biotechnology Division, National Environmental Engineering Research Institute (NEERI), Nagpur, India. I thank the Chancellor of Covenant University, Bishop David Oyedepo, the former Vice Chancellor, Prof. Aize Obayan and current Vice Chancellor, Prof C.K. Ayo and the entire management of this great university for granting a one-year study leave for this work in India.

I acknowledge my supervisor Prof. J.A. Omoleye for his extremely valuable guidance and timely directions during the course of this work and good will messages while I was in India for the experimental and analytical stages of this research. Prof. F.K. Hymore, co-supervisor to my Ph.D. degree and also my Head of Department is acknowledged for always asking about the progress of this work while in India and adding value to the thesis for early completion. I appreciate Prof. S.S. Adefila for his continuous interest in early completion of this work. I thank all members of faculty in the Department of Chemical Engineering: Engr. J.S. Udohitinah, Dr. A.M. Obande, Engr. V. Efeovbokhan, Engr. O.A. Adeeyo, Engr. A.A. Ayoola, Engr (Mrs) M.E. Ojewumi for their good wishes. To Engr. A.B. Aleji and Engr. O. Osunkoya for their valuable contributions to the success of this work. I equally extend my appreciation to other staff of the Chemical Engineering Department.

I deeply from my heart thank my guide at NEERI, India, Dr. R.A. Pandey for being a father and for the leadership roles he played to making the research stay in India a success. I thank all the scientists in EBD: Mr. D.B. Satpute, Dr. B.K. Sarangi, Dr. S.N. Mudliar, Dr. Kiran Paradkar for their contributions to the success of this study.

I would also want to thank all the research students at EBD. Their help was fundamental in its moments: Mr. Balendu S. Giri for all his encouragements and readiness to assist even at inconvenient situations, Mr. Abijit A. Gadhe and Ms. Sharvari C. Deshmukh for their technical inputs to the manuscripts of this work, Ms. Nidhi Sahu for taking me through and assisting in the microbiology part of the fermentation step. I thank Ms. Deepika Soni, Saumita Barneeje, Mr. B. Chandrashekhar for making the research stay a huge success. I thank all my friends at research scholar's home NEERI: Ravindra Gautam for his readiness to take me round Nagpur town when there was need for it, Nitin Gadem, Abhay Fulke, Jitendra Sharma. I thank my flat mates at RSH: Ankit Gupta, S.A. Praveen, and Rakesh Kadaverugu for their warmness and being accommodative.

I thank my beloved wife, Mrs. Afolajaye Omoyeni Ayeni for her prayers, encouragement, and holding forth the home while I was away for 12 months. It was not an easy thing taking care of the boys all alone. For her relying on the promises of God for us, showing that God will surely visit us even when things were not working well. Temiloluwa, thank you for everything. I also thank our children for cooperating with their mother and making necessary phone calls to wish me well during my stay in India.

I thank my mother for her regular phone calls to ask about my well being and her prayers for successful stay in India and improvements in my carrier. I thank all my siblings for the love and kindness shown to me, my wife, and our children.

I would also like to thank all Christian brethren for their continuous prayers and supports to me and my family during all stages of this work. I appreciate Pastor and Pastor (Mrs) S.A. Daramola, Pastor and Pastor (Mrs) Gbemiga Soetan, Pastor and Lady Evangelist Olusola Ayinde, Pastor and Prophetess Udumoi for all their spiritual guidance. I acknowledge the input of Dr. P. A. Aizebeokhai, Dr. (Mrs) Mojisola Usikalu, Dr. A. H. Adebayo, Mr. and Mrs. Ibukun Oluwumi to making this work a success.

Last but not the least, I give glory to God almighty from whom all blessings flow. He makes all things beautiful at His own time. I thank Him for making Jesus Christ available for mankind and making this study a huge success.

TABLE OF CONTENTS

PAGE

PAGE

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

LIST OF TABLES

TABLE PAGE

ABBREVIATIONS

ANOVA	Analysis of variance
APAWAO	Alkaline peroxide assisted wet air oxidation
APO	Alkaline peroxide oxidation
BSS	British standards specification
CCD	Central composite design
DNS	Dinitrosalicylic acid
DOE	Design of experiment
FPU	Filter paper unit
HMF	Hydroxymethylfurfural
IUPAC	International union of pure and applied chemistry
LCW	Lignocellulosic waste
NREL	National renewable energy laboratory
OVAT	One value at a time
PID	Proportional-intergral-derivative
RSM	Response surface methodology
SSF	Simultaneous saccharification and fermentation
WAO	Wet air oxidation

ABSTRACT

The utilization of lignocellulosic biomass as feedstock for the production of fuel ethanol has attracted considerable interests in the last few decades. The emergence of new technologies has provided hope for fuel ethanol potential uses. Lignocellulose is a valuable alternative energy source. The enzymatic hydrolysis of lignocellulosic biomass is constrained due to its complex structural features, so pretreatment is important to enhance its enzymatic digestibility. In this study, the influence of process parameters – temperature, air addition, hydrogen peroxide addition, and time – on the pretreatment of sawdust (a wood residue) was investigated. The optimization of the pretreatment step was performed by using the full factorial and central composite designs of experiments. The study assessed the compositional changes by applying short-term oxidative pretreatments such as alkaline wet air oxidation, alkaline peroxide oxidation, and alkaline peroxide assisted wet air oxidation methodologies, and their effects on the yields of reducing sugar. The best pretreatment condition based on the yield of the reducing sugar was the alkaline peroxide-assisted wet air oxidation at 150 ^{o}C, 1%H_2O_2, 10 bar air pressure, 45 min. The optimal 4-day reducing sugar yield was 335.35 mg equivalent glucose/g dry biomass at 40 g/L substrate concentration, 25 FPU/g dry substrate of cellulase enzyme, and 5 IU/g dry substrate of β-glucosidase. Furthermore, when considering the fermentability of the treated solids, at 2% effective cellulose loading, 9.71 g/L ethanol (23.43% theoretical ethanol yield) was obtained for pretreatment at 150 ^{o}C, 1%H_2O_2, 10 bar air pressure, and 45 min. At the optimum pretreatment condition, 0.1 g $Ca(OH)_2$/g dry biomass was enough to cause appreciable lignin removal. Lignin removal was largely dependent on temperature, and the prevailing oxidative conditions. Cellulose was highly preserved in the solid fraction, while more of the hemicellulose was solubilized/degraded. The high-lignin content of the raw material was a great obstacle to the digestibility of the treated material. The lignin remained largely undissolved in the solid fraction.

CHAPTER ONE

1. INTRODUCTION

1.1 Background of the study

Lignocellulosic biomass (e.g. sawdust obtained from wood) provides a unique and sustainable resource for environmentally safe organic fuels and chemicals. Furthermore, due to the abundance of lignocellulosic materials, its conversion to ethanol (a biofuel) is considered one of the most important uses of biomass as an energy source in the modern world especially in the United States, Europe and Asia (Kheshgi *et al*., 2000). Again, ethanol produced from biomass would be of great benefit to the transportation sector where it is assumed that $^{2}/_{3}$ of Nigeria's gasoline is consumed. Globally, fossil fuels are being threatened out of dominance over other fuels by:

1. The high international market price of fossil fuels.
2. The negative effects of fossil fuels products on the environment e.g. the release of greenhouse gases like carbon dioxide that contribute to global warming. The pollution of air, water, and soil by fossil fuels products (carbon dioxide from fossil fuel combustion accounted for nearly 80% of global warming in the 1990s) (Hileman, 1999).
3. By far, the greatest proportion of the world's energy requirements comes from petroleum exports especially in the Middle East, a region of high political tension.

These reasons have necessitated efforts at finding alternatives to fossil fuels. Lignocellulosic wastes (LCW) refer to plant biomass wastes that are composed of cellulose, hemicellulose, and lignin as well as other minor components. Both the cellulose and hemicellulose fractions are polymers of sugars, and are thereby potential sources of fermentable sugars, which can be converted into other products.

Hemicellulose can be readily hydrolysed under mild acid or alkaline conditions. The cellulose fraction is more resistant and therefore requires more rigorous treatment than the hemicellulose fraction.

Currently, the second generation bioproducts from lignocellulosic biomass such as bioethanol, biodiesel, biohydrogen and methane are increasingly being produced from wastes (residues) rather than from energy crops (jatropha, switchgrass, hybrid poplar and willow) because the latter competes for land and water with food crops that are already in high demand. The use of food crops such as corn and sugarcane to produce biofuels is increasingly being discouraged due to the current worldwide rise in food prices. In order to minimize food-feed-fuel conflicts, it is necessary to integrate all kinds of bio-waste into a biomass economy (Mahro and Timm, 2007).

Furthermore, the use of LCW offers a possibility of geographically distributed and greenhouse-gas-favourable sources of products (Rubin, 2008). The Energy Commission of Nigeria reported that the fuel-wood resource constitutes 2.8% of the total renewable energy resources in Nigeria. Biomass reserve in Nigeria is put at 80 million m^3, which equals to an equivalence of 1.645 billion tonnes of energy which is predicted to be potentially available for the next 100 years (Umar *et al*., 2000).
Lignocellulose is the most abundant renewable biomass with a worldwide annual production of $1x10^{10}$ MT (Sánchez and Cardona, 2008).

1.2 Statement of research problem

In order to provide an alternative source of energy, fuels and chemicals from traditional fossil fuel, the project aims at considering how to generate a systematic approach of utilizing wood wastes or residues (sawdust) generated from Nigerian forest reserves. Ethanol produced from sawdust can provide a cleaner environment, environmentally friendly fuel and stimulate community based jobs and economic growth.

Lignocellulosic biomass such as sawdust, poplar wood, sugar cane bagasse, herbaceous grasses, municipal wastes provide a unique and sustainable resource for environmentally safe organic fuels and chemicals.

1.3 Objectives of the study

The research was aimed to produce ethanol through the fermentation of reducing sugars resulting from the hydrolysis of pretreated sawdust. It was to explore the effects of different conditions of short term lime pretreatment on the enzymatic digestibility of shea tree (sawdust residue, a woody biomass). The objectives of the research work were:

- The design of experiments (DOE) with MINITAB statistical software package was used to determine the effect of pretreatment on raw material with lime as a pretreatment agent for the three oxygen delignification (pretreatment) methodologies to be investigated: alkaline wet air oxidation (WAO); alkaline peroxide assisted wet air oxidation (APAWAO); and alkaline peroxide oxidation (APO) methods.
- Evaluate the variations of pretreatment conditions such as time, temperature, lime loadings, oxidative conditions (using air and/or hydrogen peroxide as oxidizing agents) on the digestibility of the sawdust by cellulase enzymes. Generate suitable models for pretreatments in order to predict optimum pretreatment conditions.
- Determination of enzymatic digestibility of raw and pretreated biomass for optimized pretreatment conditions by studying the effects of substrate concentration, enzyme loading, hydrolysis time, and temperature on hydrolysis.
- Simultaneous saccharification and fermentation (SSF) was performed to determine the ethanol yield of the lime-pretreated biomass.

These data are going to be useful to determine the pretreatment conditions that maximize enzymatic digestibility of cellulose using the sawdust residues. The data when collated will become part of bank of data for future economic analysis.

1.4 Significance of the study

Most African countries manage a large area of forest lands (reserves) from which timber is harvested. The sub-Saharan African population depends mainly on wood (Cecelski *et al.*, 1979). However due to lack of technical know-how needed for using wood and wood wastes (residues) for renewable energy and the big finances involved,

there has been general apathy towards the exploitation of wood and wood wastes as important sources for renewable energy (bio-energy).

This project is then directed to show that;

- Biofuel sources are geographically more evenly distributed than fossil fuels.
- Lignocellulosic raw materials (biomass materials that have cellulose, hemicellulose binded together with an insoluble substance known as lignin) minimize the potential conflict between land use for food (and feed) production and energy feedstock production. Presently ethanol is produced from food reserve crops like cassava, beet or carrots. These are meant for human consumption. The question is "why compete with man nutritional needs when there are alternatives to produce ethanol from lignocellulosic biomass"? The raw material is less expensive and can be produced with lower input of fertilizers, pesticides and energy.
- Biofuels might also produce employment in rural areas.

Wood and wood wastes as energy sources on an industrial scale in African countries have not been adequately looked into. Industries are more interested in the more convenient and high energy content fossil fuels (Momoh, 1997). In the field of research it is well known that there has been very little input of experimental data from African countries into the global development of wood and wood residues for the production of solid, liquid or gaseous fuels.

For environmental reasons, ethanol, a clean burning fuel, constitutes the most important approach for using lignocellulosic residues. Ethanol is a better fuel than gasoline because of its excellent physicochemical characteristics (Bailey, 1996). Adding 10 percent ethanol (v/v) to gasoline increases the octane number, improves engine efficiencies through excellent antiknock properties, and oxygenates gasoline.

1.5 Scope and limitation of the study

Initial analyses for moisture, ash, extractives, lignin, and structural carbohydrates (cellulose and hemicellulose) contents in the raw sawdust were carried out. Lime oxidative pretreatments of the 2^3 full factorial design of WAO, 2^3 central composite design of APO, 2^2 central composite design of APO and APAWAO methods were

investigated in a floor type high pressure Parr reactor. The analyses before and after pretreatment were to establish the extent of degradation of the biomass and fermentable sugars produced during enzymatic hydrolysis and were related to the cellulose content, hemicellulose solubilization, and lignin removal during pretreatment.

This study was limited to sawdust from a particular type of wood (shea tree) and one type of pretreatment agent (lime). Enzyme conversion was limited to the cellulase enzyme from *Trichoderma reesei* and beta glucosidase while the microorganism for fermentation of the treated material was also limited to a single source (*Saccharomyces cerevisiae*). This research mainly focused on the pretreatment and enzymatic hydrolysis steps. The effects of various parameters such as time, temperature, substrate loading, and enzyme loading on enzymatic digestibility were evaluated. The fermentation step was carried out simply through flask experiments. The fermentation experiments were limited to a few parameters since the primary aim was to determine the fermentability of the sugars resulting from the saccharification of the raw and pretreated sawdust. The project investigated the optimum for each parameter that may affect fuel ethanol production from the lignocellulosic residue.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Sawdust as a renewable energy source

Many environmental problems such as greenhouse gases and pollution of air, water, and soil originate from fossil fuels. Fossil fuels release greenhouse gases, like carbon dioxide, that contribute to global warming. Carbon dioxide from fossil fuel combustion accounted for nearly 80% of global warming in the 1990s ((Hileman, 1999 and 2006 The U.S. Inventory of Greenhouse Gas Emissions Sinks).

Renewable energy sources, such as lignocellulosic biomass, are environmentally friendly because they emit less pollution without contributing net carbon dioxide to the atmosphere unlike fossil fuels. Large amounts of lignocellulosic wastes are continuously generated from the production of waste materials in an undeniable part of human society. Lignocelluloses may be grouped into different categories such as wood residues (including sawdust and paper mill discards), grasses, waste paper, agricultural residues (including straw, stover, peelings, cobs, stalks, nutshells, non-food seeds, bagasse, domestic wastes (lignocelluloses garbage and sewage), food industry residues, municipal solid wastes and the like (Qi *et al*., 2005 and Roig *et al*., 2006). These wastes or residues can be considered as potential energy sources.

The world oil reserves is gradually being depleted and from a 2007 estimate of world oil statistics (2005 CIA world factbook), it is assumed that in the next 50 years world fossil fuel reserves would be exhausted (Table 2.1). Bioenergy, renewable energy from energy crops and lignocellulosic residues, agricultural and wood based, are undergoing world wide economic analysis for efficient commercialization. Biofuels, derived from these sources are important choices for exploiting alternative energy and reduction of pollution gases. The US based National Renewable Energy Laboratory (NREL) states that a 10% ethanol additive to gasoline will reduce the overall pollution from a vehicle by as much as 54% (Kryzanowski, 2008). Some other advantages of biofuels over fossil fuels are: they are easily available from common biomass sources i.e. are more evenly distributed than fossil residues; they are better represented in the CO_2

Table 2.1 – A 2007 Estimate of world oil statistics (2005 CIA world fact book).

	World total	Weighted average	Highest	Lowest
Oil Reserves [a] (89 countries)	1,394,417,153,000	13,911,517,041.2	Saudi Arabia : 262,700,000,000	Ethiopia: 214,000
Oil Consumption[a] (213 countries)	85,085,664	399,463.2	USA : 20,680,000	Niue : 20
Oil Exports (Net)[b] (30 countries)	32,234,418.5	1,007,325.6	Saudi : 6,710,000 Arabia	Bahrain : 15,000
Oil Imports (Net)[b] (21 countries)	29,916,829	1,424,610.9	USA : 10,400,000	Cote d'Ivoire: 18,600
Gasoline prices		$1.00/litre	Uruguay : $1.95/litre	Turk-menistan: $0.03/litre

[a] in barrels, [b] in barrel/day

Table 2.2 – Weight percent of cellulose, hemicellulose, and lignin in wood biomass (Bailey and Ollis, 1986).

	% Cellulose	%Hemicellulose	% Lignin
Hardwood	40 – 55	24 – 40	18 – 25
Softwood	45 – 55	25 – 35	25 – 35

cycle on combustion; they are more economical than conventional fuels, they are biodegradable and contribute to sustainability with low sulphur content.

It is estimated that the total area of forest reserves in Nigeria is 10 million ha (25 millions acres) which is about 10% of the total land area of the country (1992 FAO Corporate Document Repository). Sawdust abounds in great quantities in Nigeria. It is found in many sawmill sites, waste dumps, and in burnt forms that pollute the land and the atmosphere. The sawdust is obtained from sawn wood and probably other wood wastes. Sawdust can be wastes/residue from either hardwood or softwood or the mixture of both. Softwood and hardwood vary in the percentages of cellulose, hemicellulose and lignin (Table 2.2). The cellulose and the hemicellulose fractions are the polysaccharide complex in lignocellulosic materials. Cellulose and hemicellulose are not directly available for bioconversion because of their intimate association with lignin (Sánchez and Cardona, 2008, Williams and Morrison, 1982, and Holtzapple *et al*., 1997).

Among lignocellulosic biomass, sawdust waste from the shea tree (*Vitellaria paradoxa*) can be a useful feedstock to economically produce environmentally friendly biofuels. The shea tree is typically a savannah woodland tree species. The shea is a big tree, 10–15 m tall, that can reach 25 m. It is a decidous tree. The trunk of shea tree makes excellent charcoal. It is a favoured source of wood fuel (Shea Tree, 2011) Its natural habitat stretches over Africa, south of the savannah, from the eastern part of Senegal to the north of Uganda. This stretch covers an area of over 5,000 km long and 400–750 km wide. The West African subspecies *V. paradoxa* var. *nilotica* is found in the eastern end of the range of the distribution of the species and indigenous to northern Uganda and south-western Ethiopia. In Nigeria the shea tree occurs mainly in the wild.

2.2 Alcohol production from biomass

By applying proper processes, lignocellulosic biomass can be converted to a wide variety of fuels and chemicals. In order to increase the enzymatic digestibility of lignocellulosic biomass it has to undergo a pretreatment step, after which the treated biomass is enzymatically hydrolysed to sugars by cellulase enzyme complexes. This step can alternatively be executed chemically by dilute sulphuric acid or other acids. The

resulting sugars are then fermented to ethanol by yeast fermentation (Figure 2.1) (Hahn-Hägerdal *et al*., 1996).

The Ethanol Life Cycle

Photosynthesis

$$6CO_{2(g)} + 6H_2O_{(l)} + \text{Light} \longrightarrow C_6H_{12}O_{6(l)} + 6O_{2(g)} \quad \ldots 2\text{-}1$$

Fermentation

$$C_6H_{12}O_{6(l)} \longrightarrow 2C_2H_5OH_{(l)} + 2CO_{2(g)} + \text{Heat} \quad \ldots 2\text{-}2$$

Combustion

$$C_2H_5OH_{(l)} + 3O_{2(g)} \longrightarrow 2CO_{2(g)} + 3H_2O_{(l)} + \text{Heat} \quad \ldots 2\text{-}3$$

$$\text{Light} \longrightarrow \text{Heat}$$

Cellulase enzyme complexes work together to hydrolyse cellulose (Figure 2.2) (Reczey *et al*., 1996). Cellulase systems produced by species of *Trichoderma* fungi are the most thoroughly developed and characterized at present. These systems consist of three major classes of enzymes with different substrates and products. The enzymes in the cellulase complex are endoglucanase, exoglucanase, and cellobiase. Endoglucanase randomly attacks at β-1,4-D-glucan chains in amorphous regions of cellulose or the surface of microfibrils. Exoglucanase releases cellobiose from the non-reducing ends of β-D-glucan chains. Cellobiase hydrolyses cellobiose to glucose (Lee, 1996). For the complete hydrolysis of insoluble cellulose, a synergistic action between these components is required. Since different cellulose preparations vary widely in the proportion of different components, depending on the source, growth conditions and harvesting and handling procedures, the rate and extent of their hydrolysis of cellulose substrates also vary widely (Bisaria and Ghose, 1981). A major emphasis in the

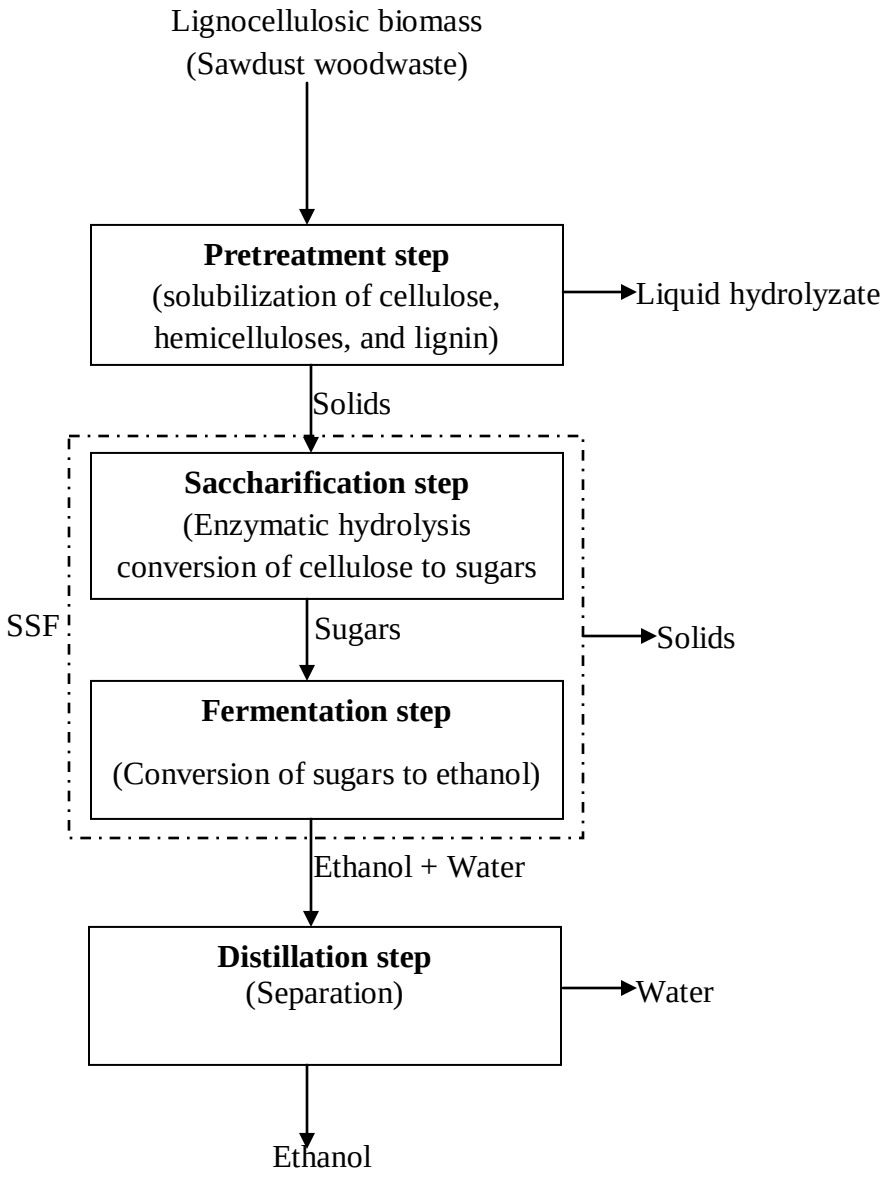


Figure 2.1 – Schematic diagram of traditional biomass conversion to ethanol.

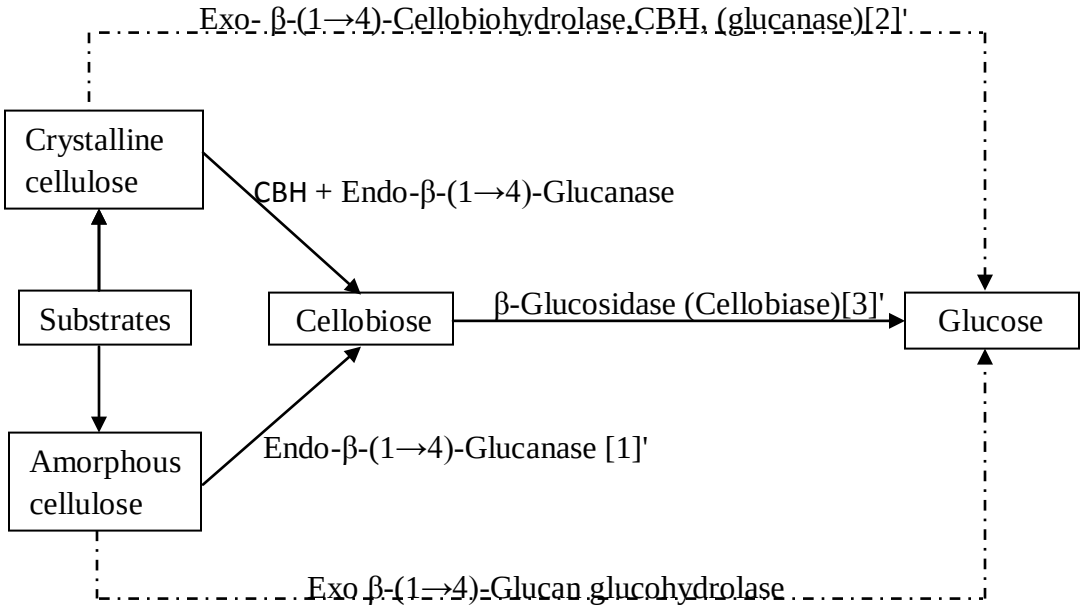


Figure 2.2 – Mode of action of cellulolytic enzymes. [1]', [2]', [3]' are the three major classes of enzymes in the cellulase complex. ⟶ Major reactions. - - - - ▸ Side pathways.

fermentation industry is ethanol production (Holtzapple *et al*., 1999). Enzymatic saccharification is advantageous because it is very selective, it can achieve high yields, and the formation of inhibitory by-product is avoided.

Cellulose hydrolysis performed in the presence of fermentative microorganisms is termed simultaneous saccharification and fermentation (SSF). Fermentation is a biological process in which enzymes produced by microorganisms catalyse energy releasing reactions that break down complex organic substrates. Finally, ethanol is recovered from the fermentation broth by distillation, or distillation combined with adsorption. Besides ethanol, fermentation can also produce chemicals such as carboxylic acids (Blasig *et al*., 1992 and Loesche, 1996) in addition to nearly 40 other chemicals and chemical feedstocks (Ladisch *et al*. 1979).

In addition to ethanol, products that are already being produced through commercial fermentation include therapeutic and research enzymes, bulk enzymes, antibiotics, lysine, monosodium glutamate, gluconic acid, lactic acid, acetic acid, citric acid, malic acid, and whole-cell yeast biomass. Of special interest in biorefining are platform intermediate chemicals from fermentation that can be converted into numerous consumer and industrial products, including succinic acid, butanol, itaconic acid, 1,3 propanediol, polyhydroxyalkanoates, and 3-hydroxypropionic acid.

2.3 Chemical structure of lignocellulosic biomass

Lignocellulosic biomass is built up of cell wall components of carbohydrates, lignin, and extractives. The most common cell wall components are the “fibres” (micro- and macrofibrils), the chemical composition of which differs significantly in all lignocellulosic materials.

The cell walls are the structural elements of biomass tissue; when they are grouped together to form e.g. a tree in wood materials, they ensure that the organism is able to withstand the natural forces to which it is exposed. The cell wall is the most defining character of the plant cell. It is a dynamic, metabolic participant in cell metabolism, growth, and structure. It is composed of polysaccharides and structural proteins.

2.3.1 The cell wall

Growing cells form the primary cell wall, composed primarily of cellulose in its matrix and sandwitching a middle lamella made of pectin between adjacent cells. Older cells may form a secondary cell wall inside the original one. The secondary cell wall is primarily made for structural strength, though metabolically active (Figure 2.3) (Lawoko, 2005). The major cell wall component, cellulose, is organized into micro-fibrils that mediate structural stability in the plant cell (Rubin, 2008). Cellulose forms the bulk of the plant cell wall, and the micro-fibrils themselves are embedded in a matrix/sheath composed of hemicelluloses, pectin, glycol-proteins, various enzymes, lignin (in some cell walls, not all), cutins, suberins, and waxes (in cells on the outside of the plant body). Each micro-fibril (about 30 by 30Å in area) is sur rounded by lignin which forms a seal with the cellulose (Figure 2.3). Thus the cellulose is protected from hydrolysis to glucose by the lignin seal as well as by its crystalline structure. Micro-fibrils are bundles of linear cellulose molecules which form crystalline entities having some amorphous regions (Ladisch *et al*., 1979).

The secondary cell wall are additional layers inside the primary cell wall laid down for compressional strength. The layers are highly lignified and formed in different planes. Moving inwards from the outside, the first layer in the wood cell is the primary wall, and three layers of secondary walls (S_1, S_2 and S_3) and, finally, there is the lumen (middle lamella) (Figure 2.3). The fibres are arranged side by side in the biomass, separated by the middle lamella. Cellulose, hemicelluloses, lignin and the other components are ordered in varying composition in the different parts of the fibre wall depending on the species of the plant.

There is still uncertainty as to the way in which all the components within the fibre wall are linked together, although it is well known that lignin is bonded chemically to the carbohydrates (Eriksson, 1980). It has later been proposed by Lawoko (2005) that there are two types of lignin: one more associated with glucomannan and one more associated with xylan. The fibres in wood must be liberated from each other in order to be available for enzymatic hydrolysis to fermentable sugars. This may be done using mechanical force, physicochemical, chemicals, biological pretreatments.

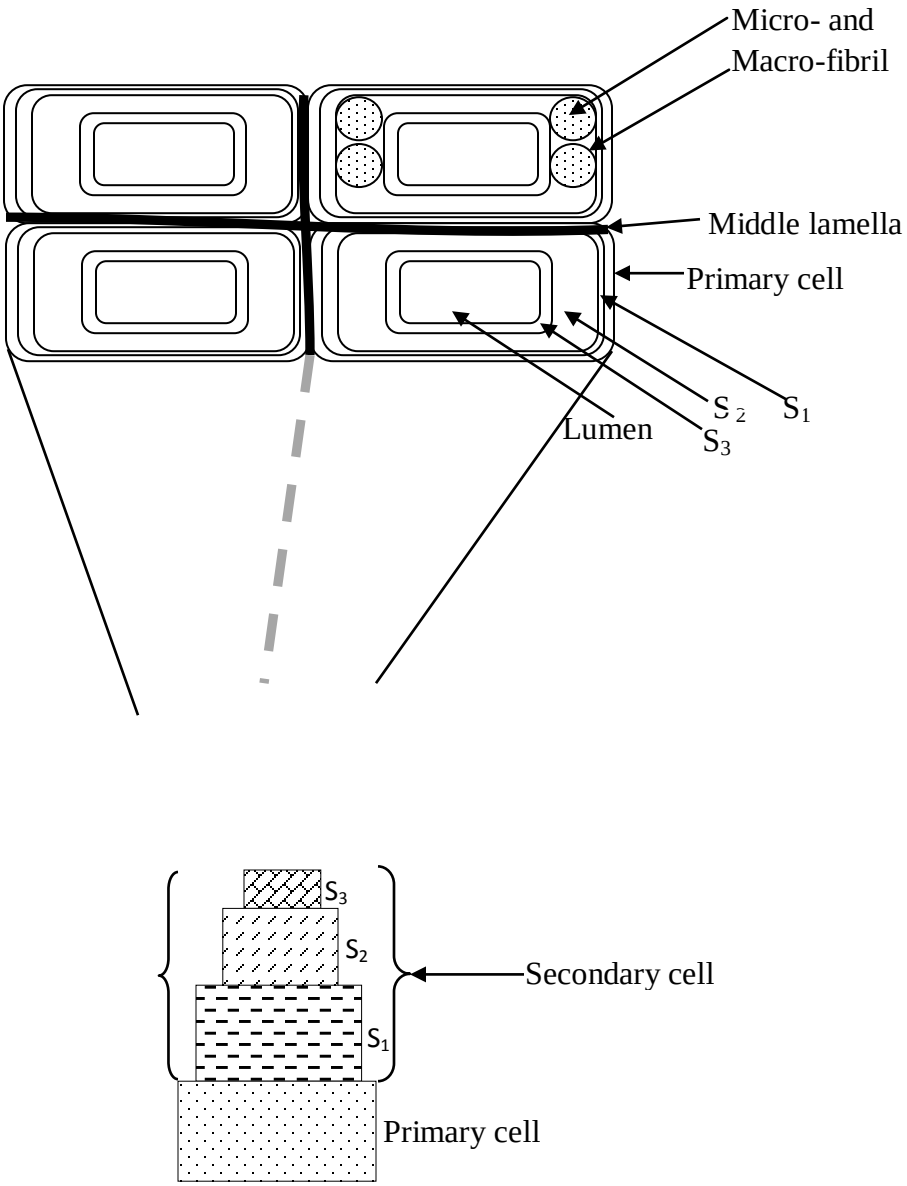


Figure 2.3 – Micro- and macro-fibrils (fibres) formation of cellulose and their positions in the wood cell wall.

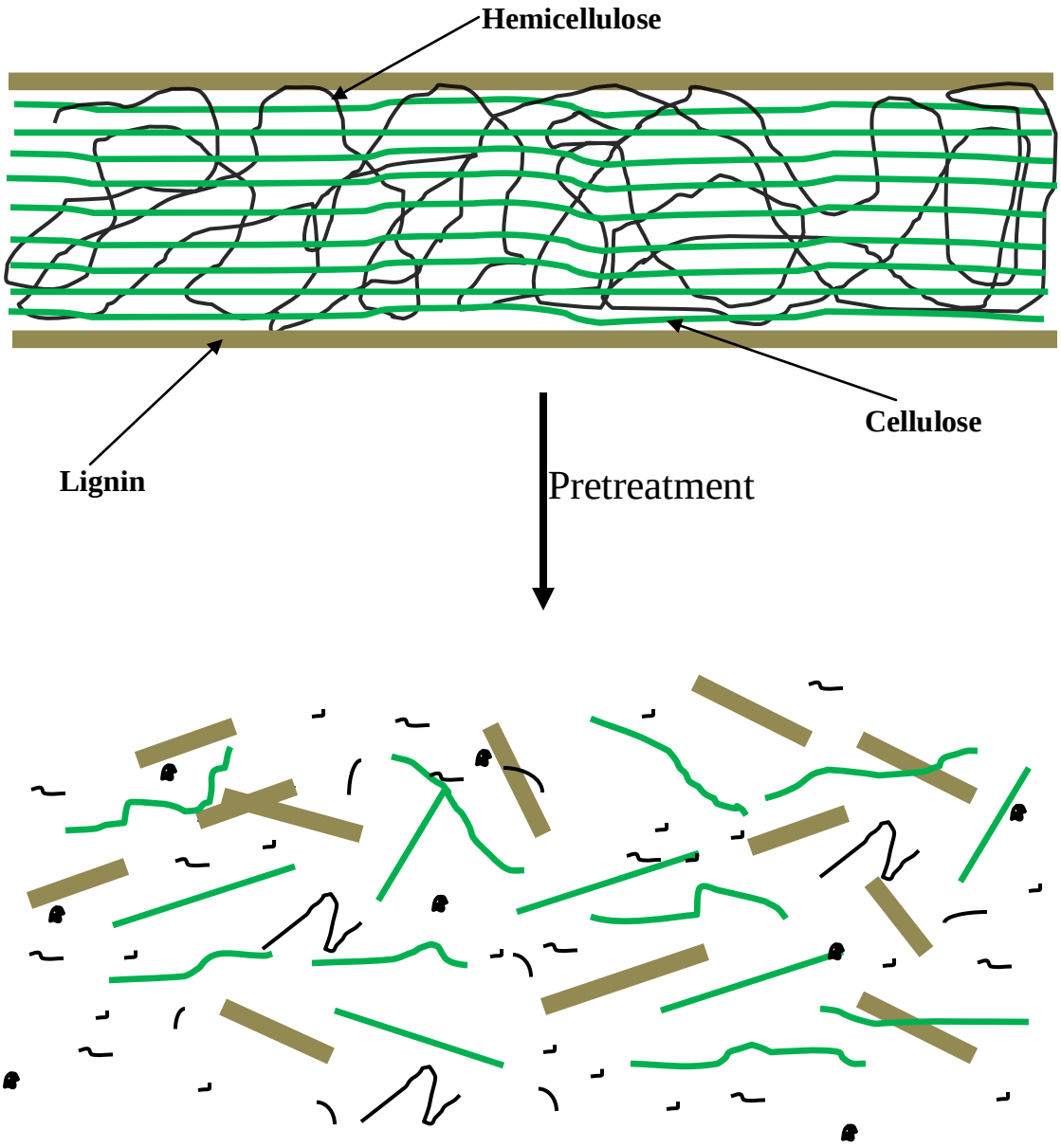


Figure 2.4 – Schematic of goals of pretreatment on lignocellulosic material (modified from Mosier *et al.*, 2005.

Many pretreatment methods have been established. Majorly, the pretreatments are meant to degrade lignin so that the polysaccharides can be available for enzymatic actions. The barrier to the production and recovery of valuable materials from LCW is the structure of lignocelluloses (Figure 2.4) (Mosier *et al*., 2005) which has evolved to resist degradation due to cross-linking between the polysaccharides (cellulose and hemicellulose) and the lignin via ester and ether linkages (Yan and Shuya, 2006 and Xiao *et al*., 2007).

The main goal of any pretreatment, therefore, is to alter or remove structural and compositional impediments to hydrolysis and subsequent degradation processes in order to enhance digestibility, improve the rate of enzyme hydrolysis and increase yields of intended products (Mosier *et al*., 2005, and Hendricks and Zeeman, 2009).

2.3.2 Cellulose

The Cellulose (40–55% of total feedstock dry matter) is a glucose polymer linked by β-1,4 glycosidic bonds with the degree of polymerization from 10,000 in native wood to 1,000 in bleached kraft pulps. The basic building block of this linear polymer is cellobiose, a glucose-glucose dimer (Figure 2.5). Cellulose has a strong tendency to form intra-and inter-molecular hydrogen bonds by the hydroxyl groups on the linear cellulose chains, which stiffen the straight chain and promote aggregation into a crystalline structure and give cellulose a multitude of partially crystalline fiber structures and morphologies (Klemn *et al*., 2005). The degree of crystallinity vary from species to species (Klemn *et al*., 1998). Cellulose samples of different origin vary widely in chain length and the degree of interaction between the chains. One water molecule is eliminated by two adjacent glucose units during linkages. The –OH groups at both ends of the cellulose chain have different chemical properties. The –OH group at the C-1 is a reducing end and the –OH group on C-4 is a non-reducing end. Hydrolysis of cellulose results in individual glucose monomer. This process is also known as saccharification. Its density and complexity resist hydrolysis without preliminary chemical or mechanical degradation or swelling. In nature, cellulose is usually associated with other polysaccharides such as xylan/or lignin. It is the skeletal basis of plant cell walls

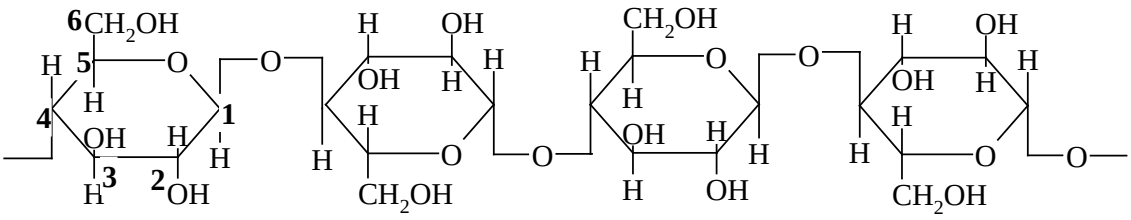


Figure 2.5 – The cellulose chain.

(Ademark *et al.*, 1998). It contains both crystalline (70%) and non-crystalline or amorphous (30%) structure.

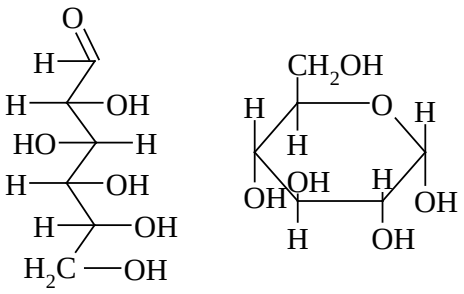
2.3.3 Hemicellulose

Hemicellulose (24–40% of total feedstock dry matter) is a short, highly branched polymer of five carbon (C_5) and six carbon(C_6) sugars. Specifically hemicellulose contain xylose (xylose has acidic group glucuronic acid which makes it more resistant to enzymatic hydrolysis) and arabinose (C_5) and galactose, glucose, and mannose (C_6) (Figure 2.6). It is more readily hydrolyzed compared to cellulose because of the branched amorphous structure. A major product of hemicelluloses hydrolysis is the C_5 sugar.

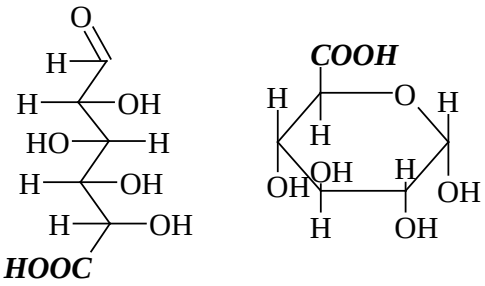
Xylan is highly substituted with acetic acid, for example, 35-70% of xylose is acetylated in hardwoods and grasses. Its branched nature renders hemicelluloses amorphous and relatively easy to hydrolyze to its constituent sugars. As the acetyl xylan fraction becomes increasingly deacetylated, it becomes more digestible, which in turn makes the cellulose fraction more accessible to cellulose enzymes and therefore more digestible. The monosaccharides released upon hemicellulose hydrolysis include a large fraction of pentoses (Mod *et al.*, 1981). The chemical and thermal stabilities of hemicellulose are lower than cellulose, due to its lack of crystallinity and lower degree of polymerization (DP of 100–200 hexose-pentose-deoxyhexose units). It is the next major component after cellulose. Xylose is always the sugar present in the largest amounts amongst the monomer units of hemicelluloses in most agricultural lignocellulosic residues. Cellulose and hemicellulose are the most abundant organic sources of food, fuel, and chemicals (Ingram and Doran, 1995). However, their usefulness depends upon their digestibility to glucose and xylose.

2.3.4 Lignin

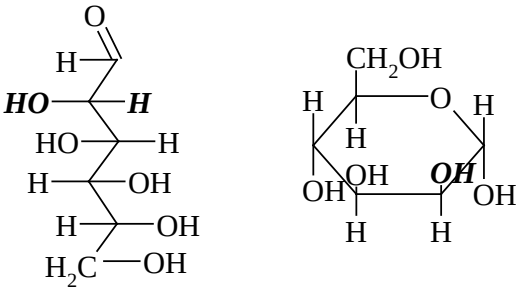
Lignin is a highly cross-linked phenyl propylene polymer and the largest non-carbohydrate fraction of lignocellulose. It’s the third major component of lignocellulosic biomass. In wood biomass it makes up 25–30% depending on the type of wood. It plays



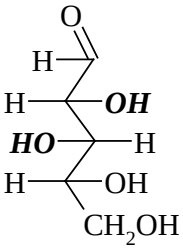
D - glucose



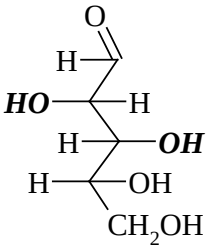
D - glucuronic acid



D - mannose



D - xylose



D- arabinose

Figure 2.6 – Hemicellulose monomer units.

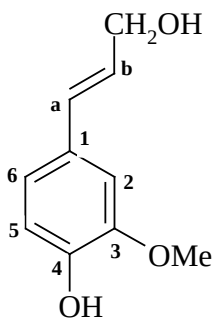
an important role in cell wall structure as a permanent bonding agent among plant cells. It is always associated with hemicellulose in the cell wall. It is constructed from three monomers: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol (Figure 2.7). Each of the monomer unit is referred to as phenylpropane unit, and has an aromatic ring with different substituents. Unlike cellulose, lignin cannot be depolymerised to its original monomers. Lignin and hemicellulose form a sheath that surrounds the cellulosic portion of the biomass. Lignin protects lignocellulose against insect attack. This complexity has made it as resistant to detailed chemical characterization as it is to microbial degradation, which greatly impedes the understanding of its effects.

Lignin has the following characteristics: no repeating structure, it is amorphous, concentrated in middle lamella, it is formed last during cell development, and it is covalently bonded to polysaccharides. Lignin is linked through covalent bonds to all the major polysaccharides in the biomass cell wall, namely; arabinoglucuronoxylan, galactoglucomannan, glucomannan, pectin and cellulose. It is also evident that the lignin polymer cross links various polysaccharides to each other. Such a network structure may play an important mechanical role for the "woody" properties of the secondary xylem.

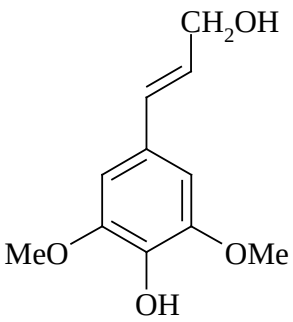
In softwoods, corniferyl alcohol is the predominant lignin monomer. However, corniferyl and sinapyl alcohol are lignin monomers found in hardwood. Both softwood and hardwood lignin also contain a small amount of p-coumaryl alcohol. The exact lignin structure is not fully understood because of the random nature of the linkages between the phenyl-propane units. These reactions are irreversible. There are many possible monomers of lignin, and the types and proportions depend on the source in nature. The concentration of lignin in the middle lamella has been proposed to be over 50%, whereas that in the secondary cell wall is estimated at less than 25% (Westermark *et al*., 1988).

2.4 Lignin-Carbohydrate complexes (LCCS)

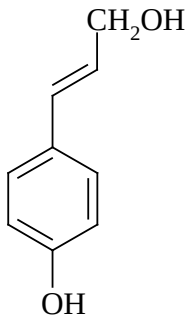
The interactions between the fibres of wood are less understood (Lawoko, 2005). However, there is evidence for covalent linkages between lignin and carbohydrate which is termed lignin carbohydrate complexes (LCCS). Different linkage types of four models for native lignin carbohydrate bonds (LC–bonds) have been proposed (Lawoko, 2005).



Corniferyl alcohol



Sinapyl alcohol



p-Coumaryl alcohol

Figure 2.7 – Distinguishing chemical structures of lignin building blocks (structure of monomer units of lignin, Me = CH_3).

The models are: benzyl ethers, benzyl esters, phenylglycosidases, and recently, acetal linkages (Figure 2.8).

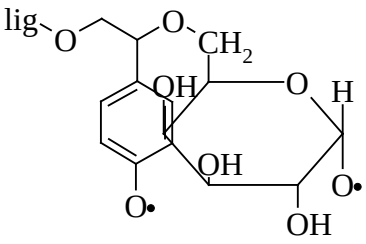
2.4.1 Lignin-Hemicellulose bonds

During the synthesis of wood, some lignin is covalently bonded to hemicelluloses. Therefore, during pretreatments lignin gets extracted along with the hemicelluloses. The lignin portion presents a problem in converting the hemicelluloses into valuable chemicals, since it can inhibit enzymes that are used for the hydrolysis of the hemicelluloses into individual sugars. If lignin is still in the mixture during the fermentation process, it can inhibit the yeast as well, which leads to a lower yield of the desired products. Therefore, lignin should be removed prior to the hydrolysis and the fermentation processes to increase the yield of the valuable chemical (Larsson *et al*. 2001 and Johansson, 1998). In addition to the three major components of cellulose, hemicelluloses, and lignin, wood also contains some pectic substances, which are a type of carbohydrate quite unlike the hemicelluloses. They are, however, present to a much lesser extent.

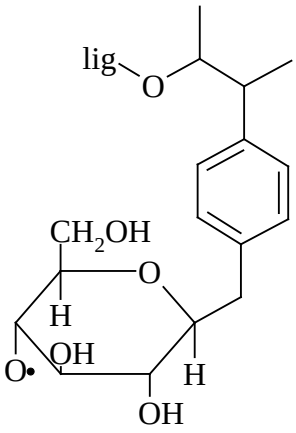
2.5 Woody biomass and non-woody lignocellulosic biomass

Woody biomass are physically larger, structurally stronger and denser than the non-woody biomass. The woody biomass can be harvested all round the year, eliminating long term storage. The high lignin content increases energy density. They have a near-zero ash content (Table 2.3). This eliminates dead load in transportation and processing.

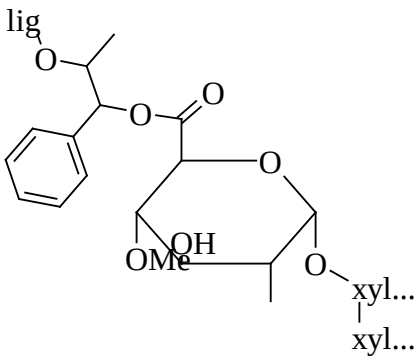
The softwood species such as douglas fir, red pine, spruce, *E. globullus* and almond tree, specifically have lower content of pentoses (xylan fraction) as shown in Table 2.3 than the non-woody biomass such as corn stover, rye grass, bagasse flour, barley hay, and rice straw. This favours its easy conversion to ethanol because fermentation of pentoses to ethanol is relatively difficult. Pentosan recovery yield is often low due to its decomposition to furfurals, a fermentation inhibitor, in thermal/chemical treatment of lignocellullosic biomass.



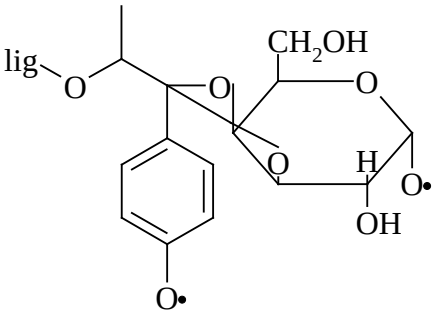
Benzylether LC - bond



Phenylglycoside LC- bond



Benzylester LC - bond



Acetal LC - bond

Figure 2.8 – Proposed model types of lignin carbohydrate linkages

Table 2.3 – Compositions of some woody and non-woody biomass, %(w/w).

	Ash	Extractives	Lignin	Arabinan	Galactan	Xylan	Glucan	Mannose	Carbohydrate	References
Woody										
Douglas fir	0.4	-	32	2.7	4.7	2.8	44.0	11.0	66.8	Pettersen, 1984
Red pine	0.4	-	29	2.4	1.8	9.3	42	7.4	62.9	Pettersen, 1984
Spruce	0.3	-	28.3	1.4	2.7	5.7	43.2	11.5	64.5	Zhu *et al.*, 2009
Olive tree	1.7	5.4	20.4	1.9	1.0	16.0	34.4	1.4	54.7	Cara *et al.*, 2006
Oak tree	-	-	24.3	0.9	1.4	20.3	45.2	4.2	72.0	Shafiel *et al.*, 2010
Non-woody										
Corn stover	9.5	5.6	21.4	3.4	-	20.8	37.5	-	61.7	Kim, 2004
JTW[a]	8.6	19.5	20.4	2.8	0.7	16.9	31.1	-	51.5	Zheng *et al.* 2007
Wheat straw	3.6	-	25.3	1.7	-	20.3	35.7	-	57.7	Chen *et al.*, 2007
Rice straw	14.9	4.1	26.3	-	-	30.1	26.5	-	56.6	Teramoto *et al.*, 2009
CWR[b]	7.4	13.7	24.4	3.3	0.8	16.5	34	-	54.6	Zheng *et al.* 2007
Barley hay	7.0	-	22.0	1.6	0.03	11.2	28.6	-	41.4	Chen *et al.*, 2007

[a]Jose Tall Wheat grass (*L. Triticoides*), [b]Creeping Wild Rye grass (*A. elongattum*)

The woody biomass materials possess more glucan fraction than the non-woody materials which means if pretreatment method is effective more sugar monomers will be readily available for bioethanol conversion. In addition, accumulation of energy varies with the type of plants; different plants consist of different energy values. The residue that has a higher carbon is said to have a higher thermal output. The elemental compositions of these two feedstocks are as follows; woody: C = 49.5%, H = 6.5%, O = 43.0%, N = 1.0%, water content = 15–20%. Its thermal value is 16,700–18,800 kJ/kg. This value varies with tree variety and part of the tree.

Non-woody: C = 40-46%, H = 4-6%, O = 43–50%, N = 0.6–1.1%, S = 0.1–0.2%, P = 1.5–2.5%. Its thermal value is 14,200–15,500 kJ/kg which is less than woody fuel (2010 FAO; A potential renewable energy development and utilization of biomass energy). As a result of these advantages over the non-woody biomass, the ethanol production of woody biomass can be enhanced with pretreatments than can overcome the strong recalcitrance to lignocelluloses (Zhu and Pan, 2010). The compositions of woody biomass therefore give a great task of breaking loose the structure for easy conversion to bioethanol.

In order to increase the enzymatic digestibility of lignocellulosic biomass it has to undergo a reliable and cost efficient pretreatment step. The pretreatment step is a unique step. Through this step the complex structure of the biomass is broken to its three major parts or fractions. The maximum yield of the ethanol is highly dependent on this step.

2.6 Pretreatment methods

Pretreatment is a crucial process step for the biochemical conversion of lignocellulosic biomass into fuel such as ethanol. It is required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars. Pretreatment has been recognized as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion (Mosier *et al*., 2005).

In order to increase the enzymatic digestibility of lignocellulosic biomass it has to undergo a pretreatment step after which the treated biomass is enzymatically hydrolysed

to sugars by cellulase and hemicellulase. The resulting sugars are then fermented to ethanol by yeast. The lignocellulosic biomass conversion to ethanol processing route is as shown in Figure 2.1.

Pretreatment involves the alteration of biomass so that (enzymatic) hydrolysis of cellulose and hemicellulose can be achieved more rapidly and with greater yields. Possible goals include the removal of lignin and disruption of the crystalline structure of cellulose. The following criteria lead to an improvement in (enzymatic) hydrolysis of lignocellulosic material:

- Increasing the surface area and porosity
- Modification of lignin structure
- Removal of lignin
- (Partial) depolymerization of hemicellulose
- Removal of hemicellulose
- Reducing the crystallinity of cellulose

In an ideal case, the pretreatment regime employed leads to a limited formation of degradation products (like HMF, furfural) that inhibit enzymatic hydrolysis and fermentation, and is also cost effective. However, these are actually the most important challenges of current pretreatment technologies. Common pretreatment techniques of biomass are listed:

- Mechanical pretreatment; milling, ultrasonic pretreatment.
- Chemical pretreatment; liquid hot water (LHW), weak acid hydrolysis, strong acid hydrolysis, alkaline hydrolysis, Organosolvolysis.
- Oxidative delignification; hydrogen peroxide, ozonolysis, wet oxidation, room temperature ionic liquids (RTIL).
- Combined chemical and mechanical pretreatment; steam explosion, ammonia fibre explosion (AFEX), CO_2 explosion, mechanical/alkaline pretreatment.
- Biological Pretreatment.

The choice of the optimum pretreatment process depends very much on the objective of the biomass pretreatment since different products are yielded. In addition, the choice of a pretreatment method should not be based only on its potential yield but also on other important parameters such as its economic assessment and environmental impact.

2.6.1 Alkaline hydrolysis

In pretreatment of lignocellulosic biomass for ethanol production, the optimum is to have pretreated products that will be available for enzymatic hydrolysis and fermentation of the sugars. The various additives explored in the pretreatment stage have a lot of affect on the effectiveness of production, most especially the high impact parameters. In alkaline pretreatment, the use of chemical agents such as; calcium hydroxide ($Ca(OH)_2$), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), ammonium hydroxide (NH_4OH), have been investigated under different conditions (Klinke *et al*., 2004, Bjerre *et al.*, 1996, Schmidt *et al.*, 1996, Chang and Holtzapple, 2000). In addition to the chemical agents are the use of pure oxygen, air, hydrogen peroxide, variation of pH, and different chemical loadings at different temperature conditions. These variations are to enhance lignin removal and the accessibility of enzymes to the carbohydrates during saccharification step.

The major effect of alkaline pretreatment is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides. In addition, alkali pretreatments remove acetyl and various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose surface (Chang and Holtzapple, 2000). However, no effect of dilute alkaline pretreatment was observed for softwoods with lignin content greater than 26% (Millet *et al*., 1976). Alkaline hydrolysis mechanism is believed to be based on the saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components, for example, lignin and other hemicelluloses (Sun and Cheng, 2002). The porosity of the lignocellulosic materials increases with the removal of the cross-links (Tarkow and Feist, 1969). Dilute NaOH pretreatment has been studied on some biomass sources such as straw (Bjerre *et al*., 1996), corn stalk, cassava bark, peanut husk (Chosdu *et al*., 1993), and softwoods (Millet *et al*., 1976).

Studies also exist for lime pretreatment on switchgrass, corn stover, wood, and municipal waste (Chang *et al*., 1997, Chang *et al*., 1998, and Kaar and Holtzapple, 2000). Due to the mild conditions, degradation of sugars to furfural, HMF and organic acids is limited. The addition of air or oxygen to the reaction mixture greatly improves the delignification, especially highly lignified materials (Chang and Holtzapple, 2000), and during ammonia pretreatment on corn cobs/stover, switchgrass (Iyer *et al*., 1996). Pretreatment of biomass with aqueous ammonia at elevated temperatures reduces lignin content and removes some hemicellulose while decrystallising cellulose. Ammonia pretreatment techniques include the ammonia fibre explosion-method (AFEX), ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA).

An important aspect of alkali pretreatment is that the biomass by itself consumes some of the alkali. The residual alkali concentration after the alkali consumption by the biomass is the alkali concentration left over for the reaction (Gossett *et al*., 1982). Alkali pretreatment can also cause solubilization, redistribution and condensation of lignin and modifications in the crystalline state of the cellulose. These effects can lower or counteract the positive effects of lignin removal and cellulose swelling (Gregg and Saddler, 1996). Furthermore, alkaline pretreatment is reported to cause changes of the cellulose structure to a form that is denser and thermodynamically more stable than the native cellulose (Pettersen, 1984).

2.6.2 Oxygen delignification

Delignification of lignocellulose can also be achieved by treatment with an oxidizing agent such as hydrogen peroxide, per-acetic acid, ozone, oxygen or air (Hendricks & Zeeman, 2009). The effectiveness in delignification can be attributed to the high reactivity of oxidizing chemicals with the aromatic ring. Thus, the lignin polymer will be converted into compounds such as carboxylic acids. Since these acids formed will act as inhibitors in the fermentation step, they have to be neutralized or removed. In addition to an effect on lignin, oxidative treatment also affects the hemicellulose fraction of the lignocelluloses complex. A substantial part of the hemicellulose might be degraded and can no longer be available for sugar production.

2.6.2.1 Hydrogen peroxide

Dissolution of about 50% of lignin and most of the hemicellulose has been achieved in a solution of 2% H_2O_2 at 30 °C. The yield of subsequent enzymatic hydrolysis can be as high as 95%. The use of H_2O_2 for delignification was demonstrated with a maximum yield at pH = 11.5 (Gould, 1984). No substantial delignification occurred below pH of 10.0. Hydrogen peroxide had no real effect on the enzymatic digestibility at pH = 12.5 or higher. It was also reported that hydrogen peroxide concentration should be at least 1% and the weight ratio between H_2O_2 and biomass should be 0.25 for a good delignification (Gould, 1984).

The delignification is probably caused by the hydroxyl ion ($HO^{\cdot}$), which is a degradation product of hydrogen peroxide with a maximum yield at pH 11.5–11.6. About half of the lignin was solubilized in this way (at the temperature of about 25 ^{o}C and duration of 18–24 h) (Gould, 1984). Alkaline peroxide oxidation also involves pretreatments carried out at mild temperatures (Gould, 1985, Saha and Cotta, 2007, and Silanikove, 1994). Alkaline peroxide oxidation is reported to partially delignify lignocellulosic materials, leaving a cellulosic residue that is highly susceptible to enzymatic digestion by cellulase (Gould, 1985). Alkaline peroxide oxidation (APO) pretreatment is also known to decrystallize cellulose (Gould, 1984). It is also known that under proper conditions hydrogen peroxide (H_2O_2) will react readily with lignin and related phenolics (Agnemo and Gellerstedt, 1979 and Lachenal *et al*., 1980) to yield an array of low molecular weight, water-soluble oxidation products (Bailey and Dence, 1969). Natural degradation of lignin can occur through a variety of different organisms. Hydrogen peroxide excreted by the organism plays an important role in the degradation (Forney *et al*., 1982).

2.6.2.2 Per-acetic acid

The use of peracetic acid at ambient temperatures as a pretreatment method for hybrid poplar and sugar cane bagasse has been investigated (Teixeira *et al*., 1999). Oxidative delignification with peracetic acid is said to be lignin selective and no

significant carbohydrate losses occurred. Similar results for digestibility at using a mixture of NaOH and peracetic acid were also achieved.

2.6.2.3 Ozonolysis

Ozone treatment focuses on lignin degradation by attacking and cleavage of aromatic rings structures, while hemicellulose and cellulose are hardly decomposed. It can be used to disrupt the structure of many different lignocellulosic materials, such as wheat straw, bagasse, pine, peanut (Neely, 1984), cotton straw and poplar sawdust (Sun and Cheng, 2002).

2.6.2.4 Wet air oxidation

Wet oxidation operates with oxygen or air in combination with water at elevated temperature and pressure (McGinnis *et al*., 1983). It was presented as an alternative to steam explosion which had become the most widely used pretreatment method (Ahring *et al*., 1999). Industrially, wet air oxidation processes have been used for the treatment of wastes with a high organic matter by oxidation of soluble or suspended materials using oxygen in aqueous phase at high temperatures (150–350 ^{o}C) and high pressure (5–20 MPa) (Jorgensen *et al*., 2007). Wet air oxidation (WAO) has been successfully applied for the treatment of wheat straw and hardwood (Schmidt *et al*., 1996, and Schmidt and Thomsen, 1998). In recent studies on alkaline wet oxidation of wheat straw, the main degradation products formed from hemicellulose and lignin were carboxylic acids, CO_2, and H_2O.

Compared to other pretreatment processes, wet oxidation has been proven to be efficient for treating lignocellulosic materials because the crystalline structure of cellulose is opened during the process (Panagiotou and Olsson, 2007). About a 65% degree of delignification could be achieved with wheat straw (Klinke *et al*., 2004). Wet oxidation of wood material has been shown to dissolve mainly the hemicellulose. One reported advantage of the wet oxidation process is the lower yields of furfural and 5-hydroxymethylfurfural, which are potential inhibitors in the fermentation step.

2.6.2.5 Alkaline peroxide assisted wet air oxidation (APAWAO)

The pretreatment method involves the use of H_2O_2 and air/or oxygen with an alkali as the pretreatment chemical agents. The H_2O_2 improves the oxidative effect of air during the wet air oxidation pretreatment. Table 2.4 also shows some of the lignocellulosic pretreatment technologies.

2.7 Chemical reactions during alkaline pretreatment

In alkaline pretreatment, oxygen/air plays a prominent role in the removal of lignin and the degradation of the carbohydrates. In normal state oxygen is a weak oxidant. Reactivity can be improved by raising the temperature and providing a reactive substrate. The efficiency results in more carbohydrate mass becoming available for enzymatic hydrolysis to fermentable sugars. The use of oxygen/air has some advantages;

1. It is a cheap chemical.
2. It reduces the consumption of other chemicals (to an appreciable level).
3. It is environmentally friendly.

Mechanistic studies of the reaction of oxygen species with carbohydrates and lignin under alkaline conditions are documented in the literature (Hausman, 1999). Reaction mechanisms of hydroxyl radical on carbohydrates and lignin, and superoxide with lignin have been proposed (Figures 2.9 – 2.12).

2.8 Oxygen species

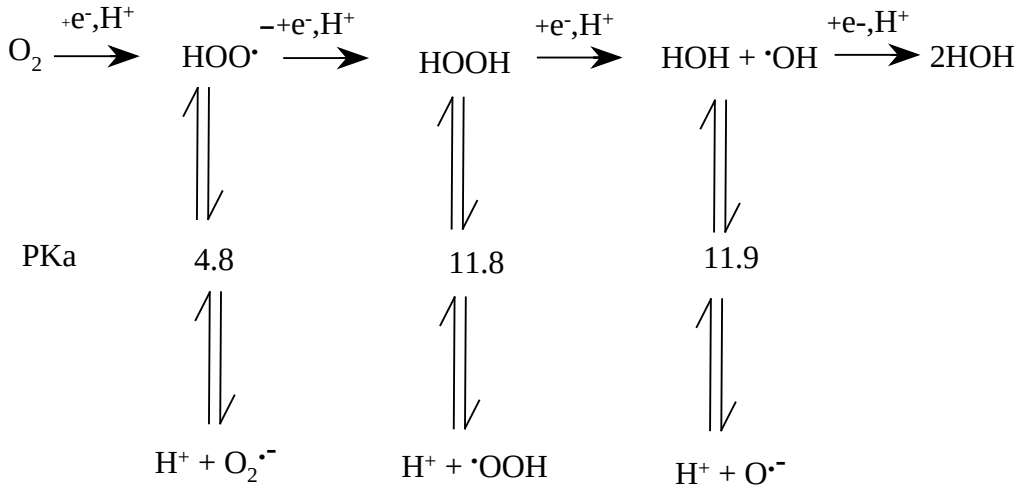
In oxygen delignification, oxygen can undergo a series of reductions to water (Figure 2.9); these reduction give rise to the various conjugate oxygen species involved in delignification. These species are; the molecular oxygen itself (O_2), superoxide radical ($O_2^{\cdot-}$) or its equilibrium hydroperoxy radical $HO_2^{\cdot}$, hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$). Molecular oxygen is a precursor of the other oxygen species which are formed by one electron and proton transfers (Gratzl, 1992). With a PKa of 4.8, the hydroperoxy radical ($HO_2^{\cdot}$) is in equilibrium with superoxide anion ($O_2^{\cdot-}$). The hydrogen peroxide(H_2O_2) is in equilibrium with the hydrogen peroxide anion($HO_2^{\cdot}$) at

Table 2.4 – Lignocellulosic biomass pretreatment methodologies.

Pretreatment	Raw materials	Advantages	Disadvantages	References
Liquid hot water	Bagasse, corn stover, olive pulp, alfalfa	Removal of hemicelluloses increases fermentable sugars Risk of degradation products is reduced	Removal of hemicelluloses Produces high fermentation Inhibitors	Weil *et al*., 1998
Dilute acid	Corn stover, Wood	Specially suitable for biomass with low lignin content Alters lignin structure with high sugar yields Low operational costs	High amount of fermentation inhibitors Corrosive and toxic	Ishizawa *et al*., 2009 Kuhad *et al*., 2009
Alkaline	Hardwood, wheat straw, peanut husk	Removes lignin with very high sugar yields. No or low fermentation inhibition. Increases surface area Decreases DP	Alkaline recovery High residue formation	Millet *et al*., 1976 Chang *et al*., 1998 Kaar and Holtzapple, 2000
Organosolv	Woody biomas	Removal of hemicelluloses and lignin increases sugar yields	High operational and invest-ment costs Extensive detoxification needed due to high concentration of inhibitors.	Zhu and Pan, 2010 Chum *et al*., 1990 Harmsen *et al*., 2010
Wet Oxidation	Wheat straw	No or low fermentation inhibitors Decrystallization of cellulose and removal of lignin	Not yet proven at pilot scale	Harmsen *et al*., 2010 Bjerre *et al*., 1996

Table 2.4 Continued

Pretreatment	Raw materials	Advantages	Disadvantages	References
Steam explosion	Poplar wood, sugar cane, bagasse	Alters lignin structure Cost effective for hardwood	High amount of fermentation inhibitors	Kuznetsov *et al*., 2002
AFEX	Wheat straw, MSW, corn stover	No or low fermentation inhibitors Removes lignin and hemicellulose	Less effective for high lignin biomass Low applicability to different biomass types Ammonia recovery	Holtzapple *et al*., 1991 Vlasenko *et al*., 1997 Mes-Hartree *et al*., 1980
CO_2 explosion	Alfalfa	Low fermentation inhibitors Removes hemicelluloses and decrystallizes cellulose	High in investment costs	Sun and Cheng, 2002
Biological	Wood wastes Bermuda grass stems	Low energy requirement Mild environmental condition	Rate of hydrolysis is very low	Itoh *et al*., 2003 Fan *et al*., 1987



$$O_2 + 4e^- + 4H^+ \rightarrow 2\,H_2O$$

Figure 2.9 – Oxygen species derived from molecular oxygen in aqueous solution (Gratzl, 1992).

PKa of 11.8. Finally, at PKa of 11.9, the hydroxyl radical($HO^{\cdot}$) is in equilibrium with its anion, superoxide anion ($O_2^{\cdot -}$) (Sjöström, 1993).

2.9 Mechanism of carbohydrates degradation

In wood pulping, two mechanisms affect the yields of carbohydrates: peeling (reaction which involves stepwise removal of the reducing end groups leading to successive removal of one sugar unit at a time) which starts at a low temperature and goes very fast, and alkaline hydrolysis (which involves the glycosidic bonds with random scission of the polysaccharide macromolecules, i.e. a random chain cleavage occurs) which starts around 140 ^{o}C. In random chain cleavage, metals such as iron and copper play an important role because it accelerates the chain cleavage reactions. They catalyze the formation of reactive radicals that randomly attack the cellulose chain, ultimately resulting in chain breakage at the point of attack. The decrease in the average length of the cellulose chain results in lower yield of the carbohydrate. These two steps have been identified as the degradation reactions responsible for carbohydrate losses (Sjöström, 1981 and Sjöström, 1993).

During the peeling reaction, primary peeling occurs at the reducing end of the carbohydrate chain and terminates with a stopping reaction or oxidation by oxygen. Secondary peeling is initiated on the reducing end of the new chain immediately following random hydrolysis by an alkali. However it is believed that the peeling reaction is less important in oxygen delignification because the reducing ends are oxidized by oxygen, preventing the peeling reaction. Cleavage of glycosidic linkages is more significant during oxygen delignification than the peeling reaction. In all, the reaction mechanism for carbohydrates degradation involves three major steps (Figure 2.10);

1. Primary oxidation by a hydroxyl radical and formation of a carbonyl intermediates at the C-2 position of a monomeric unit, Figure 2.10(d).
2. A cleavage of the glycosidic bond at C-4 by beta- alkoxy elimination, (e) .
3. Formation of a new reducing end group, (f).

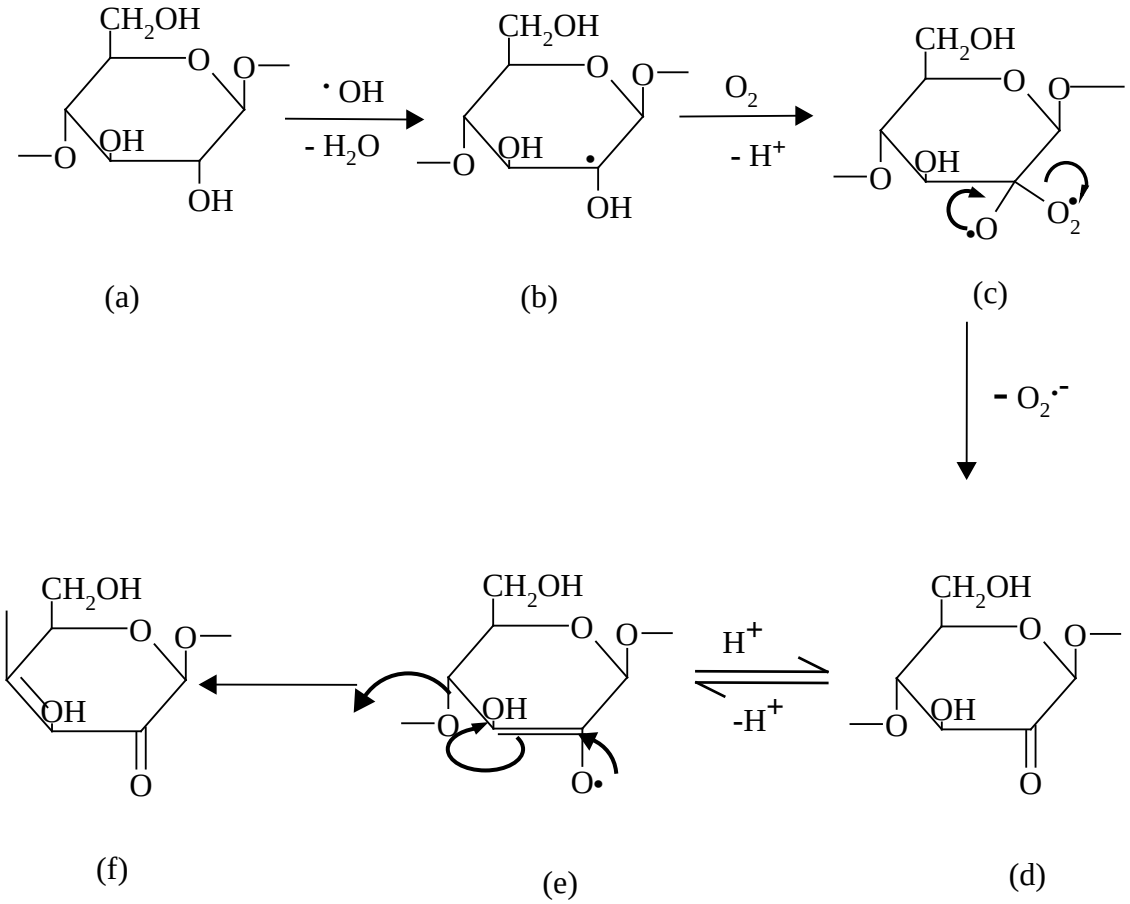


Figure 2.10 – A proposed mechanism for carbohydrate degradation by hydroxyl radical during oxidative delignification (Gierer, 1997).

2.10 Mechanism of lignin removal

The alkaline process conditions ionize free phenolic hydroxyl groups in the residual lignin to generate a phenolate ion (Figure 2.11(a)), which is considered to be the initiation of the lignin reaction. In Figure 2.11(b) oxygen as a radical reacts with the phenolic lignin structure to form a reactive intermediate called hydroperoxide. (R = H, OAryl, or alkyl group). The primary reaction of oxygen with lignin under alkaline conditions proceeds via a resonance stabilized phenoxyl radical.

The resonance stabilized intermediates (right hand side of Figure 2.11(b)) then undergo reaction with themselves (lignin condensation) or with oxygen species such as hydroxyl ($HO^{\cdot}$), hydroperoxy ($HOO^{\cdot}$) and the superoxide ($O_2^{\cdot -}$) radical to form organic acids, carbon dioxide and other small molecular weight organic products via side chain elimination, ring opening and demethoxylation reactions (Figure 2.12) (Johansson & Ljunggen, 1994). Hydroxyl radicals cannot degrade lignin, but only reacts with both phenolic and non-phenolic types of lignin. Hydroxyl radicals has insignificant effects in the ring opening of both phenolic and non-phenolic compounds. Formation of phenoxy radical does not depend on the hydroxyl radical, since it can be formed with any oxygen species that is present in the reaction mixture.

After the formation of the phenoxyl radical, it is the superoxide anion that degrade the aromatic ring to smaller compounds. The superoxide anion forms a muconic acid intermediate (Hausman, 1999).

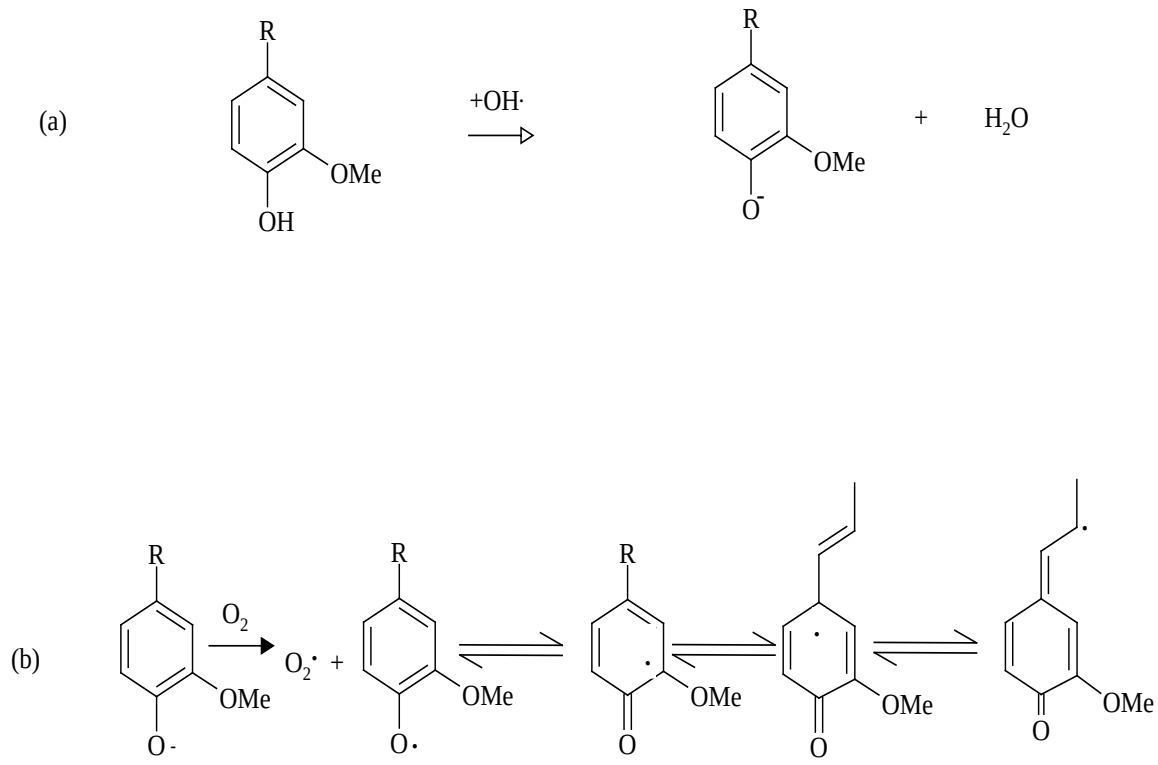


Figure 2.11 – Radical chain reactions during oxygen delignification in alkaline condition (Hausman, 1999).

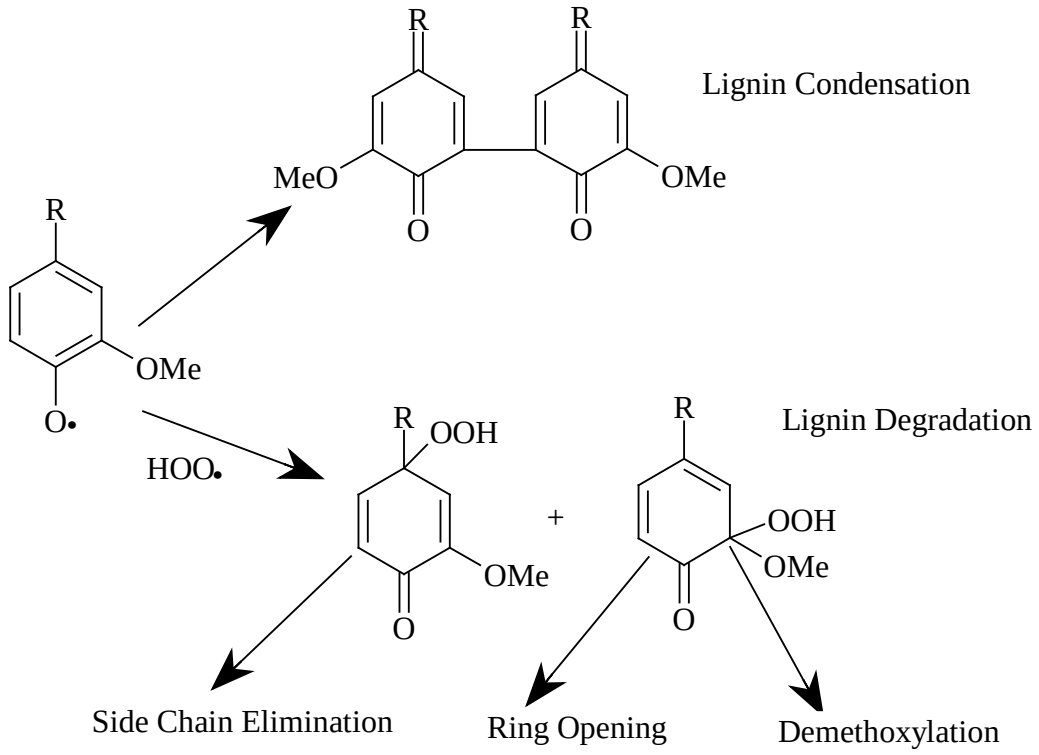


Figure 2.12 – Proposed reactions of lignin via phenoxyradical (Johansson and Ljunggen, 1994).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Statistical designs of experiments

The conventional technique for the optimization of a multi-factorial system is to deal with one-variable at a time (OVAT). However, this type of method is time-consuming, not cost efficient, and does not reveal the interactive and square effects of the factors. Statistical design of experiments (DOE) was used for the purposes of planning/designing and analyzing/interpreting the pretreatments experimental data. Statistical approach to experimental design is necessary to draw meaningful conclusions from the data. With DOE, experiments can be designed to systematically investigate the process or product variables that influence product quality. Once process conditions and product components are established, improvements to enhance the product's reliability and quality can be performed.

In the pretreatment experiments, cellulose content, hemicellulose solubilization, and lignin removal (delignification) were assigned as responses (dependent variables) while temperature, time, air pressure and/or H_2O_2 concentration were the factors or independent variables (regressor variables) influencing the responses. The relationship between these variables is characterized by a mathematical model called a regression model. The regression model is fit to a set of sample data. Generally the relative efficiency of any design increases as the number of factors increases (Montgomery, 1991).

3.1.1 Choice of experimental design

MINITAB 15 (PA, USA) statistical software package was used for the design of experiments, statistical analyses, plotting of response surfaces, and optimization of pretreatments experimental data.

Table 3.1 – Statistical 2^3- factorial design for WAO experiments.

Factor	Low level	High level
Reaction temp., X_1(^oC)	170	195
Air pressure, X_2(bar)	5	10
Reaction time, X_3(min)	10	20

Table 3.2 – Statistical 2^3- central composite design for APO experiments.

Factor	Low level	High level
Reaction temp., X_1(^oC)	120	150
Reaction time,X_2(min)	20	40
H_2O_2,X_3 (% v/v)	1	1.5

Table 3.3 – Statistical 2^2- central composite design for APO experiments.

Factor	Low level	High level
Reaction temp.,X_1(^oC)	90	120
Reaction time, X_2(min)	15	30

A two-level factorial design of experiments was adopted to optimize the pretreatment conditions for the WAO pretreatments. Three operating factors viz. reaction temperature, air pressure and reaction time (Table 3.1) were taken into consideration, to yield 8 different experiments (Appendix O) (Table O-7). The choice of these three factors were based on earlier reported studies on rice husks and wood waste (Banerjee *et al*., 2009 and Palonen *et al*., 2004). The three variables chosen were designated as X_1 (temperature), X_2 (pressure), X_3 (reaction time).

Factorial designs involve the study of the effects of two or more factors on response variables. The effect of a factor is the change in response produced by the level of the factor. This is also called the main effect. Levels are designated as "low" (-) and "high" (+). An interaction between factors also occurs in some experiments such that the difference in response between the levels of one factor is not the same at all levels of the other factors. Interactions are the driving forces in many processes. An interaction produces a form of curvature of the response surface model generated from experimental data. Factorial design is necessary when interaction may be present to avoid concluding wrongly. One disadvantage of factorial designs is that they are not capable of quantifying or even detecting curvature in the response because of their use of just two levels.

Response Surface Methodology (RSM) based on Central Composite Design (CCD) of experiments was adopted to investigate the optimum parameters of APO pretreatments such that the cellulose content, the hemicellulose solubilization, and delignification will enhance enzymatic hydrolysis. It is a design for fitting response surfaces called response surface designs or designs for quadratic models. It determines the optimum operating conditions for the system or it determines a region of the factor space in which operating requirements are satisfied. It reveals three levels in order to fit a model that can resolve curvature of the response. CCDs consist of a factorial or fractional factorial design with centre points augmented with a group of axial (or star) points that allow estimation of curvature. With the main and interactive effects of the regressor (independent) variables, the quadratic effects are also involved in the second order model.

The 2^3 central composite design was used to develop a statistical model for the optimization of process variables (Table 3.2). The 2^3 CCD contains 20 experiments

carried out in duplicate giving a total of 40 experimental runs (Table O-8). The three variables chosen were designated as X_1 (Temperature), X_2 (Time), X_3 %(v/v) H_2O_2.

The 2^2 central composite design was also used to investigate the effects of process variables at much lower reaction temperatures and time (Table 3.3). The concentration of the hydrogen peroxide was kept constant in this design. The CCD contains 13 experiments carried out in duplicate giving a total of 26 experimental runs (Table O-9). The two variables chosen were designated as X_1 (Temperature), X_2 (Time).

3.2 Preparation, storage and handling of raw material

The shea tree, *Vitellaria paradoxa* (Sapotaceae), was harvested from the forest around Idanre (6^o51'N 5^o06'E), South West, Nigeria, in early April 2010. The tree which included the bark was pruned in the forest and chopped into size (3.66 m in length, breadth of 0.30 m, and 0.05 m in thickness) at the central processing unit of the local sawmill (Ilepa, Ifo, Nigeria; 6^o49'N 3^o12'E). No reliable information about the wood age can be provided since the woods were harvested initially for commercial purposes and the sawdust used in this study was a by-product of the processing of the woods at the sawmill. Sample storage condition before delivery to the laboratory was 27 ^{o}C to 33 ^{o}C, sample condition during delivery to laboratory was air dried and thinned. The sawdust was sampled in late June, 2010 from the central processing point of the mill.

Upon arrival in the laboratory, the samples were air-dried and sieved to pass through mesh 14 and retained by mesh 80 sieve sizes (BSS specification) following the procedure as explained in Appendix A. Seventy three percent by weight of the initial sawdust was retained after the sieving process.

The moisture content and dry weight of biomass were determined as described by Ehrman (1994). Samples were dried in a convectional oven at 105 ± 5 ^{o}C for 3 h to a dry matter content of 88%. 50 g dry raw biomass was consecutively sieved with four different sizes of British standard testing sieves. The dried and sieved materials were stored in plastic bottles, capped tightly and kept at room temperature. The materials were used shortly after.

3.3 Compositional analysis of raw and pretreated sawdust

The composition of lignocellulosic biomass can vary greatly with the particular season and place where the material arises. For woody materials, the part of the tree from which the sample is taken, and the variety make the analysis to vary. The contents of cellulose, hemicelluloses, lignin, extractives, and ash in the raw biomass and solid fraction of the pretreated samples were determined through gravimetric methods. The methods used to determine each of the components are briefly discussed below. A more detailed description of each of the experimental procedures is included in the corresponding appendixes.

3.3.1 Extractives

The extractives, W_E, were quantified by Soxhlet extraction of the biomass using acetone as solvent. Extractives include non-structural components of biomass samples that could potentially interfere with the down stream analysis of the biomass sample. The method as reported by Blasi *et al*., (1999), Li *et al*., (2004), and Lin *et al*., (2010) was used to quantify the extractives in the raw and pretreated dry biomass (Appendix B). Biomass extractives were analyzed as acetone extractable compounds (Chacha *et al*., 2011).

3.3.2 Hemicellulose

Hemicellulose is a co-polymer of different C_5 and C_6 sugars that exist in the plant cell wall. There is a significant variation of the hemicellulose content of lignocellulose depending on whether it is derived from hardwood, softwood, or grasses. Hemicellulose is positioned both between the micro- and the macro-fibrils of cellulose. The approach of Li *et al*., (2004) and Lin *et al*., (2010) was also used to quantify the hemicellulose content, W_H, of both raw and pretreated dry biomass (Appendix C).

3.3.3 Ash

It is the organic residue left after ignition at 575 ^{o}C. Ash content, W_{A}, was measured based on NREL standard Procedure (Determination of Ash in Biomass)(Appendix D) (Sluiter *et al*., 2008)).

3.3.4 Determination of lignin (acid insoluble and acid soluble lignin)

The lignin content, W_{L}, separates into acid soluble material and acid-insoluble material (Klason lignin). The acid soluble lignin was quantified by UV-visible spectroscopy and the acid insoluble lignin was determined by gravimetric analysis (Appendix E). The NREL laboratory analytical procedure was applied (Sluiter *et al*., 2008).

3.3.5 Determination of cellulose content

The cellulose content, W_{C}, was calculated by difference, assuming that extractives, hemicellulose, lignin, ash, and cellulose are the only components of the entire biomass (Blasi *et al*., 1999, Li *et al*., 2004, and Lin *et al*., 2010):

$$W_{\mathrm{C}} = 100 - (W_{\mathrm{A}} + W_{\mathrm{E}} + W_{\mathrm{H}} + W_{\mathrm{L}}) \qquad \ldots 3\text{–}1$$

3.4 Reactor set up for pretreatments

The Parr reactor (pressure vessel) is made of type 316 stainless steel. The complete set up has the following parts:

- The process controller having a power switch located on the front panel. A pressure harness port, and tachometer are present for the pressure harness, communication cord set, and tachometer cable. The controller is equipped with default PID valves designed to give reasonable temperature control across a wide

temperature range. Two thermocouple ports are also present to accept either thermocouples or their extension wires.

- The power control port connects the process controller to the power controller serving as a communication link between the two controllers. Thermocouple burn-out is included in each of the controllers.
- The 4857 reactor controller is equipped with CalGrafix software. The software allows reaction variables (temperature, air pressure, stirrer revolution) to be adjusted to their set point values.
- The power controller has both heating and cooling indicators. During the process, the heating indicator lights intermittently as the temperature approaches the set point on the controller. Also during the process, the cooling indicator lights intermittently as the temperature approaches the set point on the controller.
- The heating elements are sheathed in the bomb heater. The surface of the heating elements are protected from any explosive atmosphere such that the surface will be free from igniting any flammable vapours.
- The cooling coils are made in a serpentine configuration. They are anchored with compression tube fittings screwed into the underside of the bomb head.

Variables that were reported to have shown remarkable effects in lime pretreatment, and generally lignocellulosic biomass conversion to ethanol are temperature, time, oxidation, lime concentration (Kim, 2004, Chang, 1999, and Granda, 2004). As a result, the study was carried out based on these variables.

The raw material was pretreated with lime (calcium hydroxide) in the presence of water. The 1.8 L batch reactor has a double six-blade turbine impeller and external heating element sheathed with a jacket and internal stainless steel loops for cooling. The schematic diagram of the reactor set up is shown in Figure 3.1. The reaction was controlled by a Parr PID controller model 4857. A solenoid valve adjusted the water flow through the internal coil, and regulated the temperature at the set point ($\pm 2\ ^{o}C$) with constant stirring at 200 revolutions per minute. Each reaction was terminated by running cold water through the internal loops. There was constant stirring while cooling, thereby maintaining a relatively homogenous environment. Pictures and details of the whole reactor system are shown in Figures 3.2 and 3.3.

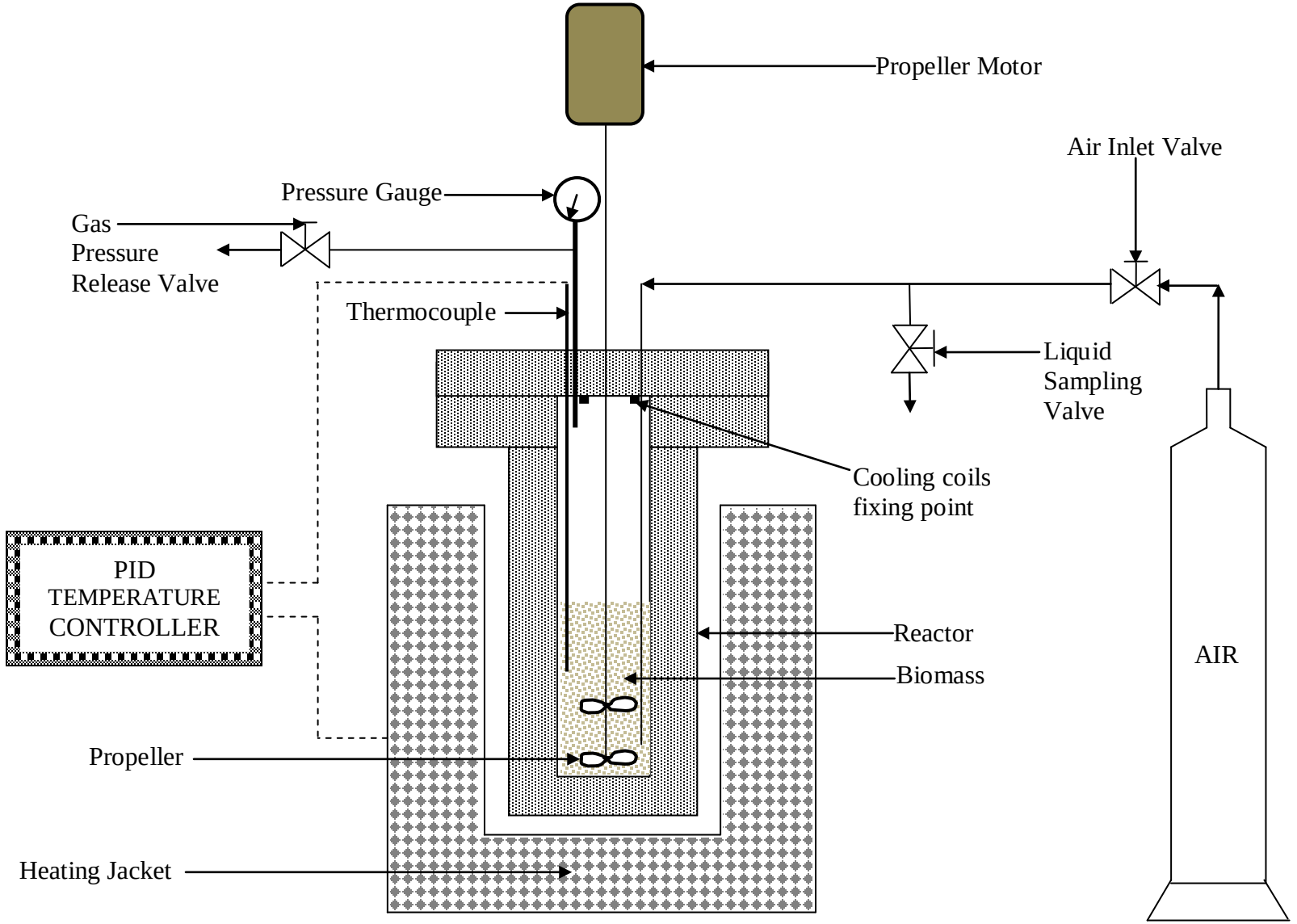


Figure 3.1 – Schematic diagram of the pretreatment reactor set up.

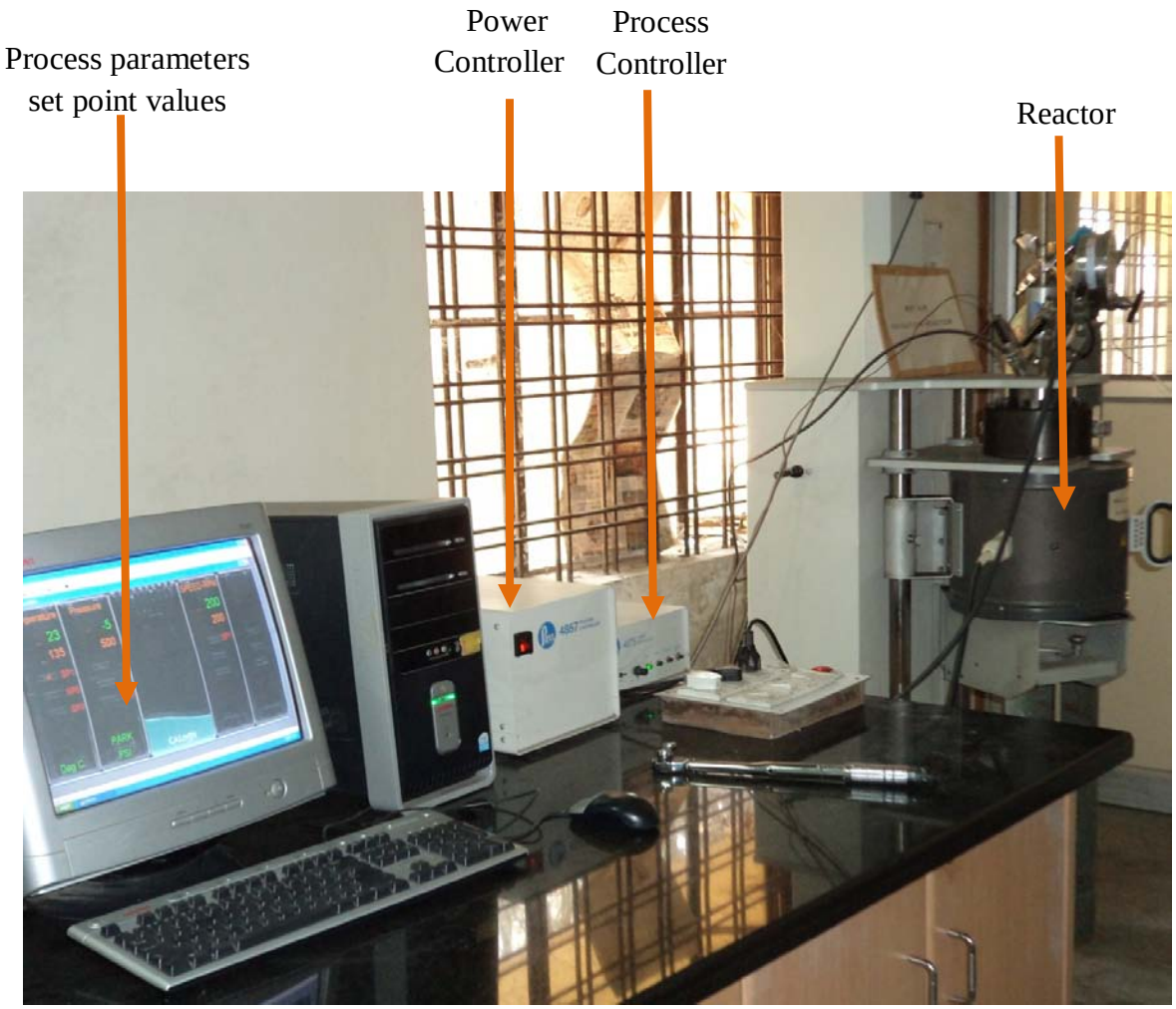


Figure 3.2 – Complete reactor set up with the process and power controllers.

(a)

(b) (c) (d)

Figure 3.3 – Pictures of reactor system. a) System with reaction vessel in place, heating jacket, and gas cylinder. b) Bomb head region of reactor with the pressure gauge, stirrer motor, sampling point, gas inlet and outlet valves, thermocouple, and solenoid. c) Cooling loops, double six blade turbine impellers, impeller shaft. d) Heating coil/element.

After the specified reaction time, the reactor and slurry were allowed to cool to ambient temperature. The pretreated slurry was separated into the solid and liquid fractions by vacuum filtration, and the solid fraction was washed with water to a neutral pH (for the alkaline peroxide oxidation pretreatments the specific lime consumptions during pretreatments were determined before washing solids with distilled water). The pH of the liquid fraction was measured and the solid fraction was dried and weighed. The compositions of the solid fraction were analyzed. The liquid fraction for WAO pretreatments was analyzed for reducing sugar content through the DNS method. Details are described in Appendix F (Raw material pretreatment procedures). Experimental conditions and key parameters for the three different experimental designs for the pretreatment process are summarized in Tables 3.1–3.3. Distilled water was utilized for pretreatment experiments.

3.5 Pretreatment method

Granda (2004) reported that both air and pure oxygen have important effect on lime pretreatment, enhancing significantly the delignification of lime pretreated bagasse at 57 ^{o}C for 2 months, with a minor difference between the two (air and pure oxygen). Also, about 65% degree of delignification could be achieved with wheat straw during wet air oxidation (WAO) pretreatment (Klinke, 2004). Dissolution of about 50% of lignin and most of the hemicellulose has been achieved in a solution of 2% H_2O_2 as an oxidizing agent at 30 °C in alkaline peroxide oxidation pretreatment (Gould, 1984). In this study, air was used in the alkaline wet air oxidation pretreatment as the oxidizing agent. For APO pretreatment, H_2O_2 was used as the oxidizing agent while a combination of air and H_2O_2 was used for the APAWAO pretreatments.

3.6 Lime as the alkaline pretreatment agent

The raw material was pretreated with lime (calcium hydroxide) in the presence of water in order to maintain an alkaline medium. The types of lime treatment that show high total sugar yields have been reported:

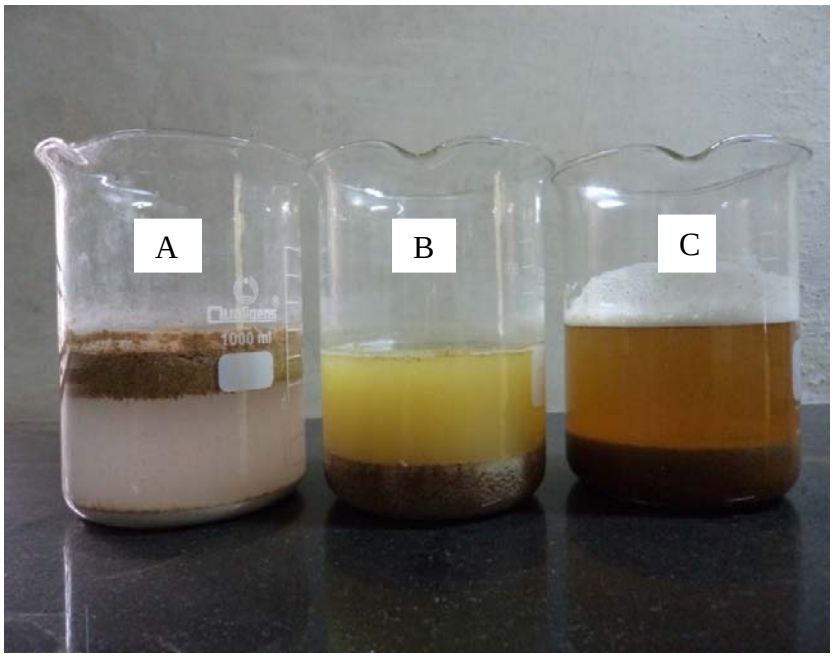
- Short term lime pretreatment which involves boiling biomass with lime loading of 0.1 g $Ca(OH)_2$/g dry biomass at temperatures of 85–150 ^oC, 0–14.8 bar oxygen pressures for 1–6 h (Chang *et al*., 1997, Chang *et al*., 1998, and Chang and Holtzapple, 2000). This removes approximately a third of the lignin and the acetyl groups from hemicelluloses (Chang *et al*., 1997 and Chang *et al*., 1998). It was also shown that oxidative lime pretreatment could be used to pre-treat high-lignin biomass.
- Long-term pretreatment which involves using the same lime loading at lower temperatures (40–50 ^oC) for 4-6 weeks in the presence of air. It was reported by Kim (2004) that long-term pretreatment removes about half of the lignin and all the acetyl groups in corn stover.
- Lime pretreatment removes lignin from biomass, thus improving the reactivity of the remaining polysaccharide. It also removes acetyl and the various uronic acid substitution on hemicelluloses that lower the accessibility of the enzyme to the hemicelluloses and cellulose surface (Kaar and Holtzapple, 2000 and Chang and Holtzapple, 2000). Lime has the following advantages: it is inexpensive; safe to handle; and can be simply recovered (Chang *et al*., 1998). This study was based on the short-term lime pretreatment.

All pretreatment experiments were carried out in the Parr reactor model 4578 (Figure 3.2) positioned at the bioprocess laboratory of National Environmental Engineering Research Institute (NEERI), Nagpur, India.

3.6.1 Specific lime consumption determination

The amount of unreacted lime in the pretreated sample was determined to estimate the lime consumption for the two CCDs alkaline peroxide pretreatment method (Appendix G). In order not to over estimate the lime consumption for the WAO pretreatment, lime consumption determination was left out since CO_2 present in air was not scrubbed so as not to interfere (though at a minimal level) with the analysis. The amounts of lime consumed during the pretreatment at each condition were determined by pH neutralization with standard solution of 5 M HCl. The appearances of the biomass

(a)



(b)

Figure 3.4 – ((a) and (b)). Slurry and solid fraction of pretreated biomass. (A): Slurry before pretreatment. (B): Slurry after pretreatment. (C): Slurry after neutralization. (D): Raw dry biomass. (E) and (F): Wet biomass after APO pretreatments at 120 ^{o}C and 135 ^{o}C respectively. (G): Wet biomass after APO pretreatment at 150 ^{o}C.

mixtures before pretreatment, pretreated slurry before and after neutralization are shown in Figure 3.4 ((a) and (b)).

3.7 Enzymatic hydrolysis

After pretreatment, the slurry was neutralized, washed with water, vacuum filtered, and the solid fraction was stored in the freezer compartment of the refrigerator and the liquid fraction stored in plastic bottles in the refrigerator for the enzymatic hydrolysis and fermentation steps. The procedure is based on the NREL standard procedure "Enzymatic Saccharification of Lignocellulosic Biomass". The step is to determine the maximum extent of digestibility possible after the enzymatic saccharification of cellulose from washed untreated or washed pretreated biomass. The focus of the pretreatment step is to methodically change the key features favouring enzymatic hydrolysis and fermentation leading to the production of ethanol.

The pretreated and washed biomass was weighed into the culture tubes. Citrate buffer (1.0 M, pH 4.8 (5 mL)) and tetracycline solution (10 mg/mL tetracycline in 70% ethanol (40 µL)) were added to the slurry to keep constant pH and prevent microbial growth respectively. The total volume of the mixture was then made up to the desired volume by adding distilled water. The tube was placed in a 130 rev/min shaking air bath and 50 ^{o}C. After 1 h incubation period, cellulase and β-glucosidase were added to the flask. The loading rates of these enzymes varied from one experiment to the other. Samples were drawn at different time intervals and the reducing sugars were measured at each time point considered in the saccharification step. The complete hydrolysis procedures are given in Appendix J. The same procedure was also applied to washed untreated biomass.

Prepare 100 mL 1 M citrate buffer (citric acid monohydrate (21.01 g/L) and sodium citrate (29.41 g/L) separately in 1 L volumetric flasks. From the 1 M solutions of citric acid monohydrate and sodium citrate, mix 27 mL of sodium citrate, and 23 mL of citric acid, dilute the mixture with 50 mL of distilled water and make up to 100 mL. The pH should be 4.8 (Adjust pH if necessary). Prepare also 10 mg/mL tetracycline in 70% ethanol solution.

3.7.1 Cellulase activity determination

The cellulase enzyme used (in form of a brown liquid) was provided by M/s Zytex, Mumbai, India. β-Glucosidase used was a white crystalline solid (Himedia Laboratories, Mumbai, India, Extra pure CAS No.: 9001-22-3, Lot 0000037747, activity of 10 UI/mg solid); it was added in some cases to completely convert cellobiose to glucose. The method describes the procedure for measurement of cellulase activity using International Union of Pure and Applied Chemistry (IUPAC) guidelines (Ghose, 1987). The procedure has been designed to measure cellulase activity in terms of "filter-paper units" (FPU) per millilitre of original (undiluted) enzyme solution. The procedures for determining the enzyme activity and the definition of filter-paper units are given in Appendix I.

3.7.2 Convertibility of the sawdust material

In this study, sawdust wood residue was pretreated with lime under oxidative conditions (effects of air pressure, hydrogen peroxide, and a combination of air and hydrogen peroxide). The enzymatic hydrolysis was investigated on the treated samples of the optimized conditions of both WAO and 2^3 CCD APO pretreatments. The variations of the optimized conditions as shown in Table O-16 were also subjected to enzymatic hydrolysis. Also investigated was the ease of enzymatic hydrolysis on pretreatments carried out on the 13 experimental runs of the 2^2 CCD APO conditions. Detailed descriptions of all the enzymatic hydrolysis studies are discussed in Appendix J.

The efficiency of enzymatic hydrolysis should be evaluated to determine the optimum condition for pretreatments. The effects of pretreatment methods used, substrate concentration, hydrolysis time and temperature, and enzyme loadings on digestibility were investigated.

For the optimized conditions of both WAO and 2^3 CCD APO pretreatments and their variations, 13 experimental runs of the 2^2 CCD APO conditions, the enzymatic saccharification was performed with 2% dry biomass loading (20 g/L), 25 FPU/g dry biomass (equivalent to 0.087 mL cellulose enzyme)[A], 12.5 IU/g dry biomass (equivalent

to 0.2 5 mL β-glucosidase)[B], incubation time of 50 ^{o}C, 130 rev/min of the shaking incubator for 3 days hydrolysis time (see footnote below). Sampling times were 2, 24, and 72 h. Reducing sugar concentration in the hydrolyzate was measured as discussed in Appendix K using dinitrosalicylic acid (DNS) reagent after the samples were centrifuged at 4000 rev/min for 5 min (Miller, 1959).

3.8 Fermentation of treated and untreated materials

Simultaneous saccharification and fermentation (SSF) involves the combination of enzymatic hydrolysis of treated sample by cellulase enzymes and fermentation of resulting sugars by yeast occurring at the same time in the same vessel. To characterize the relative fermentability of pretreated solids, samples with pretreated conditions of 150 ^{o}C, 1%H_2O_2, 10 bar air pressure, 45 min (2^3 CCD APO pretreatment optimized conditions) and 120 ^{o}C, 1%H_2O_2, 30 min (2^2 CCD APO pretreatment optimized conditions) were used. The NREL procedure, "SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation Laboratory Analytical Procedures was strictly followed.

[A]For 2% dry biomass loading and 25 FPU/g dry biomass enzyme loading in 10 mL total working volume, cellulase enzyme activity of 57.8 FPU/mL:

$$0.2 \text{ g dry biomass} \times \frac{25 \text{ FPU}}{1 \text{ g dry biomass}} \times \frac{1 \text{ mL}}{57.8 \text{ FPU}} = 0.087 \text{ mL cellulase enzyme needed.}$$

[B]For 2% dry biomass loading and 12.5 IU/g dry biomass in 10 mL total working volume, β-glucosidase activity of 10 IU/mg solid:

$$0.2 \text{ g dry biomass} \times \frac{12.5 \text{ IU}}{1 \text{ g dry biomass}} \times \frac{1 \text{ mg}}{10 \text{ IU}} = 0.25 \text{ mg}$$

Therefore, 0.25 mg of the β-glucosidase crystalline solid dissolved in 10 mL distilled water, and 0.25 mL of this volume was equivalent to 12.5 IU/g dry biomass.

CHAPTER FOUR

4. RESULTS

4.1 Compositional analysis of untreated (raw) biomass

The raw material was characterized in terms of the particle size distribution and the contents of extractives, ash, cellulose, hemicelluloses, and lignin. The characterization is necessary in order to determine the extent of changes resulting from the lime pretreatment.

The particle size distribution of the raw sawdust was determined by the weight fraction of each collection. Seventy three percent by weight of the initial sawdust was retained after the sieving process. The sieved materials were stored in plastic bottles and kept at room temperature in the laboratory. The weight content of each fraction after screening are as shown in Table 4.1. Particle fractions with small sizes, BSS -14/+20 and BSS -20/+80, were mixed together for carbohydrate and lignin compositional analysis, and for lime pretreatment. The mixed fraction contains 115 g of -14/+20 and 467 g of -20/+80. The particle sizes of the raw biomass used for this study were sieved particles passing through mesh 14 and retained by mesh 80 sieve sizes (BSS specification). The greatest weight percent was the 80 mesh size.

Table 4.2 shows the composition of the raw sawdust used in the study as weight percent of each component in the raw biomass. Major components are cellulose, hemicellulose and lignin. The total carbohydrate (cellulose and hemicelluloses i.e. the holocellulose) composition was 66.17%(w/w). This makes the feedstock a potential material for ethanol production if adequate pretreatment method is used. Other minor components were ash and the extractives.

Table 4.1 – Particle size distribution of the sawdust

Mesh Size (BSS)	Particle size (Standard mm)	Average particle size (mm)	Average weight (g)	Weight fraction	Percent weight fraction (%wt)	Standard deviation
Pan	-	-	-	-	-	-
80	0.18	0.09	24.74	0.50	50	2.5
20	0.85	0.52	11.26	0.23	23	7.5
14	1.41	1.13	13.72	0.27	27	7.1

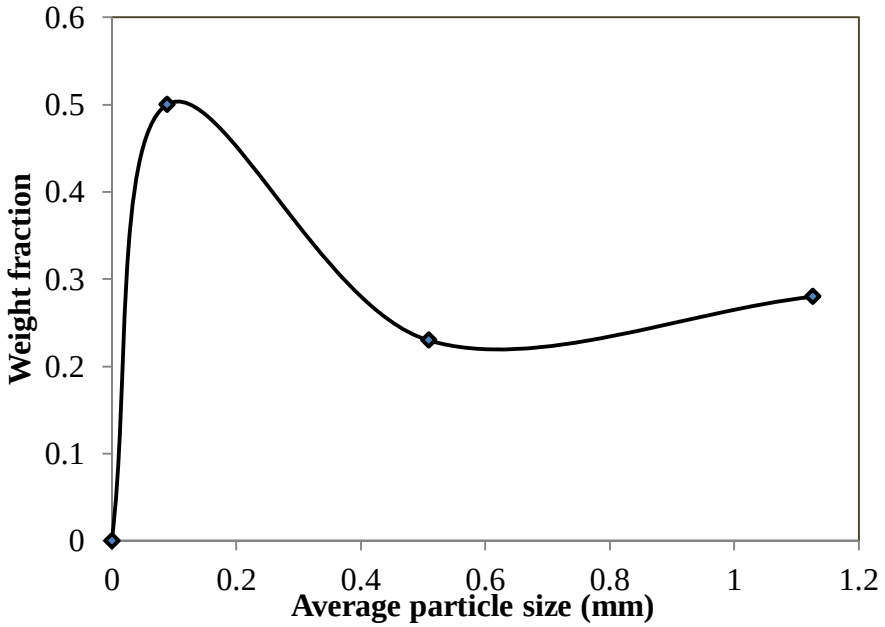


Figure 4.1 – The average particle size distribution of dry raw sawdust.

Table 4.2 – Composition of raw sawdust.

Components (g/100g dry biomass)	**Sample**			
	1	**2**	**3**	**Average[1]**
Extractives	1.92	2.07	1.68	1.89
Hemicellulose	20.70	19.93	20.30	20.31
Lignin[2]	29.61	30.39	29.70	29.90
Ash	2.09	1.98	2.06	2.04
Cellulose[3]				45.86

[1]Average of three samples

[2]Klason lignin (AIL) and acid soluble lignin (ASL)

[3]Cellulose content calculated by difference

The general definitions for the material balance for the compositional yields are given as follows:

Yield of total solids (dry biomass yield or dry matter yield):

$$Ys = \frac{\text{g pretreated dry biomass of pretreatment } i}{\text{100 g raw dry biomass}}$$

i = for each pretreatment investigated.

Yield of cellulose:

$$\%Rc = \frac{\text{g cellulose remaining after pretreatment } i}{\text{g cellulose in raw dry biomass}} \times Ys$$

Yield of hemicellulose:

$$\%R_H = \frac{\text{g hemicellulose remaining after pretreatment } i}{\text{g hemicellulose in raw dry biomass}} \times Ys$$

Yield of lignin:

$$\%R_L = \frac{\text{g lignin remaining after pretreatment } i}{\text{g lignin in raw dry biomass}} \times Ys$$

Yield of holocellulose:

$$\%R_h = \frac{\text{g holocellulose remaining after pretreatment } i}{\text{g holocellulose in raw dry biomass}} \times Ys$$

% Hemicellulose solubilization = $100 - \%R_H$

% Lignin removal(delignification) = $100 - \%R_L$

$$\%\text{ Cellulose content} = \frac{\text{g cellulose remaining after pretreatment}}{\text{g cellulose in raw dry biomass}} \times 100\%$$

4.2 Compositions of pretreated solid fraction

It has been well recognized that woody raw materials especially softwoods are generally more recalcitrant to enzymatic hydrolysis than other lignocellulosic substrates such as agricultural residues.

4.2.1 Lignin removal (delignification)

The enzymatic digestibility of biomass is affected by the pretreated methods used and the structural modification of the biomass (e.g. lignin content, acetyl group content, and crystallinity) (Chang and Holtzapple, 2000). Alkaline delignification is known to occur in three separate simultaneous phases: initial (rapid) phase, bulk (dominant) phase, and the final residual (slow) phase (Kim and Holtzapple, 2005). The breakage of phenolic α and β-aryl ether bonds in lignin has been ascribed to the initial phase, non-phenolic β-aryl ether bonds cleavage governs the bulk phase, which is the dominant phase; cleavage of carbon-carbon linkages are thought to cause lignin degradation in the residual phase (Gierer & Norén, 1980).

It was also reported (Dolk *et al*, 1989) that there is the possibility for the initial phase sometimes not to be controlled by chemical reaction but by diffusion because of its speed. It is therefore important to assess the effects of temperature, time, oxidative condition, lime consumption, holocellulose yield on lignin removal. In wood, the initial delignification stage involves a considerable amount of hemicelluloses degradation with little lignin removal, the bulk stage removes most of the lignin polymers and remaining hemicelluloses and, finally in the residual stage cellulose and remaining hemicelluloses are decomposed further while lignin removal proceeds very slowly (DeGroot *et al*., 1995). The effects of pretreatments on solid raw materials are illustrated in Figures 6.3–6.7.

4.3 Statistical optimization of the pretreatment process

The 2^3 full factorial WAO and 2^3 CCD APO pretreatments were optimized to investigate their performances for the pretreatment conditions. To investigate the

optimum parameters of pretreatments, cellulose content, the hemicellulose solubilization, and delignification (lignin removal) were chosen as the responses. The effects of process parameters on these responses were studied. The most suitable conditions to obtain enriched solid fraction for enzymatic digestibility were selected after optimizing and validating the pretreatment conditions.

4.3.1 Alkaline WAO pretreatments

The pretreated slurry was separated into solid and liquid fractions. The analyses of the solid and liquid fractions are shown in Table O-7. The content of sugars in the liquid fraction was measured after 4% weak acid hydrolysis and autoclaving at 121 °C for 1 h. The samples were filtered and neutralized with calcium carbonate after the weak acid hydrolysis. Reducing sugars in the liquid fraction were estimated with DNS assay using glucose as standard (Miller, 1959).

The model generated as a function of the three variables (designated as X_1 (temperature), X_2 (pressure), X_3 (reaction time)) on the predicted responses of cellulose content, hemicellulose solubilization, and lignin removal is a second-order polynomial and is represented as follows:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{1,2} X_1 X_2 + \alpha_{1,3} X_1 X_3 + \alpha_{2,3} X_2 X_3 \qquad \ldots(4\text{-}1)$$

The predicted responses are designated as Y associated with each factor level combinations; α_0 to $\alpha_{2,3}$ are the regression coefficients; X_1, X_2, X_3 are the factors. The optimal process parameters for WAO were estimated by MINITAB 15 software (PA, USA). The coefficients in the second-order polynomial (Eq. 4-1) were calculated by multiple regression analysis, based on the experimentally obtained data, and then the predicted responses were obtained using Eq. 4-1. The equations were validated using analysis of variance (ANOVA) method. Response surfaces were drawn to determine the individual and interactive effects of the test variables on cellulose content, hemicellulose solubilization, and lignin removal. The optimal values of each factor to optimize the

process responses were based on Multi-Objective Numerical Optimization. The order in which the experiments were carried out was randomized. All the experimental runs were performed within one week. Each experiment was replicated twice; reported results indicate the average values of the replicated experiments. The predicted values were compared with the experimentally obtained values and the data were in close agreement (Table 4.3).

4.3.2 2^3 CCD alkaline peroxide oxidation pretreatment

The central composite design was used for the alkaline peroxide oxidation pretreatments in the following sections. From literature (Gould, 1984 and Saha and Cotta, 2007), alkaline pretreatments of lignocellulosic residues using hydrogen peroxide as oxidizing agent have been evaluated at mild temperatures (e.g at room temperature). Furthermore, APO pretreatments have been investigated only with agricultural residues. In this study, response surface methodology (RSM) was adopted for the optimization of process variables in the alkaline peroxide oxidation (APO) pretreatments of the sawdust based on central composite design (CCD) of experiments. The pretreatments were evaluated at elevated temperatures as the raw material used was a woody residue with high lignin content. A 2^3 five level CCD with central and axial points was used to develop a statistical model for the optimization of process variables (Table 3.2). The CCD contains 20 experiments carried out in duplicate giving a total of 40 experimental runs (Table O-8). The three variables chosen were designated as X_1(Temperature), X_2(Time), X_3(% H_2O_2). The solid fraction was used to determine the optimized pretreatment conditions.

Table 4.3 – Predicted and experimental (validated) responses for the WAO pretreatment at 170 ^{o}C, 10 bar, and 10 min optimized conditions.

	Predicted responses	Experimental (validated) responses	Percentage error
SOLID FRACTION[a]			
Dry solid yield (g/100g)	-	86.63	-
Extractives	-	7.67	-
Cellulose content	55.79	51.76	± 7.79
Hemicellulose	57.88[b]	9.85 (57.94)[b]	± 0.10
Lignin	24.00[c]	28.60 (17.14)[c]	± 40.02
Ash	-	2.12	-
LIQUID FRACTION			
RS[d] (g/L)	6.11	6.46	± 5.42
pH	7.65	7.61	± 0.53

[a]solid compositions in %(w/w). Values show the [b]hemicellulose solubilization, and [c]lignin removal in the solid fraction. [d]Reducing sugars.

Table 4.4 – Predicted and experimental (validated) responses for the 2^3 CCD APO pretreatment at 150 ^{o}C, 45 min, 1%H_2O_2 optimized conditions.

.

	Predicted responses	Experimental (validated) responses	Percentage error
SOLID FRACTION[a]			
Dry solid yield (g/100)	-	89.37	-
Extractives	-	3.70	-
Cellulose content	53.86	58.52	± 7.96
Hemicellulose	70.00[b]	6.40 (71.84)[b]	± 2.56
Lignin	11.00[c]	30.23 (9.64)[c]	± 14.11
Ash	-	1.16	-

[a]Solid compositions in %(w/w). Values show the [b]hemicellulose solubilization, and [c]lignin removal in the solid fraction.

The model generated as a function of these variables on the predicted responses of cellulose content, hemicellulose solubilization, and lignin removal is a second- order polynomial and is represented as follows:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{1,1} X_1^2 + \alpha_{2,2} X_2^2 + \alpha_{3,3} X_3^2 + \alpha_{1,2} X_1 X_2 + \alpha_{1,3} X_1 X_3 + \alpha_{2,3} X_2 X_3 \quad \ldots(4\text{-}2)$$

The predicted responses are designated as Y associated with each factor level combinations; α_0 to $\alpha_{2,3}$ are coefficients to be estimated from regression, they represent the linear, quadratic and cross-products of X_1, X_2, X_3 on the responses; X_1, X_2, X_3 are the factors. In this design, the quadratic terms ($\alpha_{1,1}$, $\alpha_{2,2}$, and $\alpha_{3,3}$) are introduced which brought about curvatures. The coefficients in the second-order polynomial (Eq. (4-2)) were also calculated based on multiple regression analysis on the experimentally obtained data, and then the predicted responses were obtained using Eq. (4-2). The order in which the experiments were carried out was randomized.The experimental (validated) and predicted responses were found to be in close agreement, thus confirming the optimization process (Table 4.4). The material balance for the optimized APO 2^3 CCD is shown in Figure 4.2

4.3.3 2^2 CCD alkaline peroxide oxidation pretreatment

A statistical 2^2 central composite design was also performed for pretreatment on the wood waste (Table 3.3). The objective was to evaluate the influence of pretreatment temperature and reaction time at much lower operating ranges (80–130 ^{o}C and 12–35 min). From the 2^3 CCD pretreatment results it was noted that comparable values could be obtained at much more lower temperature values, thereby reducing the total costs of the process. The hydrogen peroxide concentration was kept constant for all pretreatments having established from the optimization of 2^3 CCD APO that 1% H_2O_2 concentration was adequate to cause appreciable delignification. The CCD contained 13 experiments (Table 4.5) carried out in duplicate giving a total of 26 experimental runs (Table O-9). The optimization of this process was concluded based on the highest cellulose content after pretreatments and highest lignin removal as these are related to ease of enzymatic

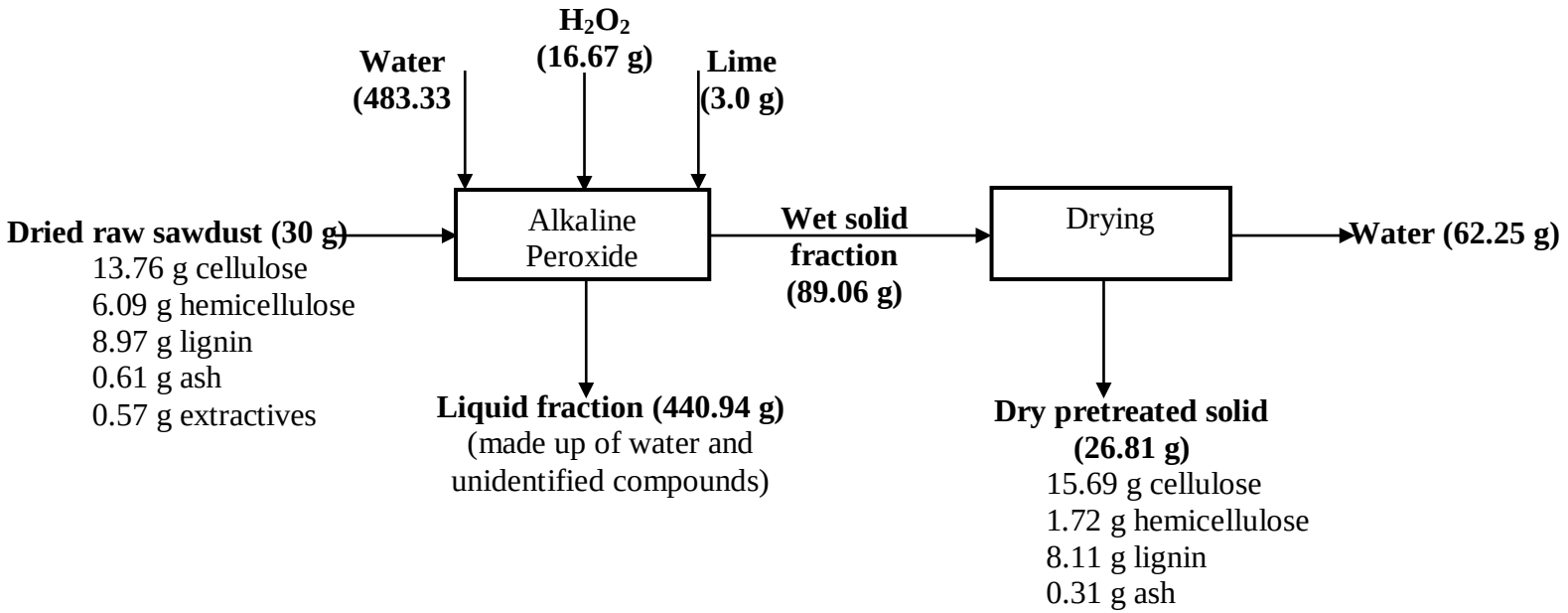


Figure 4.2 – Mass balance for the optimized alkaline peroxide oxidation pretreatment.

Table 4.5 – Cellulose content, hemicellulose solubilization, lignin removal %(w/w) after raw biomass pretreatments for 2^2 CCD alkaline peroxide oxidation.

Run order	Temperature (^oC)	Time (min)	Cellulose content	Hemicellulose solubilization	Lignin removal
1	120	15.00	59.03	70.80	14.09
2	105	11.89	58.28	65.65	7.14
3	90	30.00	60.06	58.70	13.43
4	105	22.50	60.64	60.92	14.66
5	105	33.11	55.59	58.52	9.17
6	105	22.50	58.20	62.53	14.29
7	105	22.50	58.59	62.91	16.84
8	90	15.00	59.18	59.48	12.83
9	83.79	22.50	56.69	58.10	12.65
10	105	22.50	56.24	58.67	4.96
11	120	30.00	61.12	67.93	17.20
12	105	22.50	58.03	62.26	13.59
13	126.21	22.50	58.98	68.58	6.27

Data for Figures 5.28–5.30

hydrolysis. The greater the lignin removal the more likely the enzymatic hydrolysis of the cellulose remaining in the solid fraction will proceed with ease.

4.3.4 Alkaline peroxide assisted wet air oxidation pretreatment (APAWAO)

APAWAO as an oxygen delignification process involves a combination of wet air oxidation and hydrogen peroxide oxidation. Hydrogen peroxide is added during WAO to promote the radical reactions. It was reported that WAO assisted with hydrogen peroxide gave better oxidation efficiencies at 160 ^{o}C than conventional WAO at 220 ^{o}C for treatment of aqueous wastes, thus turning a high pressure process into a medium one (Debellefontaine *et al*., 1996). At above 200 ^{o}C rapid decomposition is said to occur. APAWAO is said to be efficient for reducing the capital cost of WAO without increasing the running cost too much (Debellefontaine *et al*., 1996).

In this study, APAWAO pretreatments were considered at the optimized conditions of both WAO and APO pretreatments (2^3 central composite design). The 2^3 full factorial design of WAO pretreatment optimized conditions were 170 ^{o}C, 10 bar, and 10 min, while the 2^3 CCD APO pretreatments optimized conditions were 150 ^{o}C, 1% H_2O_2, and 45 min. The results for the variations considered are as shown in Table 4.6.

4.4 Enzymatic hydrolysis of raw and pretreated solids

The performance of any pretreatment method is reported in terms of sugars liberated during enzymatic hydrolysis rather than considering the chemical composition and physical characteristics of the biomass after pretreatment. As a result, the enzymatic hydrolysis study includes searching deeply to assessing the performances of pretreatments used. Studies on lime pretreatment with pretreatment conditions optimized for different types of lignocellulosic materials exist in the literature (Chang *et al*., 1998 and Chang *et al*., 2001). This section presents the results of the cellulose enzyme activity determination and the saccharification of the treated and untreated sawdust as described in section 3.7. Since the raw and treated biomass were subjected to different pretreatments, the results of their saccharification are presented accordingly.

Table 4.6 – Optimized conditions and alkaline peroxide/air pressure variations for WAO and 2^3 CCD APO pretreatments.

			A[a]	B[b]	C[b]	D[a]	E[b]	F[b]
			170 °C	170 °C	170 °C	150 °C	150 °C	150 °C
				1% H_2O_2[c]	1% H_2O_2 soak.[d]		1% H_2O_2[e]	10 bar 1%
		Raw	10 bar	10 bar	10 bar	1% H_2O_2	10 bar	H_2O_2 soaking[f]
		Biomass	10 min	10 min	10 min	45 min	45 min	10 min
SOLID FRACTION								
Dry biomass yield	(g)	100	86.63	75.69	85.78	89.37	79.78	82.35
Extractives	%(w/w)	1.89	7.67	3.70	0.14	3.70	3.30	3.45
Cellulose	%(w/w)	45.86	51.76	59.34	68.31	58.52	59.17	60.12
Hemicellulose	%(w/w)	20.31	9.85(57.94)[g]	8.01(70.15)	5.00(78.88)	6.40(71.84)	7.28(71.40)	4.88(80.21)
Lignin	%(w/w)	29.90	28.60(17.14)[h]	27.34(30.79)	25.1(27.99)	30.23(9.64)	28.54(23.85)	30.03(17.29)
Ash	%(w/w)	2.04	2.12	1.61	1.45	1.16	1.71	1.52

Data for Figure 5.31.

[a]At pretreatment optimized conditions and [b]APAWAO variations. [c]H_2O_2 was added just before WAO pretreatments and [e]before APO pretreatments. [d]Raw biomass was soaked for 24 h before WAO and [f]APO pretreatments. Values in parentheses show the [g]hemicellulose solubilization, and [h]lignin removal.

4.4.1 Cellulase enzyme activity

After the determination of the absorbance of the enzyme blanks, glucose standards, enzyme assay, a linear glucose standard curve using the absolute amounts of glucose standards (mg/0.5 mL) was plotted against absorbance at 540 nm as shown in Figure 4.2 (procedure and data for the plot are given in Appendix I). The data for the standard curve was found to closely fit a straight line, with the correlation coefficient for this straight line fit being very near to 1 (0.98). This graph was used to determine the concentration of reducing sugars in the sample tubes which had been incubated with cellulase enzyme solutions of varying dilutions at 50 ^{o}C for 60 min.

Table 4.7 gives data for the concentration of enzyme which would have released exactly 2.0 mg of glucose after subtraction of enzyme blank and was calculated by means of a plot of glucose liberated against the logarithms of enzyme concentration (Figure 4.3). The plot indicates that an enzyme concentration of 0.0064 will release 2.0 mg glucose from the given substrate. With this value, the filter paper units (FPU) for the given cellulose enzyme solution was calculated from the following equation;

$$\text{FPU} \equiv \frac{0.37}{\text{Enzyme concentration to release 2.0 mg glucose}} \text{ units/mL}$$

$$= \frac{0.37}{0.0064} \text{ units/mL}$$

$$= 57.81 \text{ FPU/mL}$$

Therefore, FPU for the cellulase enzyme solution was found to be 57.81 units/mL

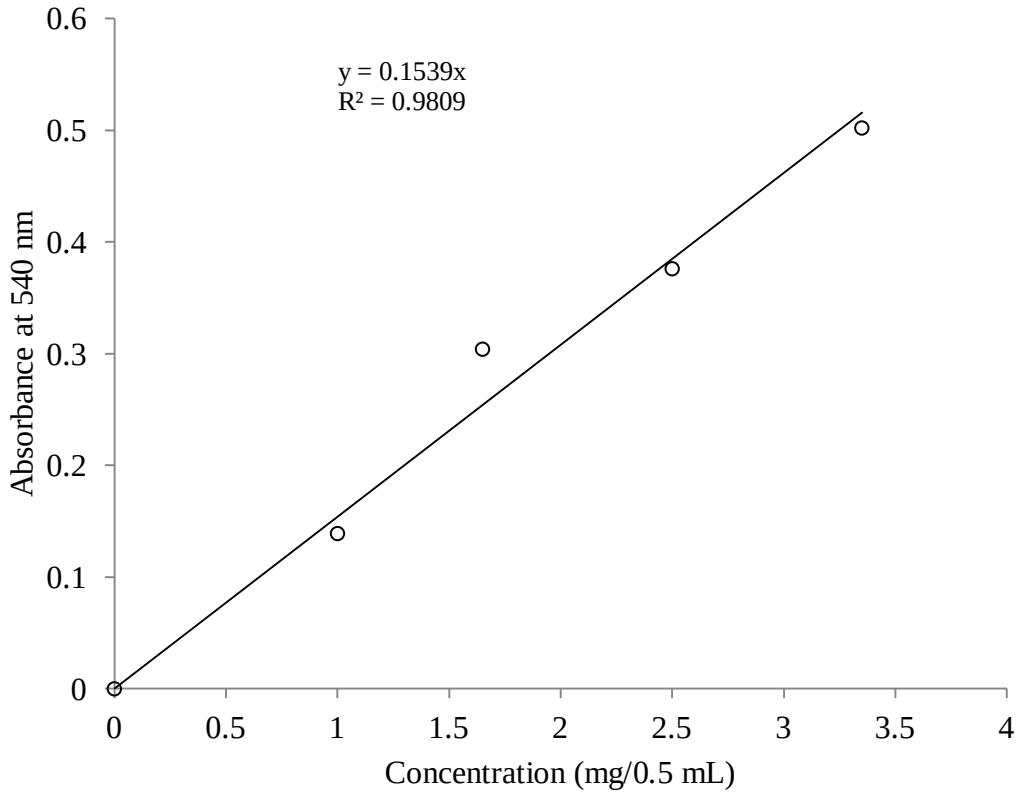


Figure 4.3 – Construction of glucose standard curve for cellulase activity determination.

Table 4.7 – Glucose concentrations of samples as determined from standard curve for cellulose enzyme activity determination.

	Diluted enzyme Conc.	Enzyme assay at 540 nm	Enzyme blank at 540 nm	Glucose conc. (mg/mL)	Enzyme blank conc. (mg/mL)	Glucose equivalent (mg/0.5 mL)
1	0.0125	0.536	0.002	3.483	0.013	3.470
2	0.0100	0.500	0.002	3.249	0.013	3.236
3	0.0075	0.352	0.000	2.287	0.000	2.287
4	0.0050	0.284	0.001	1.845	0.006	1.839
5	0.0025	0.167	0.002	1.085	0.013	1.072

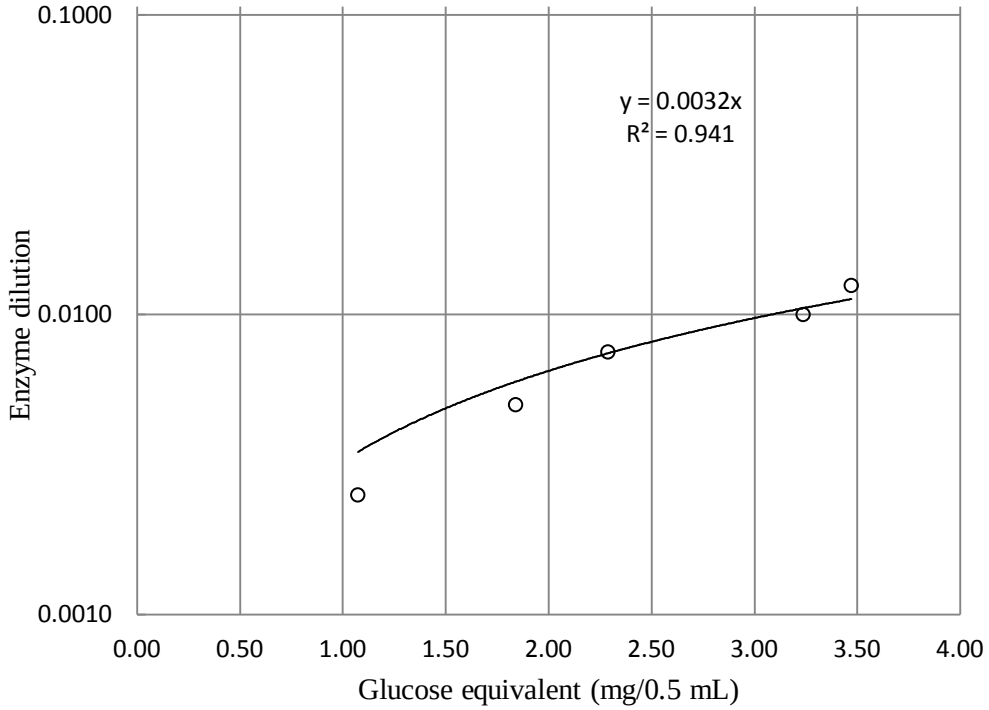


Figure 4.4 – Enzyme dilution vs. glucose concentration for cellulase activity determination.

4.4.2 Enzymatic hydrolysis of pretreated solids

Enzymatic hydrolysis is an ideal approach for converting lignocelluloses into sugars because high sugar yields can be achieved with negligible by-products. However, the enzymatic hydrolysis of lignocelluloses is affected by many factors, such as hydrolysis temperature, time, pH, enzyme loading, substrate concentration, product concentration, and biomass structural features/modification (lignin content, the presence of acetyl groups, cellulose crystallinity, degree of polymerization, surface area/pore volume of cellulose fiber, and particle size) (Converse *et al*., 1990, Sewalt *et al*., 1997, and Wong *et al*., 1988). The enzymatic digestibility of biomass is also affected by the pretreatment methods (e.g., acid and alkaline treatments). The cellulose and hemicellulose in treated biomass are generally more digestible than in untreated biomass (Kaar and Holtzapple, 2000 and Chang and Holtzapple, 2000). The IUPAC guideline (Ghose, 1987) as highlighted by the NREL procedure (Adney and Baker, 2008) was strictly followed. The absorbance of colour developed at 550 nm by liquid samples during enzyme hydrolysis was measured and compared to a glucose calibration curve to determine the concentration of reducing sugars. The data for the 3-day hydrolysis conditions and reducing sugar yields are as presented in Tables 4.8 and 4.9 respectively. Figure 4.4 shows sample preparations before enzymatic hydrolysis (Figure 4.4(a)) and fermentation. Table 4.10 shows the results of the 3-day reducing sugars yields of the 2^2 CCD APO pretreatments.

Table 4.8 – Summary of enzymatic hydrolysis conditions for pretreated biomass as specified in Table 4.6

Pretreatments	A	B	C	D	E	F
Weight of dry biomass (g)	0.658	0.607	0.662	0.685	0.702	0.698
Volume of 1-M citrate buffer (mL)	5	5	5	5	5	5
Volume of 10 mg/mL tetracycline solution(mL)	0.04	0.04	0.04	0.04	0.04	0.04
Volume of distilled water (mL)	3.965	4.016	3.961	3.938	3.921	3.925
Volume of cellulase enzyme (mL)	0.087	0.087	0.087	0.087	0.087	0.087
Volume of β-glucosidase (mL)	0.25	0.25	0.25	0.25	0.25	0.25
Total volume of mixture (mL)	10	10	10	10	10	10
Cellulase loading (FPU/g dry biomass)	25	25	25	25	25	25
β-glucosidase loading (IU/g dry biomass)	12.5	12.5	12.5	12.5	12.5	12.5
Hydrolysis time (h)	2, 24, 72	2, 24, 72	2, 24, 72	2, 24, 72	2, 24, 72	2, 24, 72

Table 4.9 – 3-d RS yields for the optimized conditions of WAO, 2^3 CCD APO pretreatments and their variations.

	A	B	C	D	E	F
Sampling Times	170 ^oC 10 bar 10 min	170 ^oC 10 bar 1% H_2O_2 10 min	170 ^oC 10 bar 1% H_2O_2 soaking 10 min	150 ^oC 1% H_2O_2 45 min	150 ^oC 10 bar 1% H_2O_2 45 min	150 ^oC 10 bar 1% H_2O_2 soaking 10 min
72 h	110.8[a]	114.87	173.52	98.59	155.66	120.01
24 h	78.96[a]	96.06	141.64	79.78	101.38	92.04
2 h	37.91[a]	24.49	54.70	39.06	53.53	48.65

Data for Figure 5.32.

[a]Reducing sugars (RS) yields (mg glucose equivalent/g dry biomass). Hydrolysis conditions: 2% (20 g/L) substrate concentration, 25 FPU cellulase enzyme/g dry biomass, 12.5 IU β-glucosidase/g dry biomass, 50 ^oC hydrolysis temperature.

(a) 

(b) 

(c) 

(d 

Figure 4.5 – Pretreated samples preparation for enzymatic hydrolysis and fermentation. (a): Pretreated samples in serum bottles for enzymatic hydrolysis. (b): Culture media and glassware set for autoclaving. (c): Cultured media in a shaking incubator. (d): Pretreated samples in fermentation flasks (air tight) with U-tube glass ready for incubation at 32 ^{o}C.

Table 4.10 – 3-d Reducing sugar (RS) yields for the 2^2 CCD APO pretreatments.

Experimental Runs	Operating conditions[a]		RS (mg equivalent glucose/g dry biomass)		
	Temperature (^oC)	Time (min)	2 h	24 h	72 h
1	120	15	32.57	64.68	81.36
2	105	11.89	27.25	81.71	128.46
3	90	30	32.59	84.59	91.89
4	105	22.5	28.92	79.09	121.84
5	105	33.11	25.76	63.53	124.37
6	105	22.5	26.20	81.10	85.82
7	105	22.5	28.44	75.18	121.33
8	90	15	30.74	79.31	87.73
9	83.79	22.5	26.34	59.39	102.82
10	105	22.5	26.34	79.14	120.77
11	*120*	*30*	*38.44*	*90.51*	*146.00*
12	105	22.5	27.13	78.67	98.85
13	126.21	22.5	33.06	87.39	128.02

Data for Figure 5.33.

[a]1%(v/v) H_2O_2 addition. Hydrolysis conditions: 2% (20 g/L) substrate concentration, 25 FPU cellulase enzyme/g dry biomass, 12.5 IU β-glucosidase/g dry biomass, 50 ^oC hydrolysis temperature.

4.4.3 Effects of substrate concentration on enzymatic hydrolysis

In this study, the influence at four substrate concentration (20, 30, 40, and 50 g/L) as well as adding or not adding supplemental β-glucosidase on digestibility were investigated. Also the same ratio of *T. reseei* and β-glucosidase was used for the different substrate loadings. Enzymatic hydrolysis procedure was performed as highlighted in Appendix J. Summary of enzymatic hydrolysis conditions for studying the effects of substrate concentration are shown in Table O-16. The results were assessed in terms of reducing sugars yield (mg equivalent glucose/g dry biomass) and also in terms of %Saccharification. Reducing sugar yields and % Saccharification are shown in Tables 4.11 and 4.12 respectively. % Saccharification (or % digestibility) as defined in Eq. 4-3 (Hui *et al*., 2009), measured the amount of the stating material (substrate) converted to simple sugars by the enzymes. In this case, the digestibility was expressed as total polysaccharides in treated substrate not as only cellulose content.

$$\% \text{ Saccharification} = \frac{\text{Reducing sugars concentration obtained (mg/g)} \times 0.9}{\text{Substrate added\{cellulose + hemicellulose\}(g/g)} \times 1000 \text{ (mg/g)}} \times 100\% \quad \ldots 4\text{-}3$$

The effects of *T. reesei* enzyme (concentrations 25, 37.5, 50, and 62.5 FPU/g of substrate), β-glucosidase (15, 22.5, 30, 37.5 IU/g of substrate), and the incubation period (2, 24, 72, and 96 h) on the hydrolysis of the treated sawdust were studied on the two optimized pretreatment conditions and results are presented in (Table 4.11 and Table 4.12). The hydrolysis temperature was 45 ^{o}C.

4.4.4 Effects of enzyme loading on enzymatic hydrolysis

The enzymatic digestibility conditions and the results of the enzyme loading study are given in Table O-17 and Table 4.13 respectively. Supplemental β-glucosidase and substrate concentration were kept constant at 5 IU/g dry biomass and 40 g/L respectively (after establishing the optimum substrate concentration for enzymatic hydrolysis and addition of β-glucosidase had little or no effect on sugar yields).

Table 4.11 – 4-d Effect of substrate concentration with corresponding increase in enzyme concentration and incubation period on the enzymatic saccharification of pretreated sawdust conditions of 150 ^{o}C, 1% H_2O_2, 10 bar, and 45 min.

	Substrate concentration (g/L)							
	20		30		40		50	
	25[a]	15[b]	37.5[a]	22.5[b]	50[a]	30[b]	62.5[a]	37.5[b]
Incubation period (h)	RS	S	RS	S	RS	S	RS	S
2	24.88	3.08	25.11	2.07	30.10	1.86	20.69	1.02
24	63.63	7.88	84.02	6.93	46.45	2.88	76.10	3.77
72	135.36	16.76	106.81	8.81	171.77	10.63	171.54	8.49
96	138.69	15.19	178.02	14.69	358.45	22.19	328.86	16.28
With no supplemental β-glucosidase[c]								
2	28.50	3.53	42.90	3.54	23.85	1.48	30.37	1.50
24	73.94	9.25	55.51	4.58	113.76	7.04	79.02	3.91
72	110.42	13.67	200.96	16.58	200.56	12.41	182.44	9.03
96	147.23	18.23	207.20	17.10	347.20	21.49	296.64	14.68

Data for Figures 5.34–5.37.

[a]cellulase enzyme loading (FPU/g dry biomass). [b]β-glucosidase loading (IU/g dry biomass). [c]cellulase enzyme loadings remained the same as with supplemental β-glucosidase. Enzymatic hydrolysis conditions: incubation temperature 45 ^{o}C, RS–Reducing sugars (mg equivalent glucose/g dry biomass), S–Saccharification (%).

Table 4.12 – 4-d Effect of substrate concentration with corresponding increase in enzyme concentration and incubation period on the enzymatic saccharification of pretreated sawdust conditions of 120 ^{o}C, 1% H_2O_2, and 30 min.

	Substrate concentration (g/L)							
	20		30		40		50	
	25[a]	15[b]	37.5[a]	22.5[b]	50[a]	30[b]	62.5[a]	37.5[b]
Incubation period (h)	RS	S	RS	S	RS	S	RS	S
2	38.97	5.97	19.73	2.01	33.86	2.59	33.76	2.07
24	44.72	6.85	52.69	5.38	48.35	3.70	25.54	1.56
72	95.33	14.59	69.28	7.07	113.20	8.66	80.12	4.91
96	81.02	12.40	84.55	8.63	85.46	6.54	98.59	6.04
With no supplemental β-glucosidase[c]								
2	43.95	6.73	60.17	6.14	38.09	2.92	49.81	3.05
24	68.97	10.56	66.87	6.82	93.43	7.15	144.05	8.82
72	141.14	21.60	133.97	13.64	147.97	11.32	153.54	9.40
96	117.94	18.05	81.59	8.33	84.27	6.45	75.21	4.60

Data for Figures 5.38–5.39.

[a]cellulase enzyme loading (FPU/g dry biomass). [b]β-glucosidase loading (IU/g dry biomass). [c]cellulase enzyme loadings remained the same as with supplemental β-glucosidase. Enzymatic hydrolysis conditions: incubation temperature 45 ^{o}C, RS–Reducing sugars (mg equivalent glucose/g dry biomass), S–Saccharification (%).

Table 4.13 – 4-d Effect of enzyme loading on the reducing sugar yield of treated sawdust.

Pretreatment conditions	150 °C, 1%H_2O_2, 10 bar, 45 min				120 °C, 1% H_2O_2, 30 min			
	Reducing sugar yield (mg equivalent glucose/g dry biomass)[a]							
Hydrolysis time (h)	2	24	72	96	2	24	72	96
Enzyme loading[b]								
10	20.71	34.13	93.91	122.04	31.06	65.81	107.00	148.46
25	21.62	111.62	139.53	335.35	29.66	79.08	131.96	164.88
40	23.81	120.87	156.71	351.23	30.77	83.35	136.74	174.35
50	24.88	145.42	183.37	365.62	36.04	88.89	141.19	186.07

Data for Figure 5.40 and Figure 5.41.

[a]constant loading of 5 IU β-glucosidase/g dry biomass was supplemented in the enzymatic hydrolysis, substrate concentration: 40 g/L. [b]cellulase enzyme loadings in FPU/g dry biomass.

Table 4.14 – 4-d Effect of substrate concentration with corresponding increase in enzyme loading and incubation period on the enzymatic saccharification of pretreated and untreated sawdust.

	Substrate concentration (g/L)							
	20		30		40		50	
	25[a]	15[b]	37.5	22.5	50	30	62.5	37.5
Incubation period (h)	RST[c]	RSU[d]	RST	RSU	RST	RSU	RST	RSU
2	24.88	8.93	25.11	11.83	30.10	9.66	20.69	9.31
24	63.63	15.15	84.02	16.16	46.45	17.30	76.10	16.87
72	135.36	10.48	106.81	7.65	171.77	15.91	171.54	12.84
96	138.69	11.99	178.02	15.32	358.45	17.73	328.86	16.19

Data for Figure 5.42.

[a]cellulase loading and [b]β-glucosidase loading. [c]Reducing sugars yield (mg equivalent glucose/g dry biomass) for treated biomass at 150 °C, 1% H_2O_2, 10 bar, and 45 min and [d]Reducing sugars yield for untreated biomass at the same enzymatic hydrolysis conditions.

4.4.5 Enzymatic hydrolysis of untreated and washed only biomass

The untreated and washed raw biomass was used as the control for comparing the enzymatic digestibility of the treated sawdust. Enzymatic digestibility of pretreatment conditions 150 ^{o}C, 1%H_2O_2, 10 bar, and 45 min were compared with untreated biomass, results are shown in Table 4.14. The substrate concentration variation (20, 30, 40, and 50 g/L) with corresponding increase in enzyme loadings and supplemental β-glucosidase on digestibility were investigated.

Cellulase enzyme was added at loading rates (FPU/mL) 25, 37.5, 50, and 62.5 as described in Appendix J. The activity of the cellulose enzyme was determined as described earlier (Appendix I).

Sugar concentration as equivalent amount of glucose per unit of dry biomass was determined by the DNS method (Appendix K). The 4-day reducing sugar yields of untreated and pretreated sawdust are plotted against substrate concentrations as illustrated in Figure 5.42. Chang *et al.*, (2001) investigated the effects of pretreatment time and temperature of poplar wood (lignin content of about 25%) on enzymatic hydrolysis and concluded that oxidative lime pretreatment enhanced the reducing sugar yields from 62 mg equivalent glucose/g dry biomass (untreated) to as much as 622 mg equivalent glucose/g dry biomass (treated at 150 ^{o}C and 6 h).

4.5 Simultaneous saccharification and fermentation

Considering a total sample size of 50 g (total working volume for fermentation is therefore 50 mL), the desired cellulose effective loadings of 2% (w/w) and 3% (w/w) were calculated to be equivalent to 1.0 g and 1.5 g of cellulose respectively in treated substrate. The calculations for the biomass and enzyme loadings needed for the fermentation step are given in Appendix L. Fermentation conditions for the biomass loadings (i.e. 2 and 3%) including the control flasks are shown in Table O-18. The cellulase loading (filter paper activity = 57.8 FPU/mL) was 25 FPU/g cellulase and the concentration of yeast (*Saccharomyces cerevisiae*) inoculum was 10% (v/v).

The *Saccharomyces cerevisiae* (kindly provided by Purti Power and Sugar Limited (distillery division) Bela, Tah Umrer District, Nagpur, India was maintained on PDA slant in a test tube and kept in the freezer until it was ready for use) was cultivated in 250 mL Erlenmeyer flasks with seals containing MYPD (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, and 10 g/L glucose) medium for 10 h maintained at pH of 4.8 as described in Appendix M according to NREL procedure, "Determination of the fermentability of the pretreatment solids" (Dowe and McMillan, 2008). Total working volume for the culture medium was 100 mL. 5 mL of inoculum cultured for 10 hours was added into 50 mL sterilized medium for the fermentation of the pretreated solids. The pH of the medium was adjusted with 0.05 M citrate buffer.

Pretreated samples were subjected to fermentation for 72 h at 32 ^{o}C in a shaker incubator at 130 rev/min (Dowe and McMillan, 2008). The experiment was performed in duplicate. The concentration of ethanol was measured for the 72 h period. The ethanol content was measured using the dichromate assay (Appendix N) (Bennet, 1971). The fermentation was also carried out in 250 mL Erlenmeyer flasks. The fermentation lock or bubble trap consisted of rubber stopper through which a glass tube was inserted. The mouths of the flasks were wrapped with aluminum foil (Figure 4.4(b) to (d)).

The fermentability of the pretreated solids was characterized by the equation (Dowe and McMillan, 2008):

$$\% \text{ theoretical ethanol yield} = \frac{[EtOH]_f - [EtOH]_0}{0.51\,(f\,[\text{Biomass}]\,1.111)} \times 100\% \qquad \ldots 4\text{-}4$$

Where:

$[EtOH]_f$ = Ethanol concentration at the end of the fermentation (g/L) minus any ethanol produced from the enzyme and medium.

$[EtOH]_0$ = Ethanol concentration at the beginning of the fermentation (g/L) which should be zero.

[Biomass] = Dry biomass concentration at the beginning of SSF (g/L)

f = Cellulose fraction of the dry biomass (g/g).

0.51 = Conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast.

1.111 = Converts cellulose to equivalent glucose.

The ethanol yield was calculated as a percentage of the theoretical yield on the basis of the total effective cellulose in the pretreated material, that is, the % theoretical ethanol yield can also be given as % cellulose conversion. The control fermentation (without biomass samples) was performed and the result was subtracted from the test fermentations for each biomass loading.

Table 4.15 shows the ethanol concentrations and ethanol yields (% of theoretical) for the two oxidative pretreatment conditions and 96 h fermentation time chosen for study.

Table 4.15 – Ethanol concentration and yields during SSF of pretreated sawdust.

Pretreatment Conditions	Biomass loading (%)	Ethanol concentration (g/L)	Ethanol yield (% of theoretical)[a]
150 °C 1% H_2O_2 10 bar air pressure 45 min	2(123.2)[b]	9.71	23.43
	3(184.8)	10.08	18.27
120 °C 1% H_2O_2 30 min	2(100.4)	7.35	24.53
	3(150.6)	7.79	17.33

[a]% cellulose conversion. [b]Values in parentheses are dry biomass concentrations at the beginning of SSF (g/L).

CHAPTER FIVE

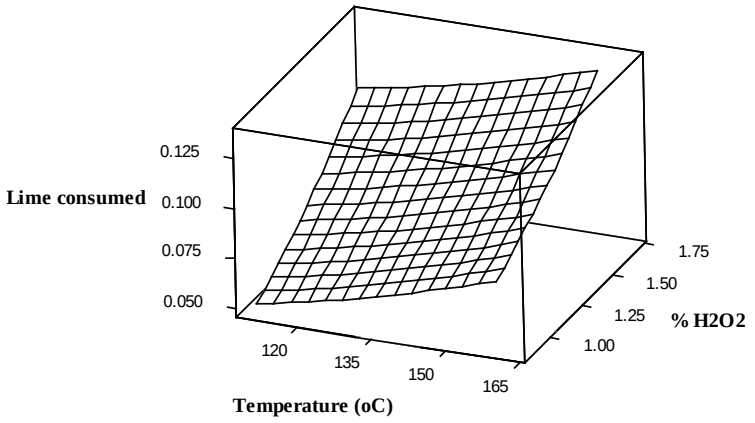
5. DISCUSSIONS

5.1 Characterization of untreated (raw) biomass

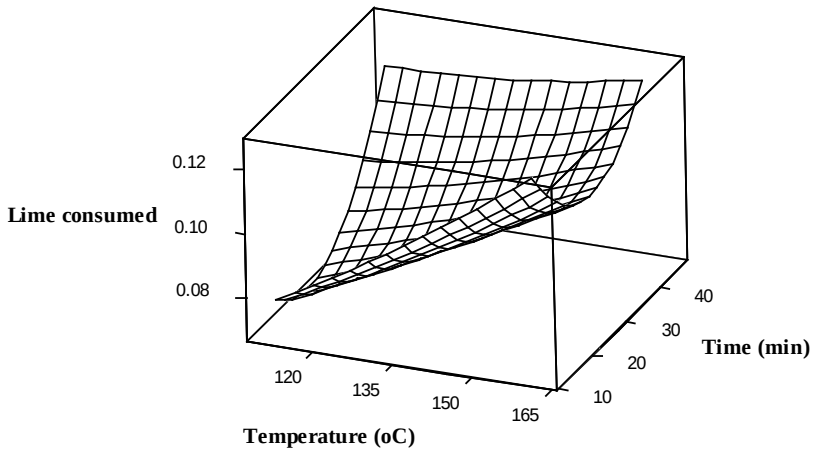
The fractions of small particles (≤ 0.853 mm), BSS +20 and +80, are 73% (w/w) and large particles (> 0.853 mm and ≤ 1.41 mm) BSS +14 are 28% in the total particle compositions (Table 4.1). It means that small particles are more distributed than the large particles in the raw sawdust composition (Figure 4.1). BSS +80 take half of the particles distribution with about 50% (≤ 0.177 mm), BSS +20 (> 0.177 mm and ≤ 0.853 mm) was 23% while the larger particles fraction BSS +14 was 28%0(853 mm and ≤ 1.410 mm). In all, there were 495 g of 0.177 mm, 225 g of 0.841 mm, and 280 g of 1.410 mm particles.

The raw material was rich in carbohydrates thereby useful for ethanol production, however, the lignin content was high (29.90%). High lignin content may stand as great obstacle to enzymatic hydrolysis of treated materials.

In general, lime consumption increased with increasing temperature. Procedure for the determination of lime consumed during pretreatment is as highlighted before (Appendix G). During the 2^3 CCD pretreatments, the minimum amount of lime consumed was 0.0578 g $Ca(OH)_2$/g raw biomass (Pretreatment 10) (Table O-1). The maximum amount recorded was 0.1251 g $Ca(OH)_2$/g raw biomass (Pretreatment 5). The average lime consumption was 0.10 g $Ca(OH)_2$/g dry biomass for the 20 pretreatments. This value for specific lime consumption agrees with other studies on lime pretreatment of some lignocellulosic biomass (Kim and Holtzapple, 2005 and Chang *et al*., 2001). Chang *et al*., (2001) established that lime consumption increased with temperature but maximum lime consumed did not exceed 0.10 g $Ca(OH)_2$/g dry biomass. The surface plot of lime consumed vs. temperature and $\%H_2O_2$ in Figure 5.1(a) revealed a situation that more lime was consumed at higher temperatures and as concentration of the hydrogen peroxide increased. However, the rate of lime consumption was more at shorter duration (Figure 5.1(b)), and decreased with increasing time. The highest lime consumption was recorded during the first 10 min.



(a)



(b)

Figure 5.1 – Surface plots of lime consumed g $Ca(OH)_2$/g raw biomass: (a) vs. temperature and % H_2O_2, (b) vs. temperature and time for 2^3 CCD APO design.

For the 2^2 CCD, lime consumption also increased with increased temperature, though the consumption was minimal (with a maximum consumption of 0.0395 and minimum of 0.0177) (Table O-2). As expected, the consumption of lime was smaller in the 2^2 CCD than the 2^3 CCD because the 2^2 CCD pretreatments were operated at lower temperatures and decreasing lime loadings (Figure 5.2). Kaar and Holtzapple (2000) also reported that no advantage was observed for increasing lime loading above 0.1 g $Ca(OH)_2$/g dry biomass for corn stover pretreatment at 120 ^oC for 5 h. Specific lime consumption increased with increasing temperature and short pretreatment time.

5.2 Changes of biomass during pretreatment

During oxidative pretreatment of biomass, a complex mixture of products are reported to be produced (Klinke *et al*., 2002). Degradation to carbon dioxide and salts such as calcium carbonate are said to be deposited at the surface of lignocelluosic biomass (Klinke *et al*., 2002). These promote compositional changes in biomass subjected to oxidative alkaline pretreatments.

The weight fractions of the pretreatment step are changed due to the solubilization of components (hemicellulose) during the alkaline pretreatments. During this process, some holocellulose (cellulose and hemicellulose) are removed by the action of hydroxide ion. In addition, delignification (lignin removal) of the solid mass also occurs.

5.2.1 Pretreatment yields

After pretreatment, the content of the reactor was transferred into a 1000 mL beaker. The biomass slurry was neutralized (Appendix G), washed and the percent pretreatment yield (dry biomass content or the recovery yield) determined (Appendix H) from the mass balance at different pretreatments. The pretreated material (dry solid weight) was subjected to analyses for the extractives, ash, cellulose, hemicelluloses, and lignin contents. A detailed explanation of these procedures can be found in the corresponding appendixes (B–E). The oxidative pretreatment designs and the pretreatment compositions are as shown in Tables O-3 to O-9.

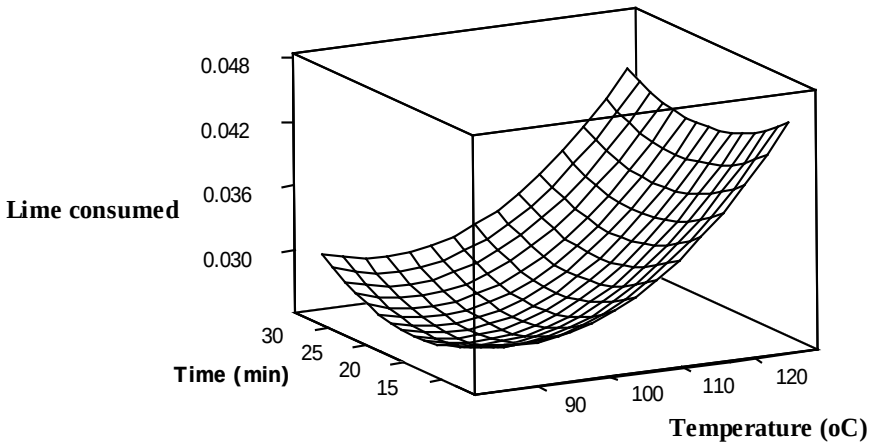


Figure 5.2 – Surface Plot of lime consumed (g $Ca(OH)_2$/g dry biomass) vs. time and temperature for 2^2 CCD APO design.

The goals of any pretreatment method are to have more holocelluloses remain in the lignocellulosic fiber matrix, and lignin removed as much as possible.

5.2.2 Holocellulose removal in the pretreated solids

The recalcitrant nature of lignocelulosic biomass to pretreatment is reflected in this study. From the compositions of the treated raw materials for the three pretreatment designs, the joint contributions of cellulose and lignin recoveries accounted for the high dry matter (dry total solids) yields in the solid residues (Tables O-7 to O-9). It showed that the lignin remained largely undissolved. However, subjecting the substrate to some pretreatment variations could increase its suitability for efficient enzymatic hydrolysis.

For the 2^3 full factorial WAO pretreatments, holocellulose yield also increased as more lignin is removed, the increase was more at lower temperature (170 ^oC). At higher temperature (195 ^oC) there was degradation (loss) of holocellulose (Figure 5.3). In the 2^3 CCD APO pretreatment, yield of holocellulose increased with lignin removal. The increase in pretreatment yield was favourable between 135–150 ^oC. Lignin removal and holocellulose yield started decreasing beyond 150 ^oC (Figure 5.4). Also, holocellulose yield increased as more lignin is removed at high temperature for the 2^2 CCD APO pretreatment (Figure 5.5).

Lignin removal increased as more lime was consumed at higher temperatures, though with minimal increases. However, lignin removal started decreasing as more lime was consumed at longer periods (Figures 5.6 and 5.7) (Table O-6). Therefore, more lignin was removed at temperatures 120–150 ^oC and short pretreatment reaction time (30–40 min).

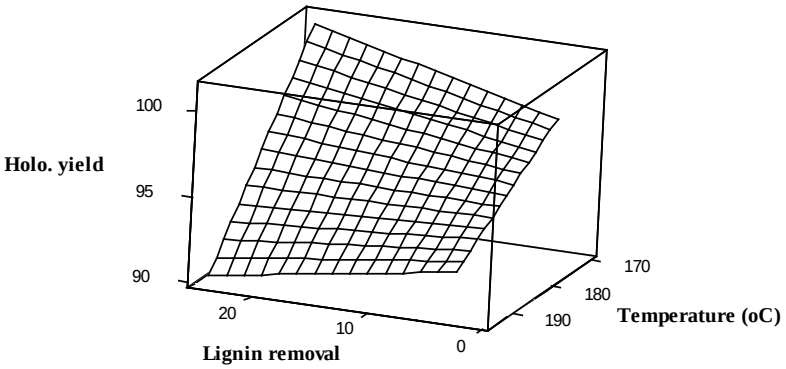


Figure 5.3 – Surface plot of pretreatment yield of holocellulose (g recovered/100g in raw biomass) vs. lignin removal %(w/w) and temperature for 2^3 full factorial WAO pretreatments.

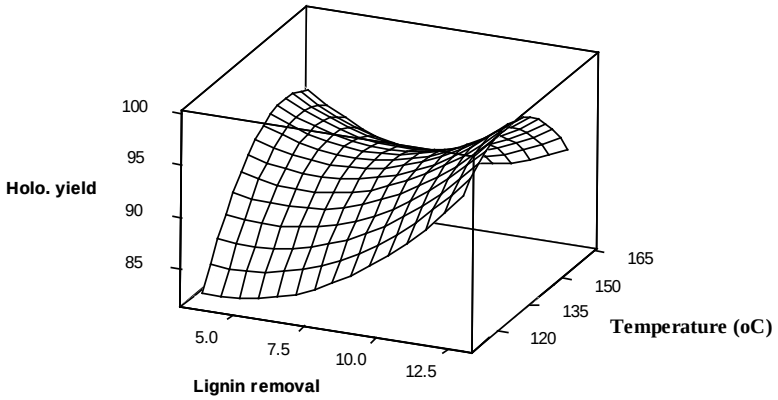


Figure 5.4 – Surface plot of pretreatment yield of holocellulose (g recovered/100g in raw biomass) vs. lignin removal %(w/w) and temperature for 2^3 CCD APO pretreatments.

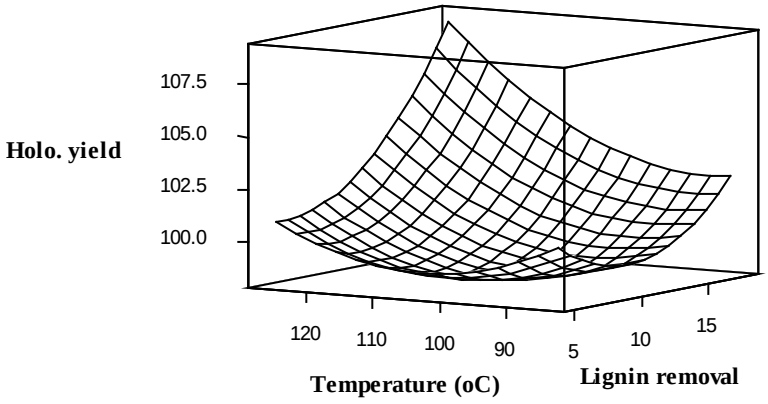


Figure 5.5 – Surface plot of pretreatment yield of holocellulose (g recovered/100g in raw biomass) vs. lignin removal %(w/w) and temperature for 2^2 CCD APO pretreatments.

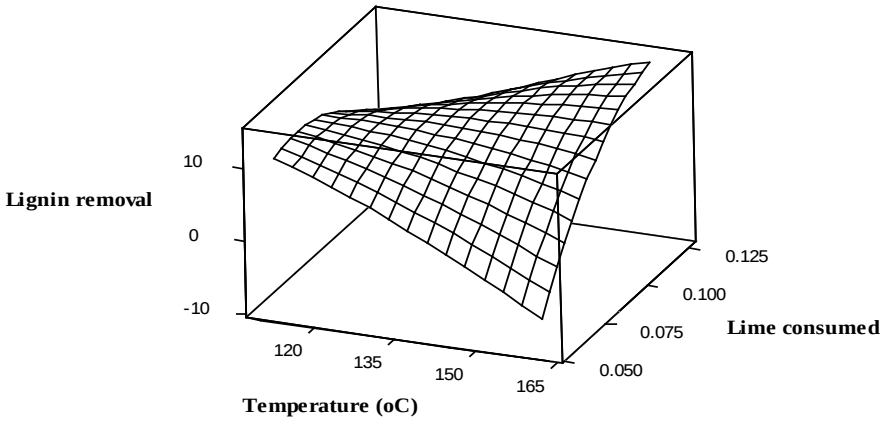


Figure 5.6 – Surface plot of lignin removal %(w/w) vs. lime consumed (g $Ca(OH)_2$/ g raw biomass) and temperature.

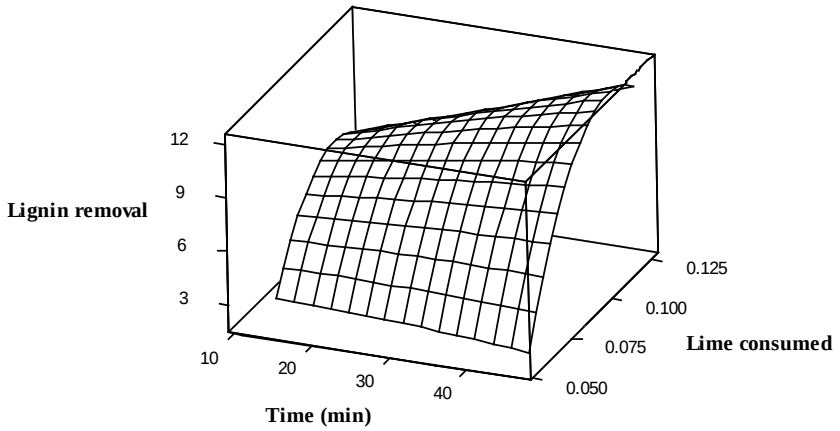


Figure 5.7 – Surface plot of lignin removal %(w/w) vs. lime consumed (g $Ca(OH)_2$/ g raw biomass) and time.

5.3 Alkaline WAO pretreatments optimization

The WAO pretreatment was aimed at fractionating the wood biomass into a solid fraction containing as much cellulose and as less lignin as possible; and a liquid fraction containing solubilized hemicellulose the best preserved as possible (Martín *et al*., 2007). The WAO pretreatment was used to study the enzymatic convertibility (at optimized pretreatment conditions) of the high-lignin sawdust using lime as the chemical catalyst as against the traditional use of Na_2CO_3 or H_2SO_4 (Bjerre *et al*., 1996, Klinke *et al*., 2002, and Varga *et al*., 2003).

The percentage of dry biomass recovered in the solid fraction after WAO pretreatment experiments ranged from 74% to 92% by weight of dry biomass and it was higher for pretreatments at 170 ^oC than for pretreatments at 195 ^oC (Table O-7). Higher temperature (195 ^oC), when combined with high pressure and/or reaction time, was found to result in excessive biomass charring which was apparent from the black colour of the solid material in pretreatments (VI), (VII) and (VIII). In the pretreatment conditions (I) to (VI) cellulose recovery of the solid fraction increased. At severe conditions (VII) and (VIII), cellulose recovery decreased indicating undesirable cellulose degradation and these occurred with decreasing pH values. The thermal degradation of cellulose is said to begin on increasing the temperature from around 195 ^oC to 200 ^oC (McGinnis *et al*., 1983). Hemicellulose recovery varied with all the conditions. High values of hemicellulose recovery occurred at high temperature pretreatments (VII) and (VIII). Increased cellulose content in the pretreated biomass ranged from 52% to 57% by weight compared with the initial raw biomass of 46%. Cellulose content decreased at elevated temperature and high air pressure to 43% due to degradation of cellulose. Cellulose enrichment was due majorly to hemicellulose solubilization and a small percentage of lignin removal. Hemicellulose solubilization varied from 37% to 64% by weight of dry biomass. The lignin removal was very low in all the conditions with the highest value of 24% (pretreatment (III)). This may be attributed to the high lignin content.

Palonen *et al*., (2004) discovered that after wet oxidation pretreatment of softwood (*Picea abies*) using sodium carbonate as pretreatment agent, the cellulose content increased to 64% from an initial 44%, hemicellulose content in the solid fraction

reduced to about 2% from an initial 18%, and lignin content only reduced to as little as 24% from an initial 28.5% (w/w). Relative content of cellulose of the WAO treated softwood increased owing to solubilization of the hemicellulose and part of the lignin fraction. The variation in values of this study to that of Palonen *et al*., (2004) (since both raw materials are woody) might be differences in pretreatment conditions such as the alkaline agent, temperature values, and raw material sources. On the other hand, lignocellulosic material with low lignin content such as sugarcane bagasse showed that the percentage of dry solid recovered after pretreatment ranged from 51 to 80% (signifying that more of lignin and hemicellulose were solubilized) (Martín *et al*., 2007). The sugarcane bagasse cellulose content increased to 70% from an initial 43%, the hemicellulose solubilized between 93–94%, and 40–50% of lignin was solubilized. This means that low lignin content of biomass increases the pretreatment yields of lignocellulosic biomass.

The second-order polynomials obtained were as follows:

Cellulose content %(w/w) =

$$84.485 - 0.184X_1 + 5.45X_2 - 0.858X_3 - 0.027X_1X_2 + 0.0079X_1X_3 - 0.1113X_2X_3$$

$R^2 = 0.9888$ (R^2 adjusted = 0.9214) …(5-1)

Hemicellulose %(w/w) =

$$-32.07 + 0.597X_1 + 15.568X_2 + 0.358X_3 - 0.092X_1X_2 - 0.0034X_1X_3 - 0.079X_2X_3$$

$R^2 = 0.9913$ (R^2 adjusted = 0.9389) …(5-2)

Lignin removal %(w/w) =

$$-155.91 + 0.69X_1 + 19.58X_2 + 1.57X_3 - 0.08X_1X_2 + 0.003X_1X_3 - 0.25X_2X_3$$

$R^2 = 0.9999$ (R^2 adjusted = 0.9995) …(5-3)

When the values from X_1 to X_3 were substituted in the above equations, the predicted responses were obtained (Table O-10).

Sugars in the liquid fraction as reducing sugars increased at high temperature pretreatments (Table O-7). This was as a result of cellulose degradation/solubilization from the solid fraction at the high temperature (195 ^oC). Pretreatment (VII) gave the highest reducing sugars value of 25 gL^{-1} while low temperature (pretreatment (III)) gave

the lowest value of 6 gL^{-1}. The pH of the liquid fraction of the pretreated biomass showed a progressively decreasing trend with increasing temperature, pressure, and reaction time. The decreasing trend was reflected at high temperature range with the lowest pH of 6.24 (pretreatment VIII).

Multiple regression analysis was also performed on the experimentally obtained data for the concentration of reducing sugar, and pH of the liquid fraction. The regression coefficients obtained were used to build the model equations to predict the response *Y* as explained in Eq. (4-1). The second order polynomials obtained were:

Reducing sugars (gL^{-1}) = $14.429 + 5.311X_1 + 1.353X_2 + 0.576X_3 + 0.651X_1X_2 - 2.179X_1X_3 - 0.075X_2X_3$

($R^2 = 0.971$) …(5-4)

pH = $6.98 - 0.458X_1 - 0.108X_2 - 0.138X_3 + 0.065X_1X_2 + 0.070X_1X_3 - 0.175X_2X_3$

(R^2 = 1.000) …(5-5)

The closeness of the predicted and experimental values of reducing sugars and pH (Table O-10) is consistent with the high R^2 values in Eqs. (5-4) and (5-5). Under these experimental conditions, it was revealed that more hemicellulose is solubilized (to the liquid fraction) than lignin removal.

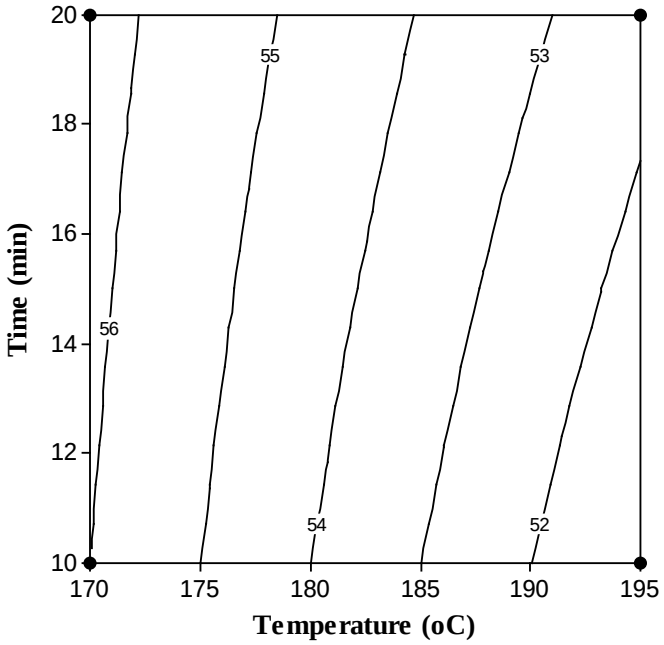
The statistical treatment combinations of the test variables along with the measured response values, corresponding to all combinations are summarized in Table O-11. The *P*-values are used as tools to check the significance of each of the coefficients in the models, which in turn, may indicate the patterns of the interaction among the variables. The larger the magnitude of *T* and smaller the *P*-value, the more significant is the corresponding coefficient. Temperature is marginally significant on cellulose content (P = 0.098), pressure also is marginally significant on hemicellulose solubilization (P = 0.079). All the main effects and the interaction effects are significant on lignin removal ($P < 0.05$); except for temperature and time interaction (Table O-11).

The summary of analysis of variance (ANOVA) representing the results is given in Table O-14. ANOVA is required to test the significance and adequacy of the models.

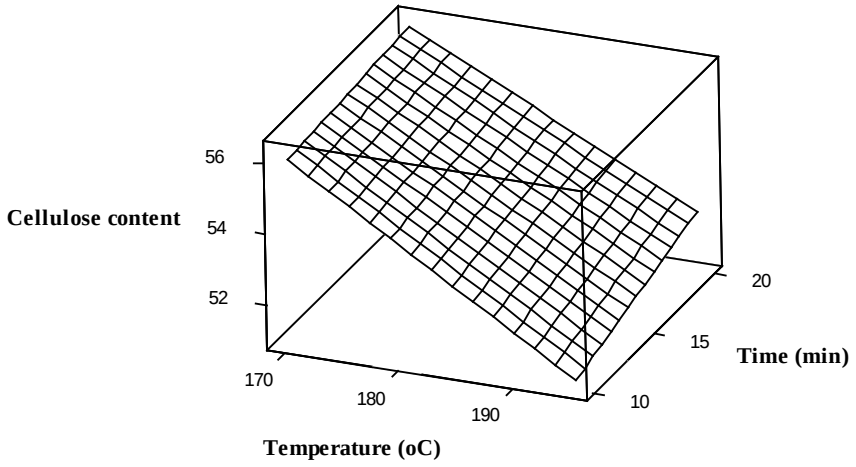
The Fisher's variance ratio (*F*-value) is the measure of variation in the data about the mean. Here the ANOVA of the multiple regression revealed that the quadratic models derived from the factorial design could adequately be used to predict the responses as evident from the high *F*-values. In addition, the multiple correlation coefficients (R^2) of the regression equations obtained from ANOVA were 0.989 for cellulose content, 0.991 for hemicellulose solubilization, and 0.999 for lignin removal. This means that the models fitted well with the experimental data. The R^2-value for cellulose content implies that the sample variation of 98.9% is attributed to the factors, and also indicates that only 1.1% of the total variation is not explained by the model. For hemicellulose solubilization (R^2 = 99.1%), only 0.9% of the total variation is not explained by the model. R^2-value for lignin removal (99.9%) reveals that 0.1% of the total variation is not explained by the model.

Two-dimensional contour plots and three-dimensional response surface curves were plotted to study the interactions between the various parameters in WAO pretreatment (solid and liquid fractions) of the sawdust material and were used to determine the optimum levels of each factor required to obtain maximum responses. Effects of individual factors on cellulose content, lignin removal, and hemicellulose solubilization %(w/w) of the solid fraction, and on reducing sugars and pH of the liquid fraction are shown in Figures 5.8–5.18

Figures 5.8–5.10 show the contour and surface plots of the interaction of the three pretreatment factors on cellulose content. The pressure should be kept at high value (10 bar), the reaction time maintained at low value (10 min), and the temperature also kept at a low value (170 ^{o}C) to obtain the maximum value of cellulose content in the solid fraction. Figures 5.11–5.13 show the surface and contour plots of effects of the pretreatment variables on hemicellulose solubilization. Reaction time (between 10 min and 15 min), air pressure (between 7.5 bar and 10 bar), and reaction temperature varying between 170 ^{o}C and 180 ^{o}C should yield maximum hemicellulose solubilization. Also, shorter reaction time, and air pressure of about 10 bar favoured more delignification of the solid fraction (Figures 5.14–5.16). Figure 5.17 shows the contour and surface plots of the interactive effects of reaction time and temperature on reducing sugar content.

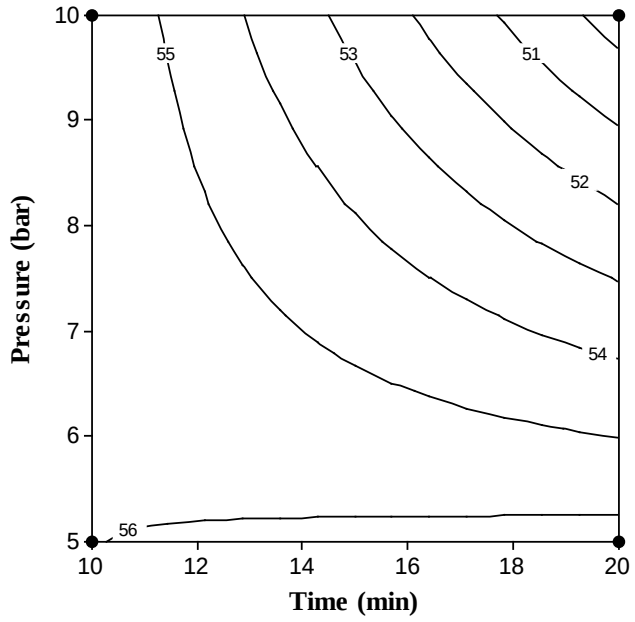


(a)

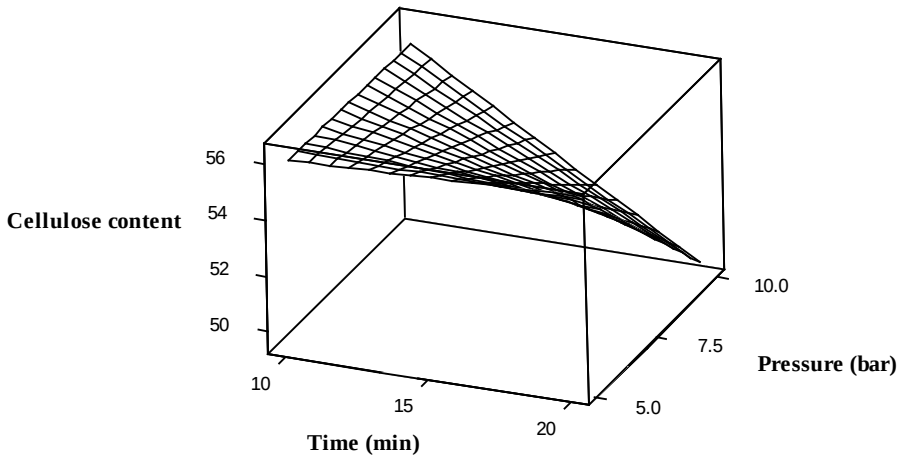


(b)

Figure 5.8 – (a) contour plot, (b) surface plot of cellulose content %(w/w) vs. time and temperature for 2^3 full factorial WAO pretreatment.

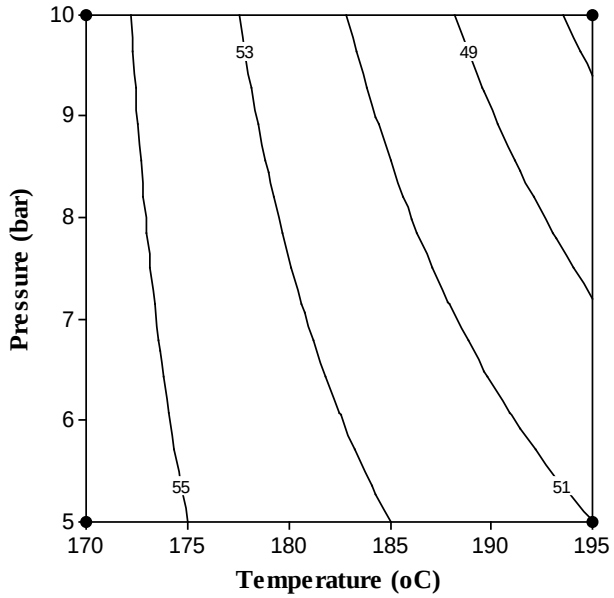


(a)

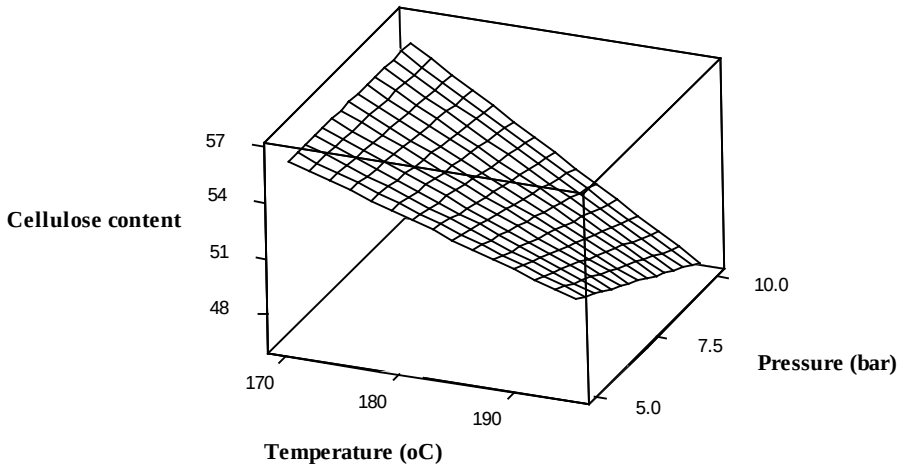


(b)

Figure 5.9 – (a) contour plot, (b) surface plot of cellulose content %(w/w) vs. pressure and time.

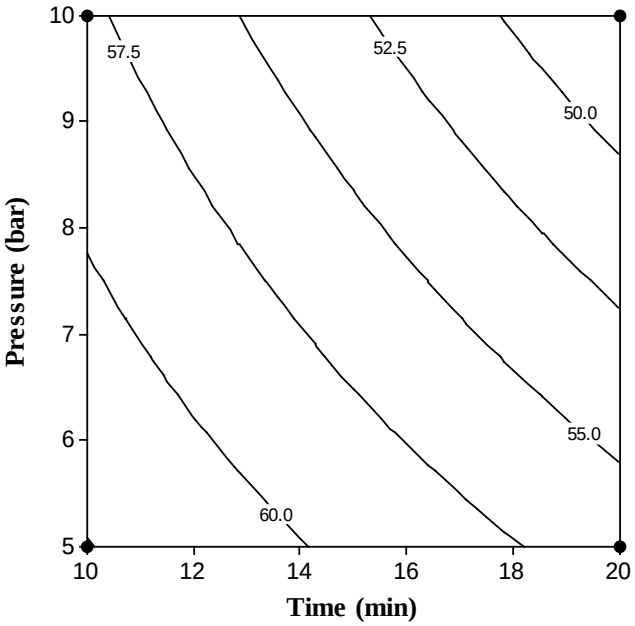


(a)

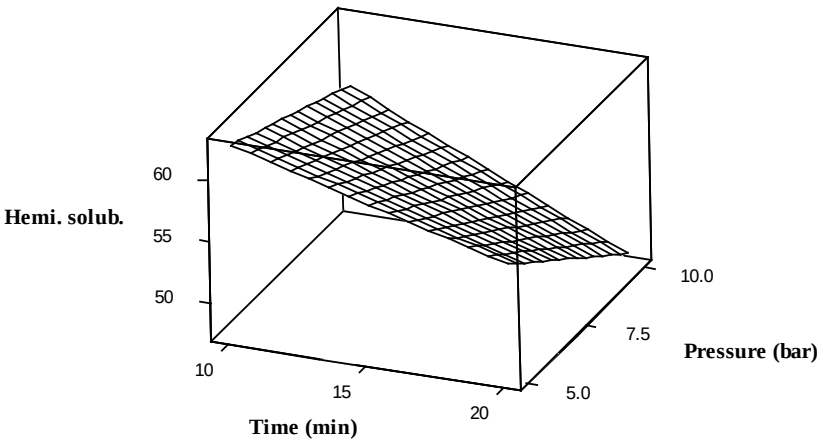


(b)

Figure 5.10 – (a) contour plot, (b) surface plot of cellulose content %(w/w) vs. pressure and temperature.

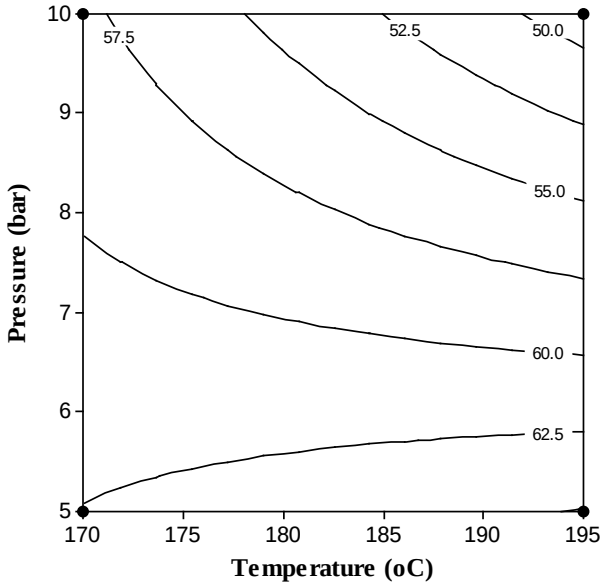


(a)

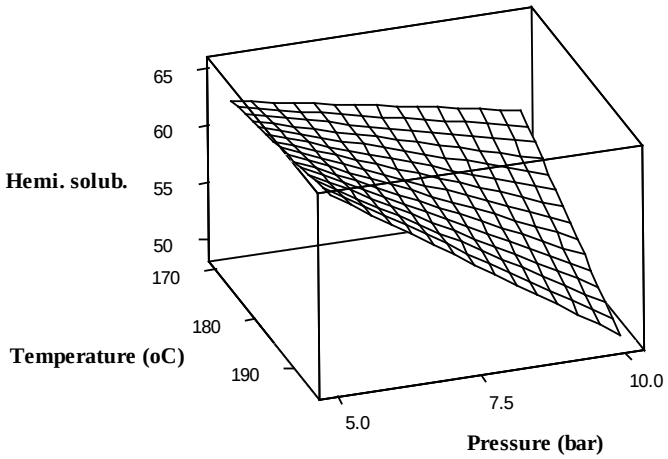


(b)

Figure 5.11 – (a) contour plot, (b) surface plot of hemicellulose solubilization %(w/w) vs. pressure and time.

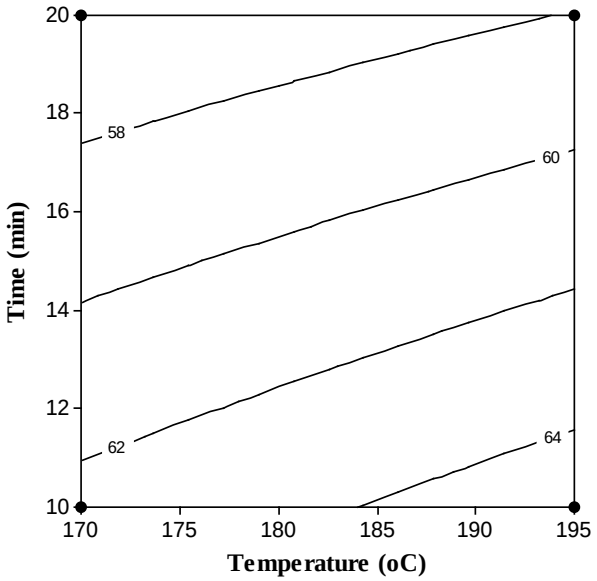


(a)

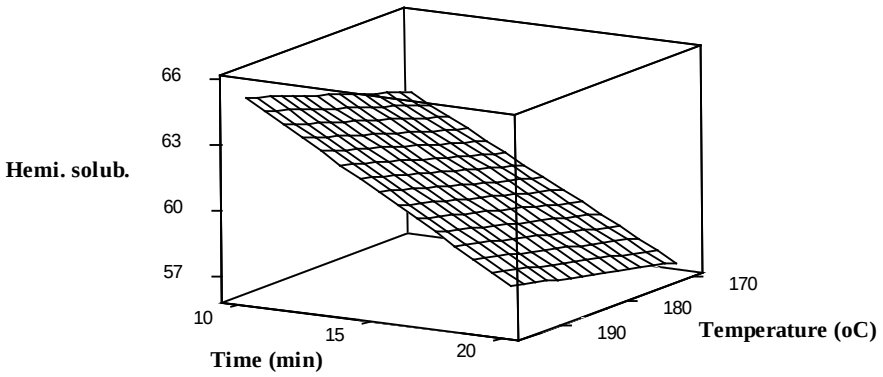


(b)

Figure 5.12 – (a) contour plot, (b) surface plot of hemicellulose solubilization %(w/w) vs. pressure and temperature.

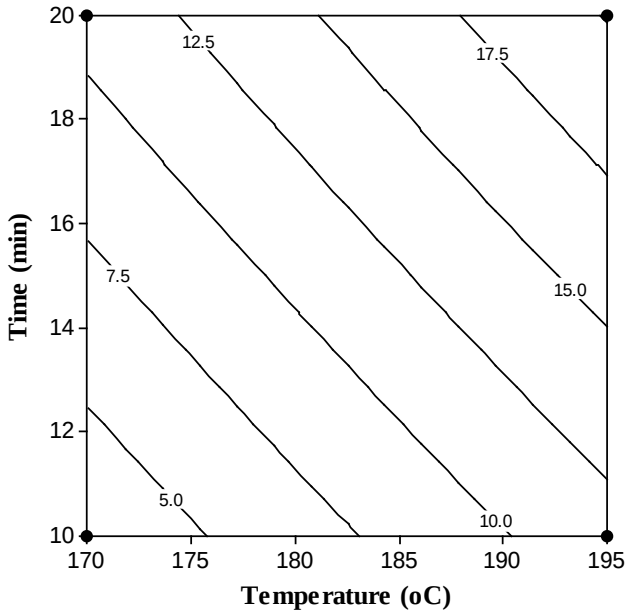


(a)

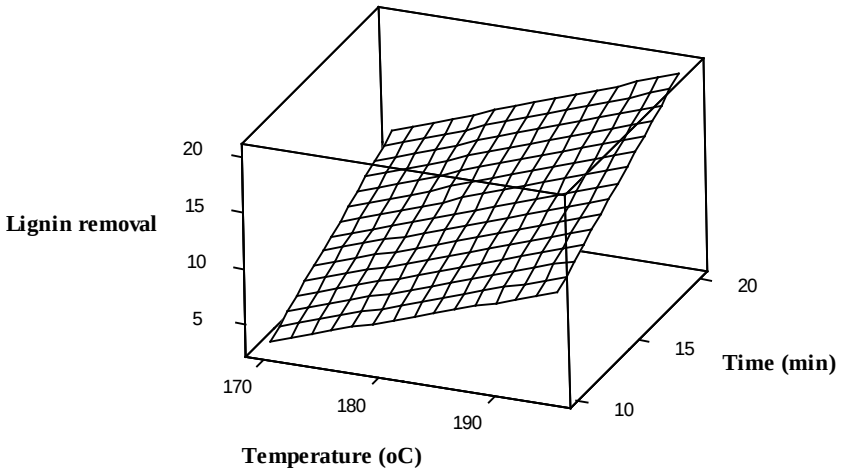


(b)

Figure 5.13 – (a) contour plot, (b) surface plot of hemicellulose solubilization %(w/w) vs. time and temperature.

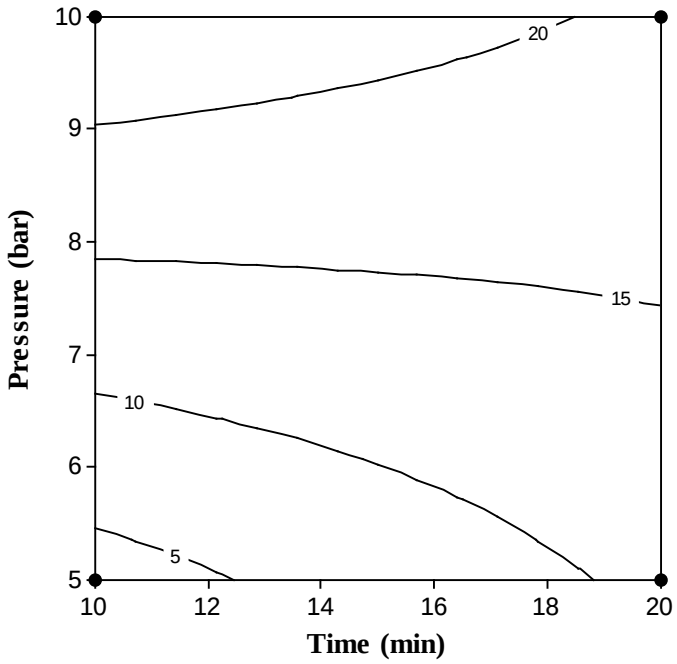


(a)

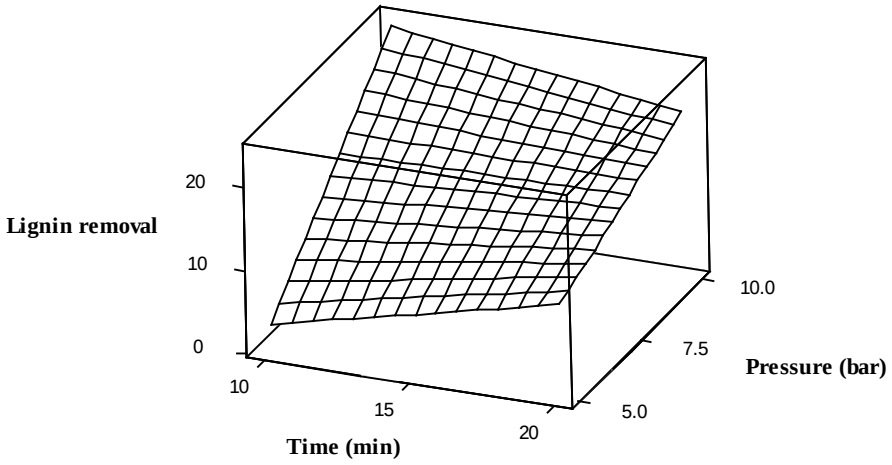


(b)

Figure 5.14 – (a) contour plot, (b) surface plot of lignin removal %(w/w) vs. time and temperature.

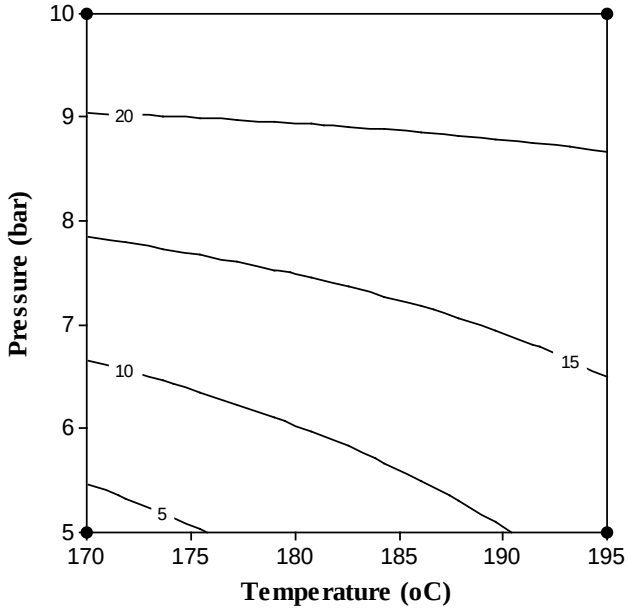


(a)

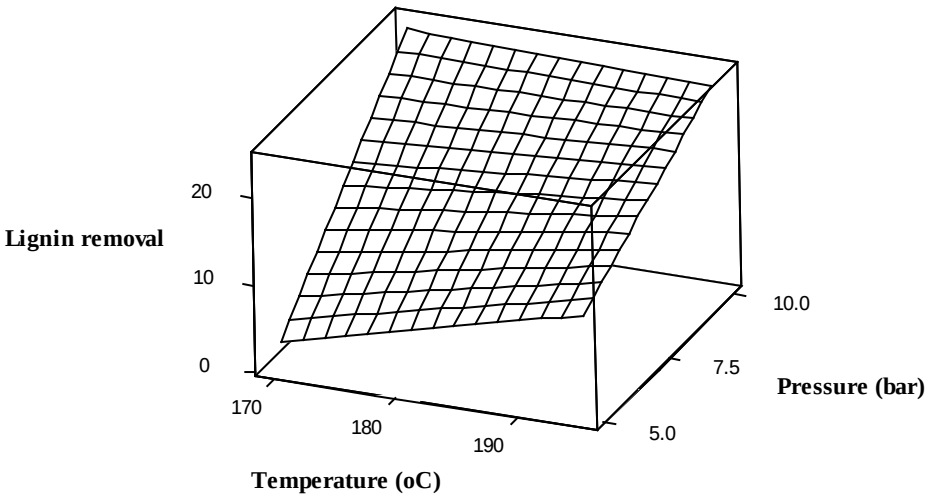


(b)

Figure 5.15 – (a) contour plot, (b) surface plot of lignin removal %(w/w) vs. pressure and time.

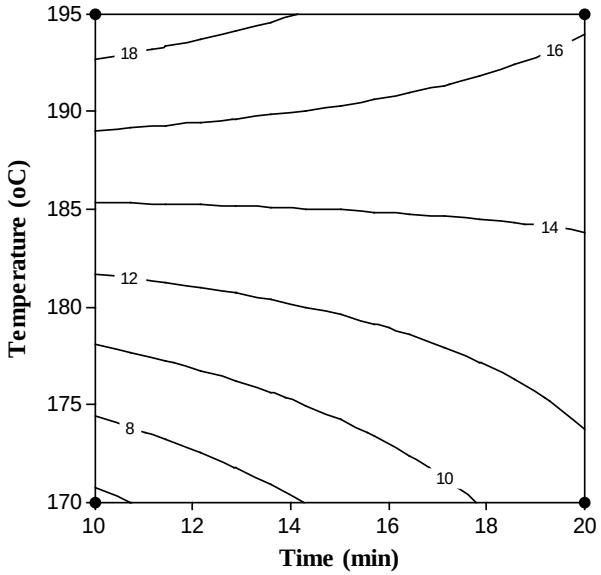


(a)

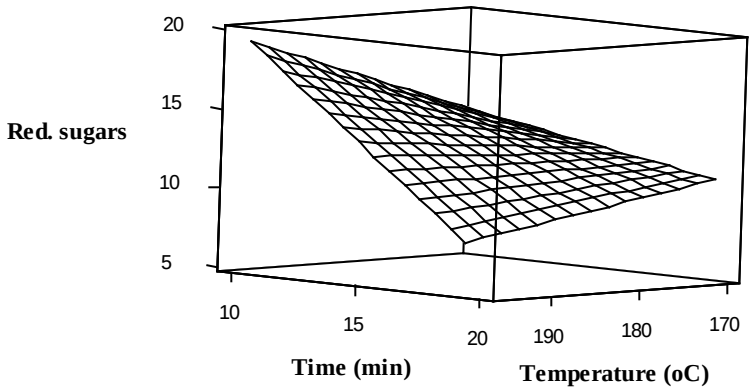


(b)

Figure 5.16 – (a) contour plot, (b) surface plot of lignin removal vs. pressure and temperature.

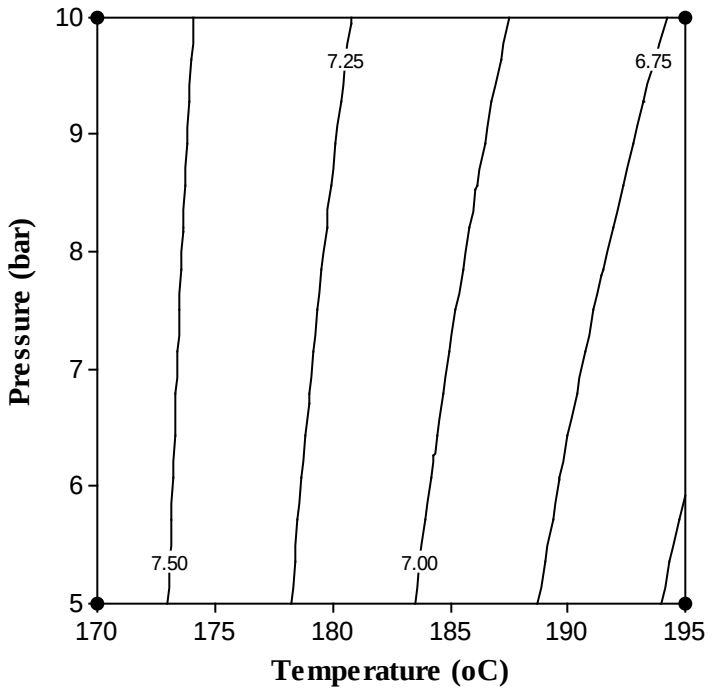


(a)

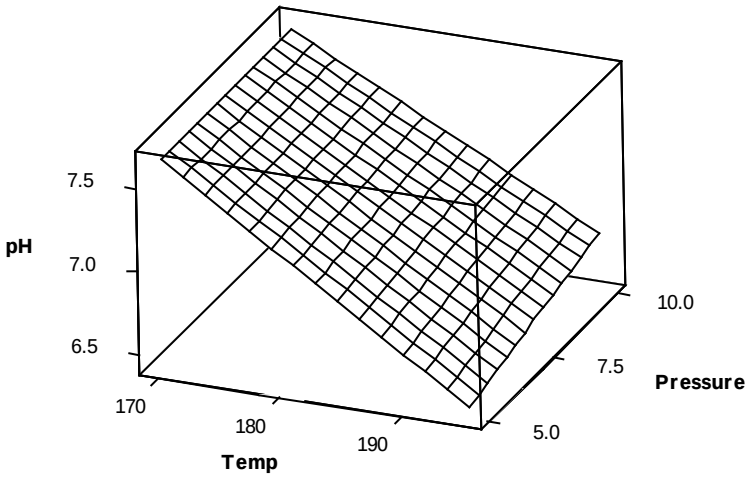


(b)

Figure 5.17 – (a) contour plot, (b) surface plot of reducing sugars (g/L) vs. temperature and time.



(a)



(b)

Figure 5.18 – (a) contour plot, (b) surface plot of pH vs. pressure and temperature.

Reducing sugars concentrations were found to be higher at high temperature and short time conditions (VII). However lower concentrations of reducing sugars (which has to correspond to increase in cellulose content, and a higher lignin removal in the solid fraction) will be preferable to higher concentration. For optimization purpose, a low pH value which should correspond to higher cellulose content in the solid fraction is preferable. Figure 5-18 shows the surface and contour plots of the interaction of pressure and temperature on pH.

The model equations for the various responses (Eqs. (5-1) to (5-5)), and the response surface and contour plots were utilized in determining the optimum WAO conditions so as to obtain a solid fraction with high cellulose content, low lignin and hemicellulose, and a liquid faction with low concentrations of reducing sugars, at a reasonably mild pH. With all these constraints in mind, the optimum cumulative responses were obtained at 170 ^{o}C, 10 bar, and 10 min (Table 4.3). Additional sets of WAO experiments at these specific conditions were performed to validate the optimized conditions. The experimental (validated) and predicted responses were found to be in close agreement, thus confirming the optimization process.

The WAO pretreatments were investigated at elevated temperatures of 170 ^{o}C and 195 ^{o}C. The results after pretreatments showed that biomass degradation occurred at these temperatures. Operating at such high temperature is not economical since the pretreatment yields were not high to correspond to the processing costs. Pretreatments carried out at much lower temperature would not require such a high pressure vessel. For this reason, this study also evaluated pretreatments at much lower temperatures by using response surface methodology of which central composite design of experiments is one.

5.4 2^3 CCD alkaline peroxide oxidation pretreatments optimization

Percentage of dry matter recovered in the solid fraction ranged from 84 to 95%. High dry matter recovery corresponded to very low lignin removal. For example, pretreatment 9 with a dry matter yield (total dry solid) of 95% showed only 4% lignin removal while pretreatment 3 with dry matter yield of 86% showed 13% lignin removal (Table O-8). Cellulose recovery in the solid residues with an average value of 102%

(pretreatments 1-20) proved the ability of the studied process for removing hemicellulose with negligible cellulose degradation. The joint contributions of cellulose and lignin recoveries also accounted for the high dry matter yields in the solid residues.

Hemicellulose recovery varied with all the conditions. It can be noted that the conditions of pretreatment 15 (150 ^{o}C, 40 min, 1% H_2O_2) corresponded to the maximum hemicellulose solubilization of up to 70%. Increased cellulose content in the pretreated solids ranged from 47–56% from the initial raw biomass of 46%. Cellulose content decreased at 120 ^{o}C (pretreatment 10) to 43% corresponding to a decrease in lignin removal. The cellulose enrichment was due majorly to hemicellulose solubilization and a small percentage of lignin removal. The lignin removal was very low in all the conditions with the highest value of 16% (pretreatment 22), which was due to the high lignin content of the woody residue. Silanikove (1994) reported that CaO-hydrogen peroxide and NaOH-hydrogen peroxide treatments of cotton straw at room temperature for 25 h caused 50–58% reduction in the lignin content. This is high compared to the value arrived at for the raw material used in this study. Cotton straw has an appreciable low lignin content. The lignin content varies between 15–25% depending on the source and species (Silanikove, 1994).

Also NaOH-alkaline hydrogen peroxide of wheat straw produced a maximum of about half of the lignin as water soluble degradation products (Gould, 1984). Study showed that oak shavings solubilized only when suspension was subjected to strong mechanical agitation (Gould, 1984).

It was also noted that the materials pretreated at low temperatures (110.5–135 ^{o}C) appeared light brown in colour while solids pretreated at 150 ^{o}C and 159.5 ^{o}C were deep brown in colour. This pretreatment also revealed that more hemicellulose was solubilized than lignin removal. Although little lignin removal was achieved in this process, favourable effects for further enzymatic hydrolysis are expected to be caused by the alkaline peroxide oxidation process, including dramatic increase in the degree of hydration of the cellulose polymer (Gould, 1984) especially for agricultural residues.

Application of CCD on the pretreatment process generated the following second order polynomial equations for cellulose content, hemicellulose solubilization and lignin removal;

Cellulose content %(w/w) =

$$88.274 + 0.1305X_1 - 0.879X_2 - 45.449X_3 - 0.003X_1^2 + 0.004X_2^2 - 3.307X_3^2 + 0.005X_1X_2 + 0.382X_1X_3 - 0.066X_2X_3$$

$$(R^2 = 0.955) \quad \ldots(5\text{-}6)$$

Hemicellulose solubilization %(w/w) =

$$771.776 - 10.324X_1 - 2.420X_2 + 45.736X_3 + 0.037X_1^2 - 0.006X_2^2 - 24.719X_3^2 + 0.016X_1X_2 - 0.079X_1X_3 + 0.507X_2X_3$$

$$(R^2 = 0.973) \quad \ldots(5\text{-}7)$$

Lignin removal %(w/w) =

$$57.845 - 1.030X_1 - 0.095X_2 + 22.566X_3 + 0.0033X_1^2 + 0.005X_2^2 - 20.2914X_3^2 - 0.002X_1X_2 + 0.203X_1X_3 + 0.126X_2X_3$$

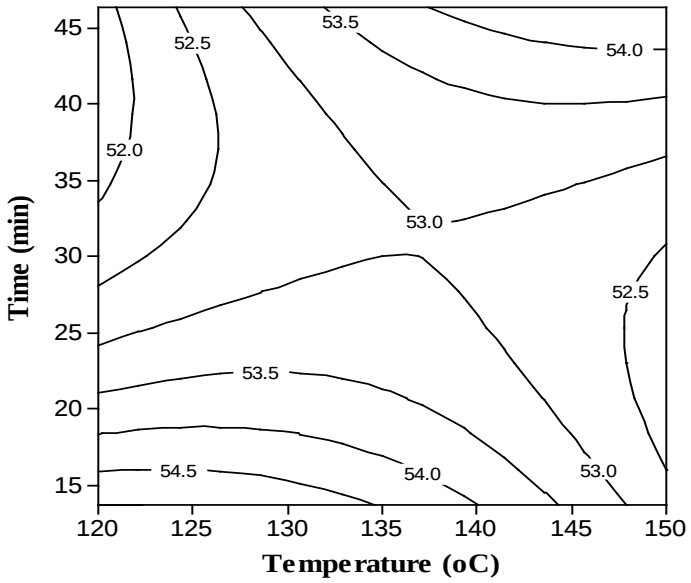
$$(R^2 = 0.840\ ) \quad \ldots(5\text{-}8)$$

When the values from X_1 to X_3 were substituted in the above equations, the predicted responses were obtained.

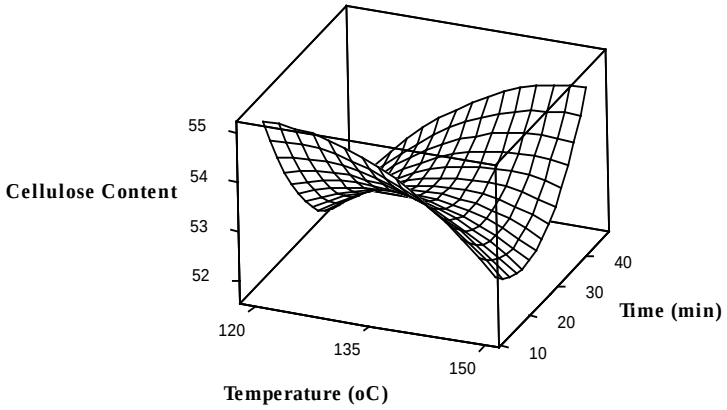
The statistical treatment combinations of the test variables corresponding to all combinations are summarized in Table O-13. The *p*-values were used as a tool to check the significance of each of the variables as well as their interactive and quadratic effects. In general, the smaller the value of *p* (< 0.05) and the larger the magnitude of *t*-value, the more significant is the corresponding coefficient term. It was observed that time and H_2O_2 main effects, time and temperature quadratic effects, temperature interactions with time and H_2O_2 were statistically significant on cellulose content. All the main, quadratic, and interactive effects are significant on hemicellulose solubilization except temperature and H_2O_2 interaction. For lignin removal, only H_2O_2 quadratic effect was statistically significant. In addition, the multiple correlation coefficients (R^2) of the regression equations obtained were 0.9549 for cellulose content, 0.9732 for hemicellulose solubilization, and 0.8404 for lignin removal. These values mean the models for the

responses fitted well with the experimental data. The R^2-value for cellulose content means that the sample variation of 95.5% is attributed to the factors, and also indicates that 4.5% of the total variation is not explained by the model. For hemicellulose solubilization 2.7% of the total variation is not explained by the model. R^2-value for lignin removal revealed that 16% of the total variation is not explained by the model.

The summary of analysis of variance (ANOVA) representing the results is discussed in Table O-15. ANOVA is required to test the significance and adequacy of the models. The Fisher's variance ratio (*F*-value) is the ratio of the mean square owing to regression to the mean square owing to error (Montgomery, 1991). It is the measure of variation in the data about the mean. The ANOVA for the three regression models indicates that models are very significant as evident from the calculated *F*-values and very low *P*-values ($P = <0.003$). Large *F*-value demonstrates that most of the variations in the responses can be explained by the regression model equations. The ANOVA table also shows a term for residual error, which measures the amount of variation in the response data left unexplained by the model. Thus, the form of the models chosen to explain the relationship between the factors and responses can be concluded to be correct. Two-dimensional contour plot and three-dimensional response surface curves were plotted to study the interactions between the various parameters in APO pretreatment of the sawdust material and were used to determine the optimum levels of each factor required to obtain maximum responses. Effects of individual factors on (%w/w) cellulose content, lignin removal, and hemicellulose solubilization of the solid fraction are shown in Figures 5.19–5.27. The plots were obtained by holding the third variable at mid-point value. Figures 5.19–5.21 show the contour and surface plots of the interactive effects of temperature, reaction time, $\%H_2O_2$ on cellulose content to obtaining maximum responses. Figure 5.19 shows the effects of time and temperature on cellulose content. Maximum cellulose content was likely to occur between 120–135 ^oC and 15–20 min or 140–150 ^oC and 40–45 min. Figure 5.20 also shows that maximum cellulose content is achievable at between 1.00–1.25% H_2O_2 and 140–150 ^oC. Hemicellulose solubilization maximum response should also be between 0.9–1.15 $\%H_2O_2$ and 30–45 min (Figures 5.22–5.24). Maximum lignin removal was likely to occur at 145–150 ^oC and 40–45 min Figures 5.25–5.27.

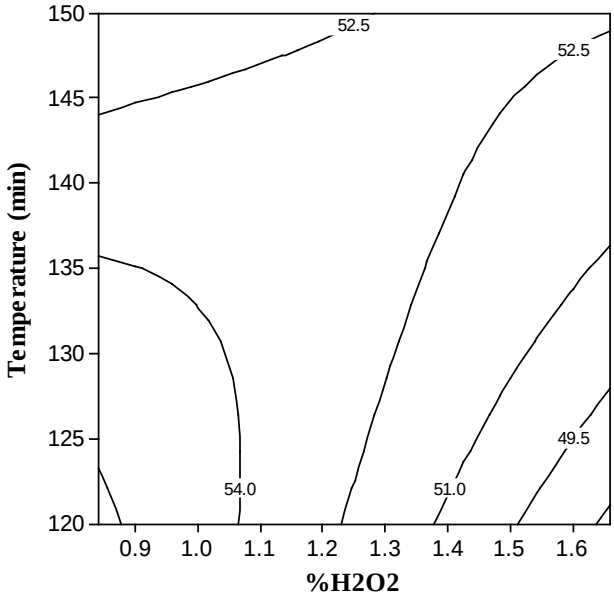


(a)

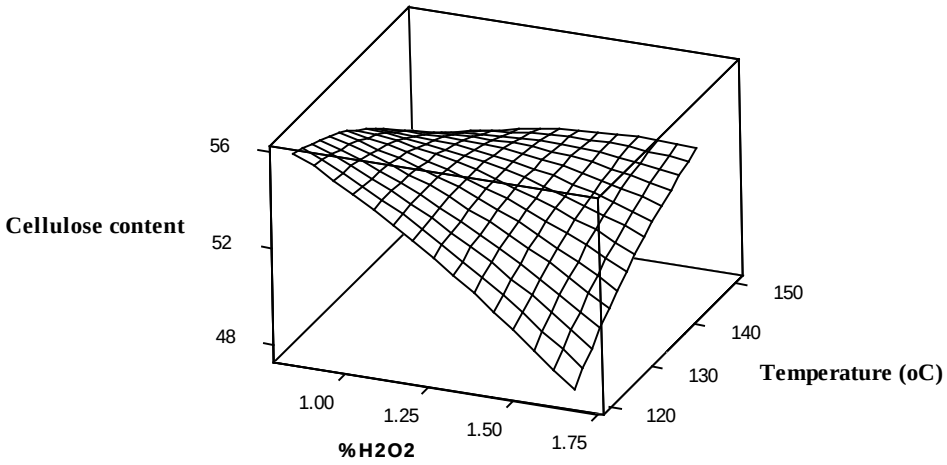


(b)

Figure 5.19 – (a) contour plot, (b) surface plot of cellulose content %(w/w) vs. time and temperature for 2^3 CCD APO pretreatment.

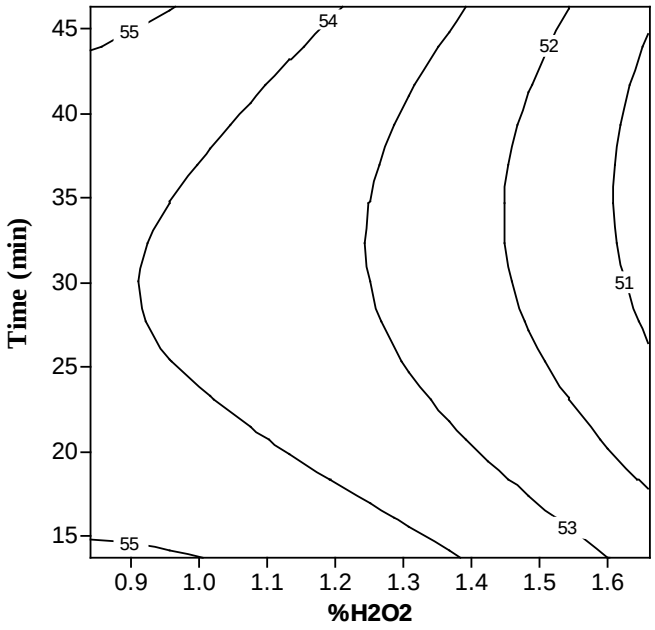


(a)

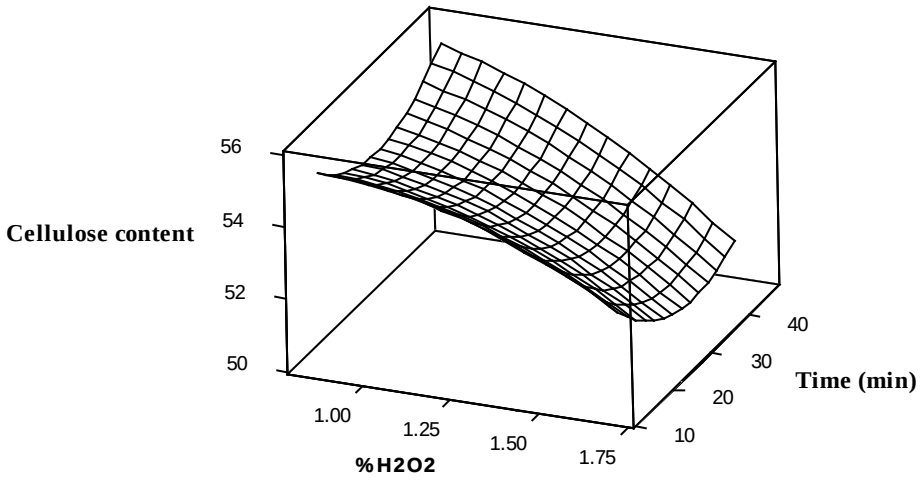


(b)

Figure 5.20 – (a) contour plot, (b) surface plot of cellulose content %(w/w) vs. temperature and $\%H_2O_2$.

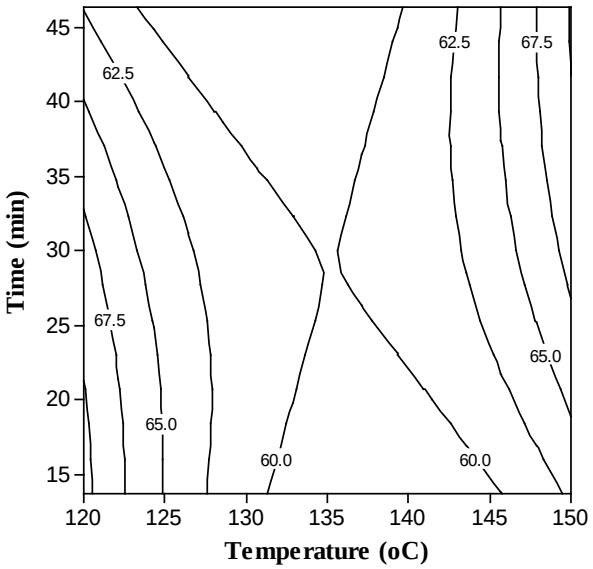


(a)

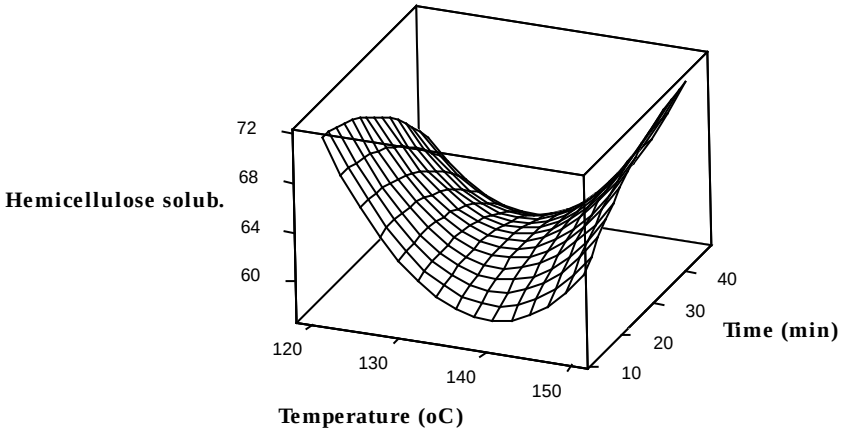


(b)

Figure 5.21 – (a) contour plot, (b) surface plot of cellulose content %(w/w) vs. time and $\%H_2O_2$.

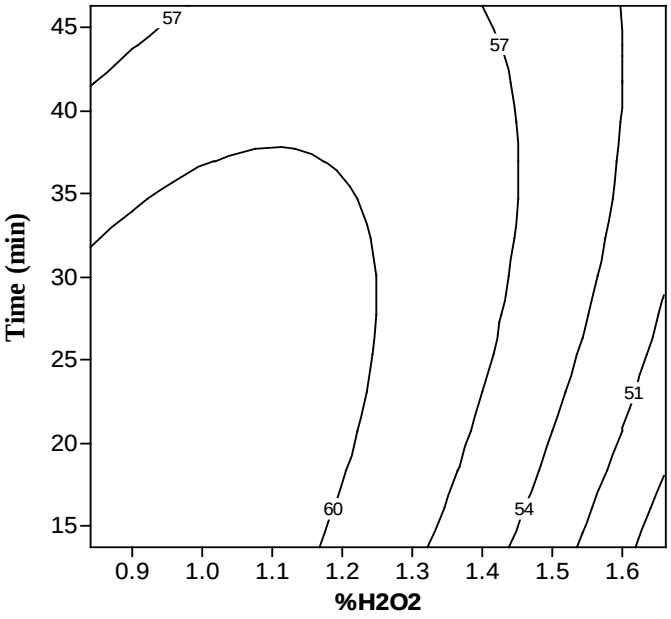


(a)

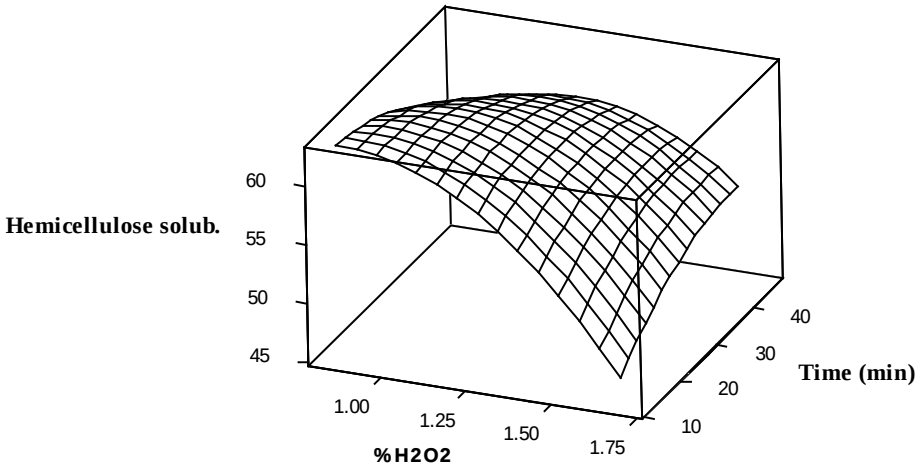


(b)

Figure 5.22 – (a) contour plot, (b) surface plot of hemicellulose solubilization vs. time and temperature.

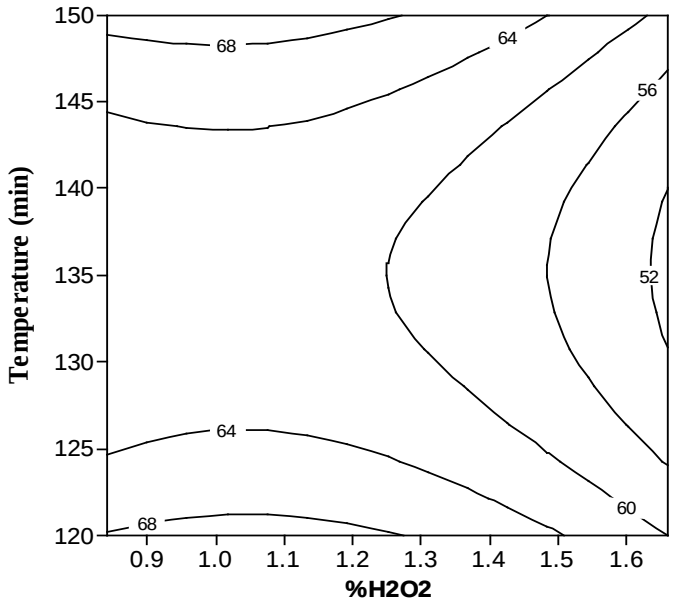


(a)

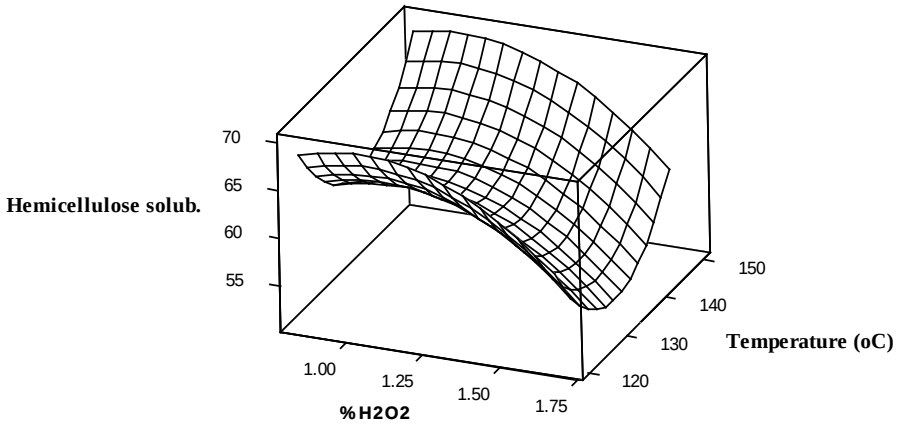


(b)

Figure 5.23 – (a) contour plot, (b) surface plot of hemicellulose solubilization %(w/w) vs. time and $\%H_2O_2$.

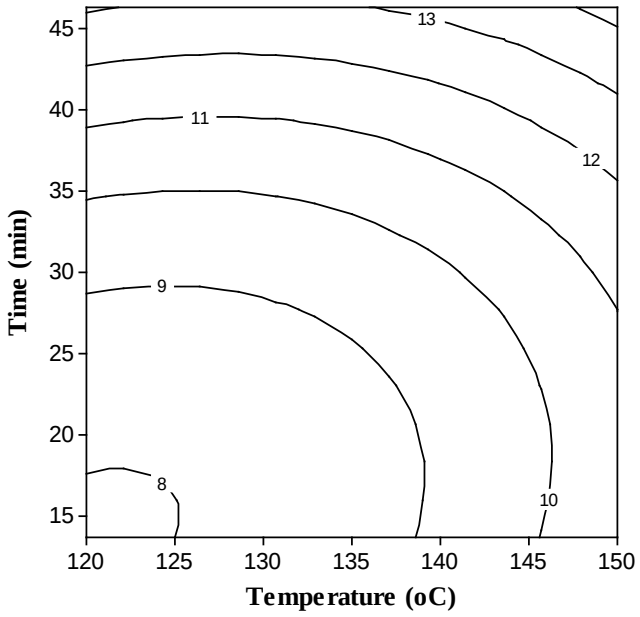


(a)

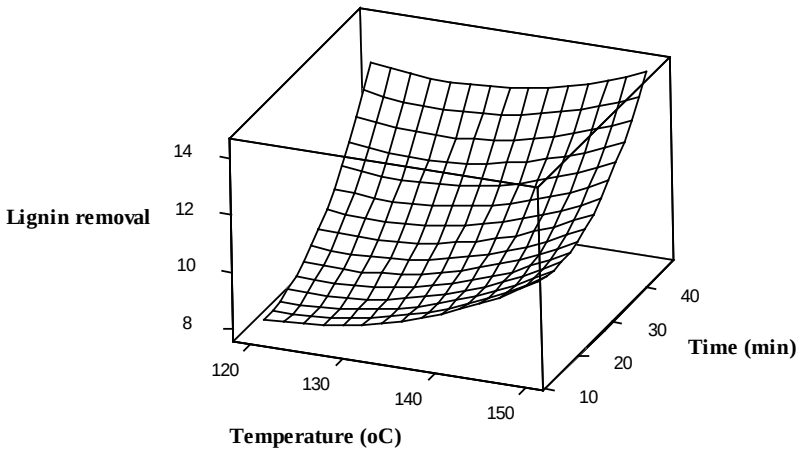


(b)

Figure 5.24 – (a) contour plot, (b) surface plot of hemicellulose solubilization %(w/w) vs. temperature and $\%H_2O_2$.

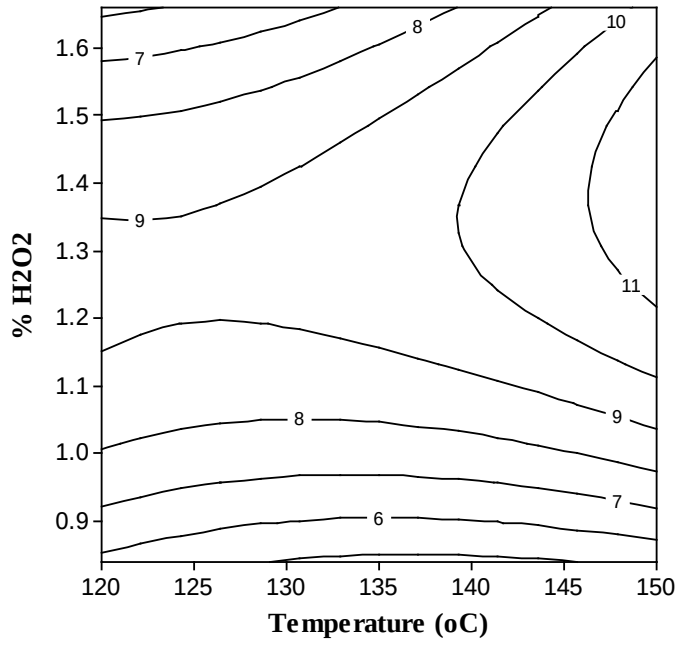


(a)

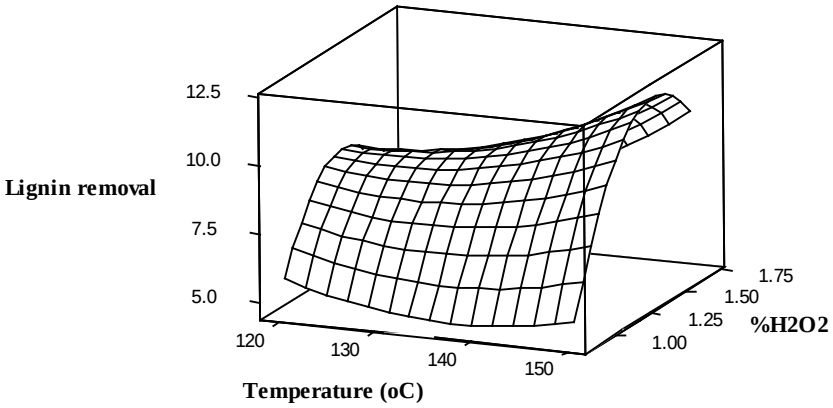


(b)

Figure 5.25 – (a) contour plot, (b) surface plot of lignin removal %(w/w) vs. time and temperature.

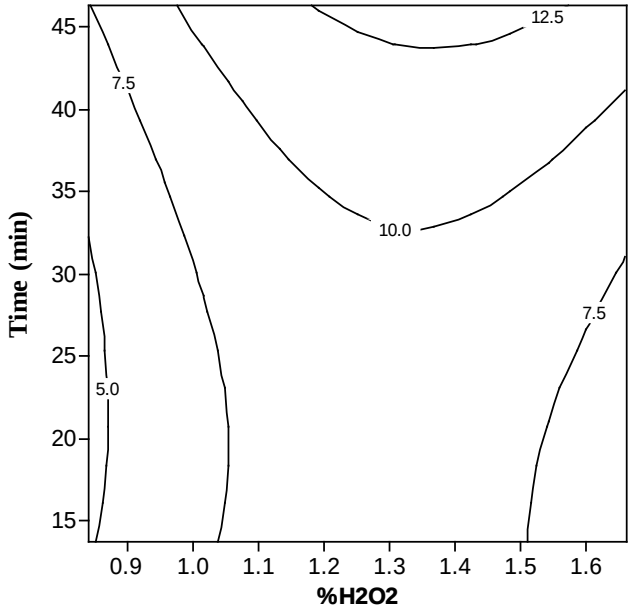


(a)

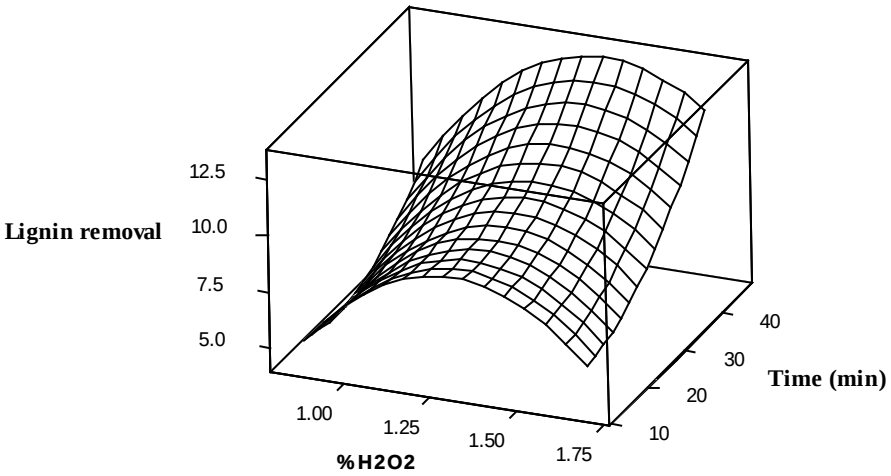


(b)

Figure 5.26 – (a) contour plot, (b) surface plot of lignin removal %(w/w) vs. $\%H_2O_2$ and temperature.



(a)



(b)

Figure 5.27 – (a) contour plot, (b) surface plot of lignin removal %(w/w) vs. time and $\%H_2O_2$.

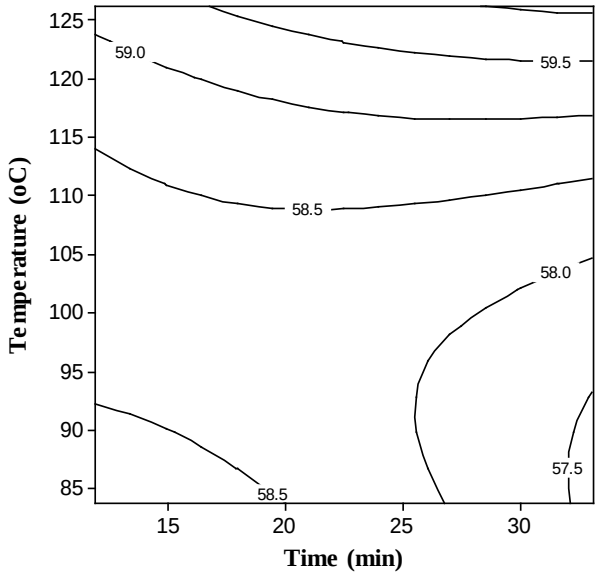
The optimal values of each factor to optimize the process responses were also based on Multi-Objective Numerical Optimization. The model equations for the various responses (Eqs. (5-6) to (5-8)), and the response surfaces and contour plots were utilized in determining the optimum APO conditions so as to obtain a solid fraction with high cellulose content, low lignin and appreciable hemicellulose. The optimum cumulative responses were obtained at 150 ^{o}C, 45 min, and 1% H_2O_2 (Table 4.4). The predicted responses were 53.86% cellulose content (desirability = 0.9638), 70.00% hemicellulose solubilization (desirability = 0.8573), and 11.00% lignin removal (desirability = 0.9994). A validation of results from the models and regression equation was performed and compared with the predicted values.

5.5 2^2 alkaline peroxide oxidation pretreatment optimization

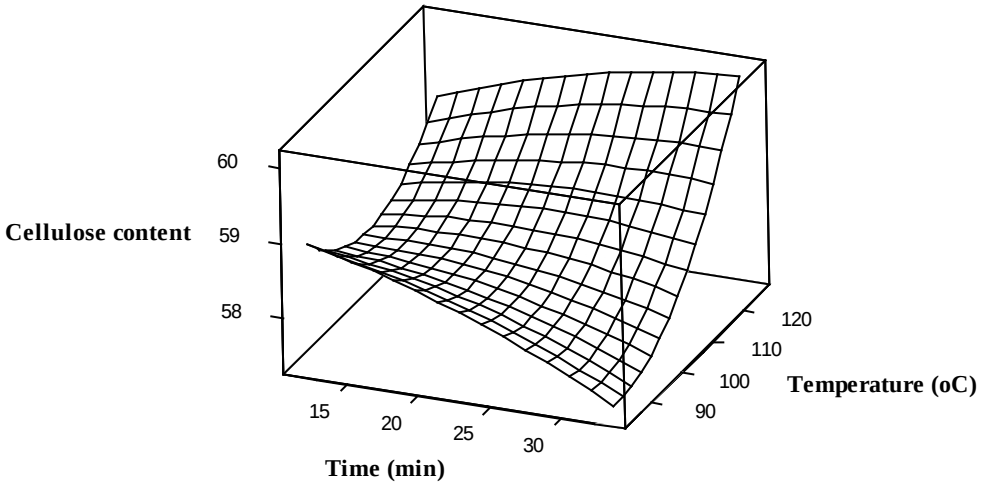
Percentage of dry matter (dry biomass) recovered in the pretreated solid ranged from 86 to 95%. Pretreatment 17 had the highest cellulose recovery of 126% (Table O-9). The high dry matter recovery in the solid residues was as a result of the undissolved lignin and the cellulose content remaining after pretreatment. Pretreatment 1 (120 ^{o}C and 15 min) corresponded to the maximum hemicellulose solubilization of up to 71% (Table 4-5), meaning that 29% hemicellulose was recovered in the solid fraction. Increased cellulose content in the pretreated solids ranged from 56–61% from the initial raw biomass of 46%. The lignin removal was very low in all the conditions with the highest value of 17%, that is, 83% of the lignin content in the solid fraction remained un-dissolved (Pretreatment 11, Table O-9). Contour and three-dimensional response surface curves were plotted to study the interactions between the two parameters in APO pretreatment of the sawdust material.

Figures 5.28–5.30 show the effects of temperature and time on cellulose content, hemicellulose solubilization, and lignin removal of the solid fraction of the treated biomass. Cellulose content was highest (60%) at around 25–35 min reaction time. Cellulose content also increased with increased temperature, (Figure 5.28). The hemicellulose solubilization was highest (70%) around 115–125 ^{o}C but decreased at higher pretreatment time. The optimum may be realized at around 20–25 min and a

temperature of about 120 ^{o}C, Figure 5.29. The lignin removal is also highest at around 20–25 min and 110–120 ^{o}C (Figure 5.30). Therefore, the optimum pretreatment can be taken to be at around 120 ^{o}C and 25 min.

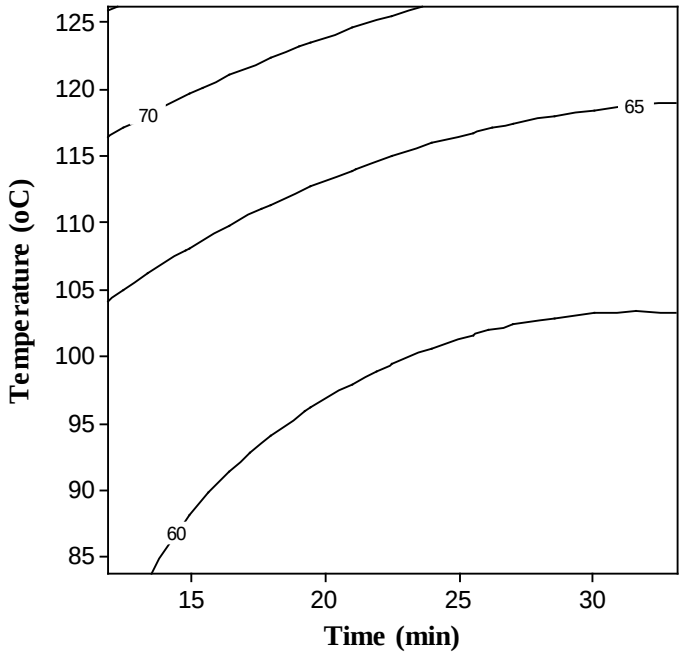


(a)

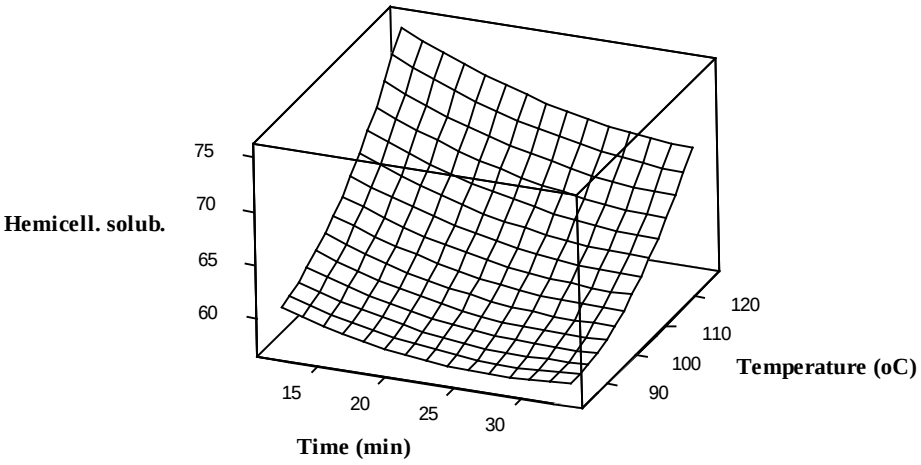


(b)

Figure 5.28 – (a) contour plot, (b) surface plot of cellulose content %(w/w) (2^2 CCD APO pretreatment) vs. temperature and time.

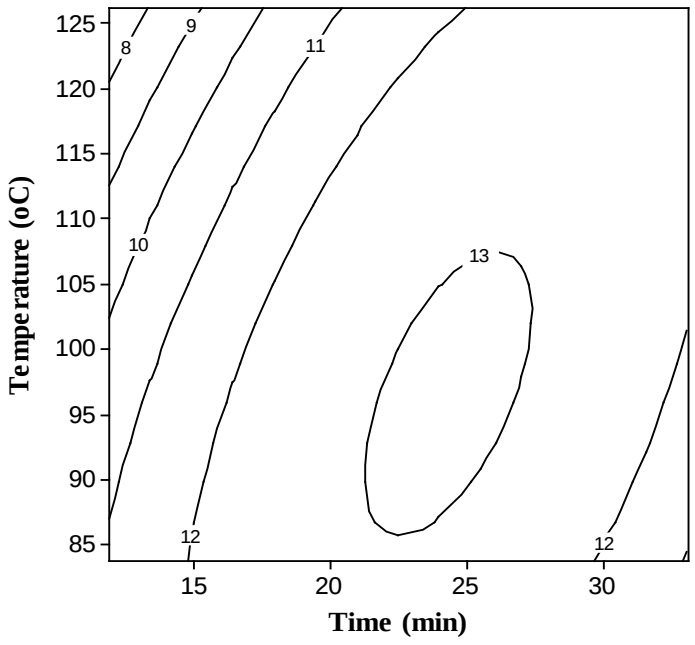


(a)

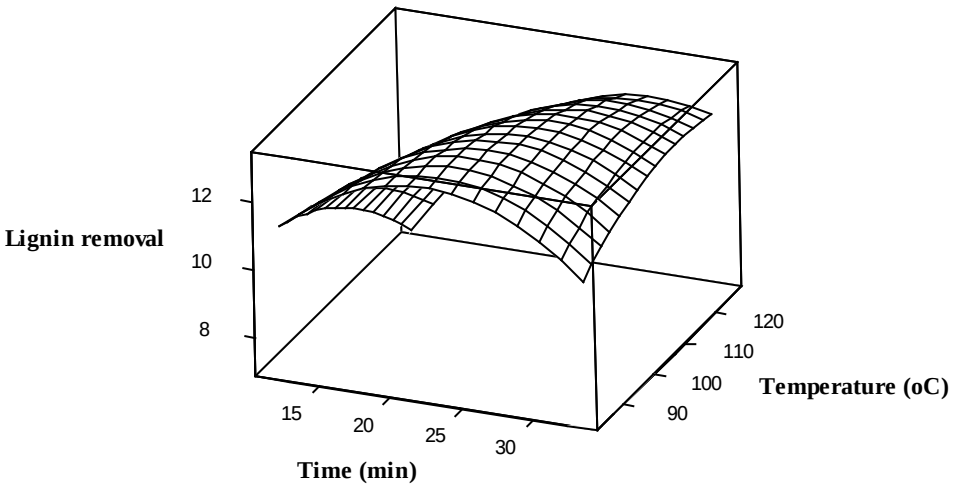


(b)

Figure 5.29 – (a) contour plot, (b) surface plot of hemicellulose solubilization %(w/w) (2^2 CCD APO pretreatments) vs. temperature and time.



(a)



(b)

Figure 5.30 – (a) contour plot, (b) surface plot of lignin removal %(w/w) (2^2 CCD APO pretreatment) vs. temperature and time.

5.6 Alkaline peroxide assisted WAO pretreatments

The APAWAO pretreatments as shown in Figure 5.31 revealed that cellulose was better preserved in the solid fraction. Hemicellulose solubilization and lignin removal increased in all the variations considered. For example, WAO pretreatment at 170 ^{o}C 10 bar air pressure, and 10 min (pretreatment A) (Table 4.6), cellulose content was 52%, hemicellulose solubilization 58%, and lignin removal 17% for the validated responses, but with 1% H_2O_2 addition during pretreatment, cellulose content increased to 59%, hemicellulose solubilization was 70%, and lignin removal increased to 31% (w/w) (Pretreatment B). For APO pretreatment at 150 ^{o}C 45 min and 1%H_2O_2, cellulose content was 59%, hemicelluloses solubilization 72%, lignin removal 10% (Pretreatment D). With the addition of 10 bar air pressure during pretreatment, cellulose content was also 59%, hemicellulose solubilization 71%, and lignin removal was 24%(w/w)(pretreatment E), more of the lignin was removed. These variations also pointed to the fact that at lower temperature of 150 ^{o}C, pretreatment yields was comparable to yields at higher pretreatment temperature of 170 ^{o}C, and more delignification occurred with the combination of air pressure and hydrogen peroxide as oxidizing agents.

These observations showed that holocellulose (especially the cellulose) can be recovered in the oxidative lime pretreatment process. The delignification and polysaccharide degradation especially hemicellulose accounted for the solubilization of biomass during pretreatment.

The level of delignification of the sawdust wood residue after the pretreatment stages revealed that the addition of air pressure/or hydrogen peroxide alone (as oxidizing agent) made the major part of the lignin content undissolved. A combination of both oxidizing agents during pretreatments improved delignification.

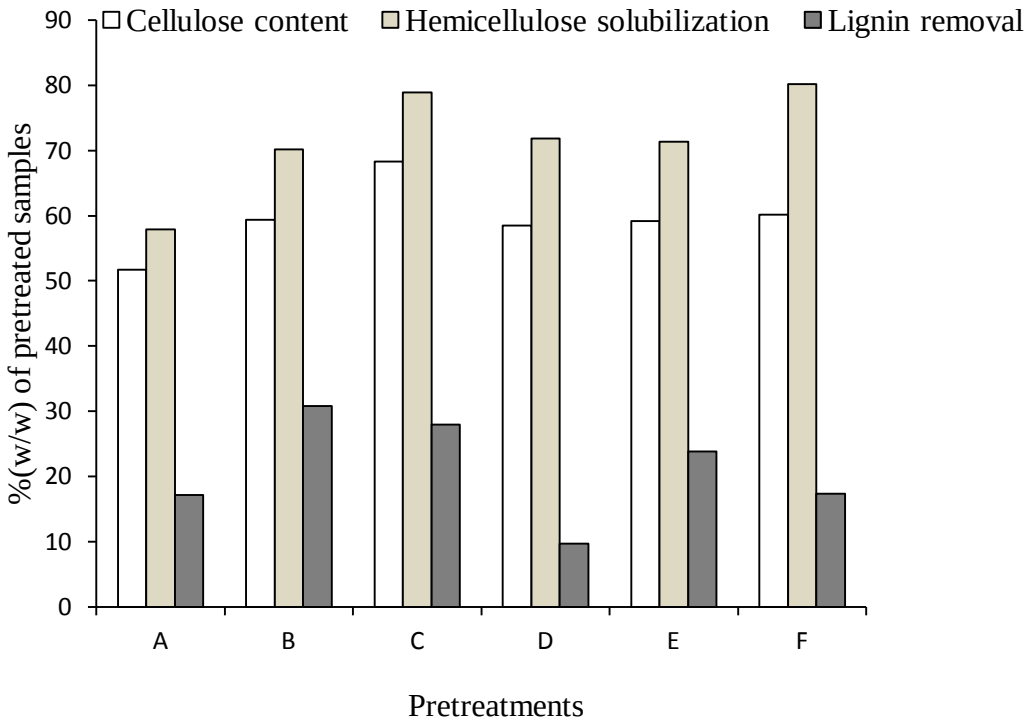


Figure 5.31 – Correlations of pretreated samples with variations of experimental conditions (WAO (A), APO (D), and APAWAO (B, C, E, and F)) pretreatments compared (from Table 4.6).

5.7 Enzymatic hydrolysis of treated solids

The reducing sugar yield was calculated as mg equivalent glucose/g dry biomass with the appropriate formula (see Appendix K). The reducing sugar yields of the time-zero samples determined the sugar contents of the enzymes and were subtracted from the sugar yields at other time point.

Table 4.9 shows the 3-day reducing sugar yields for the optimized conditions and their variations (illustrated in Figure 5.32). The reducing sugar yields (mg equivalent glucose/g dry biomass) after hydrolysis of pretreated sawdust were considered at 50 ^{o}C hydrolysis temperature and 72 h hydrolysis time. It can be seen from Figure 5.32 that under operational conditions used, maximum reducing sugar yield after 3-day hydrolysis was 173.52 mg equivalent glucose/g dry biomass for pretreatment C (170 ^{o}C, 1%H_2O_2 biomass soaking for 24 h, 10 bar air pressure, and 10 min). Pretreatment E (150 ^{o}C, 1%H_2O_2, 10 bar air pressure, 45 min) produced 155.66 mg equivalent glucose/g dry biomass).

The 3-day reducing sugars yield for the 2^2 CCD APO pretreatments was highest with pretreatment 11 (146.00 mg equivalent glucose/g dry biomass) at 120 ^{o}C, 1%H_2O_2, and 30 min (some of the results are illustrated in Figure 5.33). The trend in the reducing sugars yield for the 2^2 CCD APO pretreatments is similar to the trend in Figure 5-32. Pretreatments 11, 12, and 13 appeared that the sugar yields are likely to increase beyond the third day. There was a general increase in sugars yields as the saccharification time increased from 2 to 72 h. The low reducing sugars yields under these experimental conditions may be primarily due to the low lignin removal from the pretreatment step caused by the high lignin content in the raw biomass. Also, β-glucosidase is said to be inhibited by glucose (Holtzapple *et al*., 1990). Inhibition of the enzymes by the end products negatively affects cellulose hydrolysis. However, the optimal conditions change with the hydrolysis residence time (Tengborg *et al*., 2001), hydrolysis temperature, and are also dependent on the source of the enzymes. The concentration of cellulases has a high impact on the conversion of the cellulose. Lignin content in lignocellulosic biomass

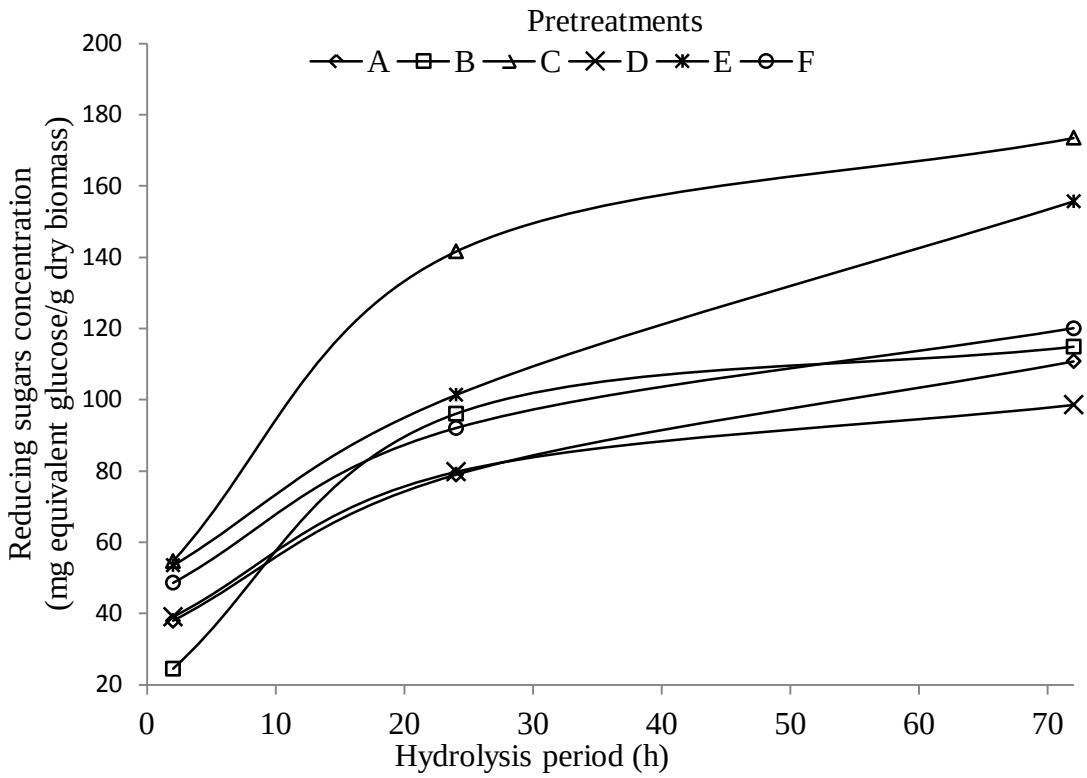


Figure 5.32 – 3-d reducing sugar yields for the optimized pretreatment conditions and their variations (from Table 4-6). Hydrolysis conditions: 2% (20 g/L) substrate concentration, 25 FPU cellulase enzyme/g dry biomass, 12.5 IU β-glucosidase/g dry biomass, 50 ^{o}C hydrolysis temperature, pH 4.8.

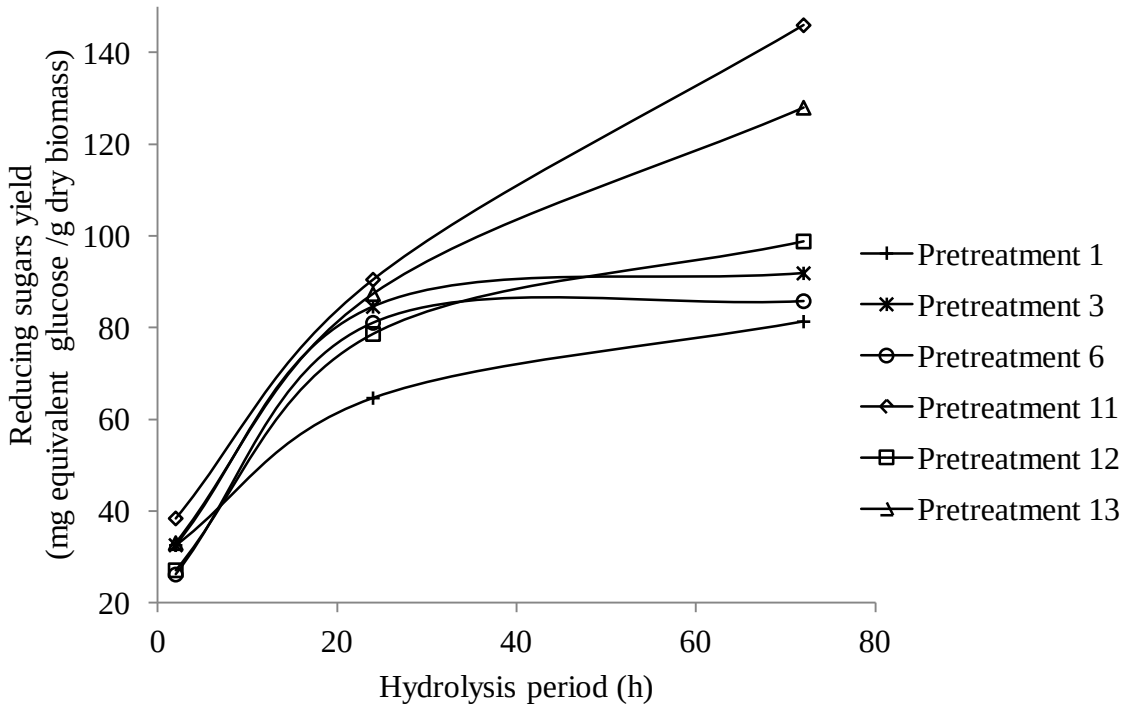


Figure 5.33 – 3-d reducing sugar yields for the 2^2 CCD APO pretreatments (Table 4–10). Hydrolysis conditions: 2% (20 g/L) substrate concentration, 25 FPUcellulase enzyme/g dry biomass, 12.5 IU β-glucosidase/g dry biomass, 50 ^{o}C hydrolysis temperature, pH 4.8.

has a great influence on digestibility of the material. Saha and Cotta (2007) using NaOH-hydrogen peroxide treatment on rice hulls (with lignin content of 18.7%) at room temperature achieved total sugars yield of 353 mg/g dry biomass in 120 h (74% conversion of treated substrate).

The "optimal" temperature for enzymatic hydrolysis using *Trichoderma reesei* is generally considered to be 50 ^{o}C (Kaar and Holtzapple, 2000). 50 ^{o}C was specified in the filter paper assay (Ghose, 1987) because this is the "optimal" temperature. For short reactions (e.g. reaction time of 60 min), such as the filter paper assay, 50 ^{o}C is a desirable temperature because thermal denaturation of the enzyme is insignificant, and the hydrolysis rate per unit of active enzyme increases at the higher temperature. However, for longer hydrolyses, lower temperatures should be considered. Therefore, it was very important to carry out under the enzymatic hydrolysis the reducing sugar yields as well as the %Saccharification (also called %digestibility or % conversion) of pretreated biomass by considering longer hydrolysis time (4-day) and lower hydrolysis temperature (45 ^{o}C); these issues are discussed in the following sections.

For the investigations, pretreatment E (Figure 5.32) and pretreatment 11 (Figure 5.33) were used to study the effects of hydrolysis temperature and time, substrate concentrations, enzyme loadings on reducing sugar yields. Pretreatment E was the optimized conditions for the 2^3 CCD APO pretreatment and it was evaluated at a lower temperature than pretreatment C (Figure 5.32). Pretreatment 11 (2^2 CCD APO pretreatment) produced the maximum reducing sugars yield from the enzymatic hydrolysis conditions considered (25 FPU cellulase/g dry biomass loading, 20 g/L(2%) substrate concentration, pH 4.8, 3-d hydrolysis time, 50 ^{o}C, 12.5 IU β-glucosidase/g dry biomass).

5.8 Substrate concentration on enzymatic hydrolysis

Substrate concentration is an important factor in the enzymatic hydrolysis of lignocellulosic biomass, because it influences the rate and extent of hydrolysis, and thussignificantly influences the economic potential of the overall process (Zhu, 2005,

Huang *et al*. 1991, and Ortega *et al*., 2001). Tengborg *et al*., (2001) reported that there is an inverse relationship between substrate concentration and hydrolysis yield. Cellulolytic enzymes are known to be inhibited by hydrolysis end-products such as cellobiose, glucose, or both. The inhibitory effect of cellobiose can be alleviated by adding supplemental cellobiase (β-glucosidase) that converts cellobiose into glucose.

The pretreated sawdust material after saccharification for pretreatment conditions 150 ^{o}C, 1%H_2O_2, 10 bar air pressure, and 45 min with 25 FPU/g substrate and 2 h reducing sugars yield was 24.88 mg/g (% Saccharification = 3.08) while it increased to 138.69 mg/g in 96 h (% Saccharification = 15.19). Results showed that reducing sugars yields increased for 20, 30, 40 g/L substrate concentrations considered up to the fourth day of hydrolysis. It also showed that more reducing sugars are likely to be produced if the hydrolysis time was increased beyond 96 h (illustrated in Figure 5.34). This also showed that the enzymes are still active to cause more hydrolysis at the reduced hydrolysis temperature of 45 ^{o}C. However, for 50 g/L substrate concentration and 62.5 FPU/g dry biomass loading, the sugars yield was 328.86 mg glucose equivalent/g dry biomass (% Saccharification = 16.28) which was lower than sugars yield at 40 g/L and 50 FPU/g dry biomass loading (358.45 mg glucose equivalent/g dry biomass)(% Saccharification = 22.19). In this case, end products such as cellobiose, glucose or both might have accumulated at the high substrate loading thereby inhibiting the hydrolysis process.

Furthermore, reducing sugars yields without supplemental β-glucosidase were comparable to when the enzyme was added. For example, at 40 g/L substrate concentration and 50 FPU/g substrate enzyme loading, the reducing sugars yield at the end of 96 h with β-glucosidase addition was 358.45 mg/g (% Saccharification = 22.19) while at the same condition but without β-glucosidase addition reducing sugars yield was 347.20 mg/g (% Saccharification = 21.49) (Figures 5.36 and 5.37). This is just about 3.1% lower. In other words, considering the economy of the process, enzymatic hydrolysis in the absence of β-glucosidase generally gave better results to hydrolysis when there was β-glucosidase supplements (Figures 5.34–5.37). The yields in reducing sugars with no beta-glucosidase addition may be due to the reduction in the inhibitive effects of the enzyme on glucose.

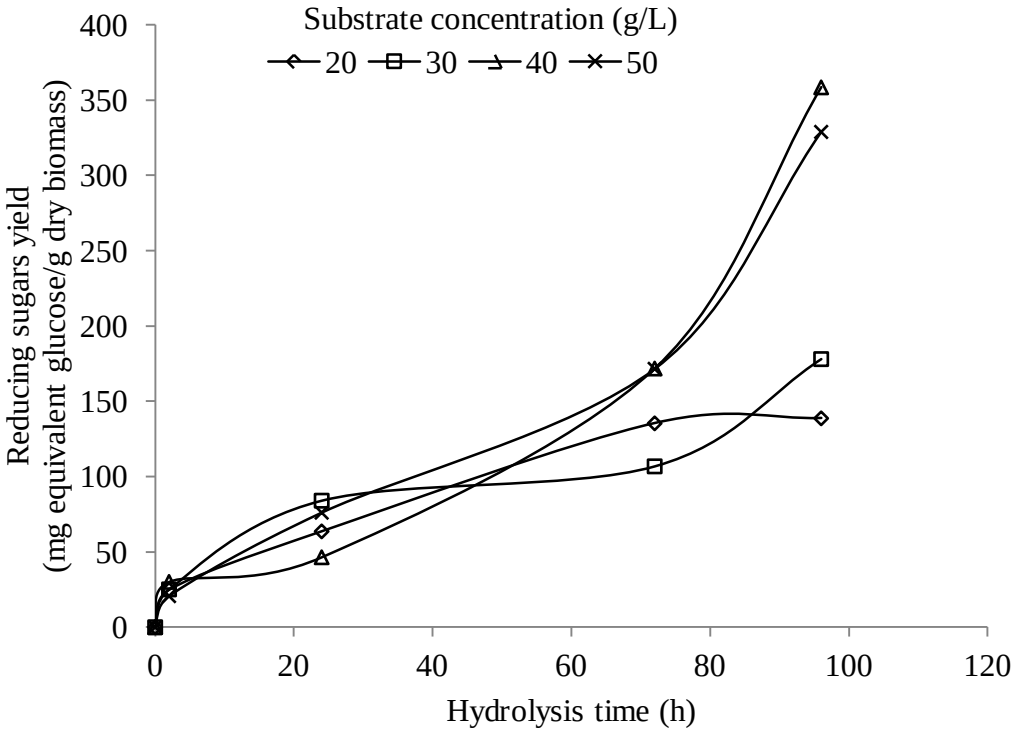


Figure 5.34 – 4-d Effect of time and substrate concentration on sugars yield with supplemental β-glucosidase. Pretreatment conditions: 150 ^{o}C, 1% H_2O_2, 10 bar, 45 min. Enzyme hydrolysis conditions: 20 g/L (25 FPU cellulase, 15 IU β-glucosidase), 30 g/L (37.5 FPU cellulose, 22.5 IU β-glucosidase), 40 g/L (50 FPU cellulose, 30 IU β-glucosidase), 50 g/L (56.3 FPU/g dry biomass, 37.5 IU β-glucosidase/g dry biomass). 45 ^{o}C hydrolysis temperature, pH 4.8.

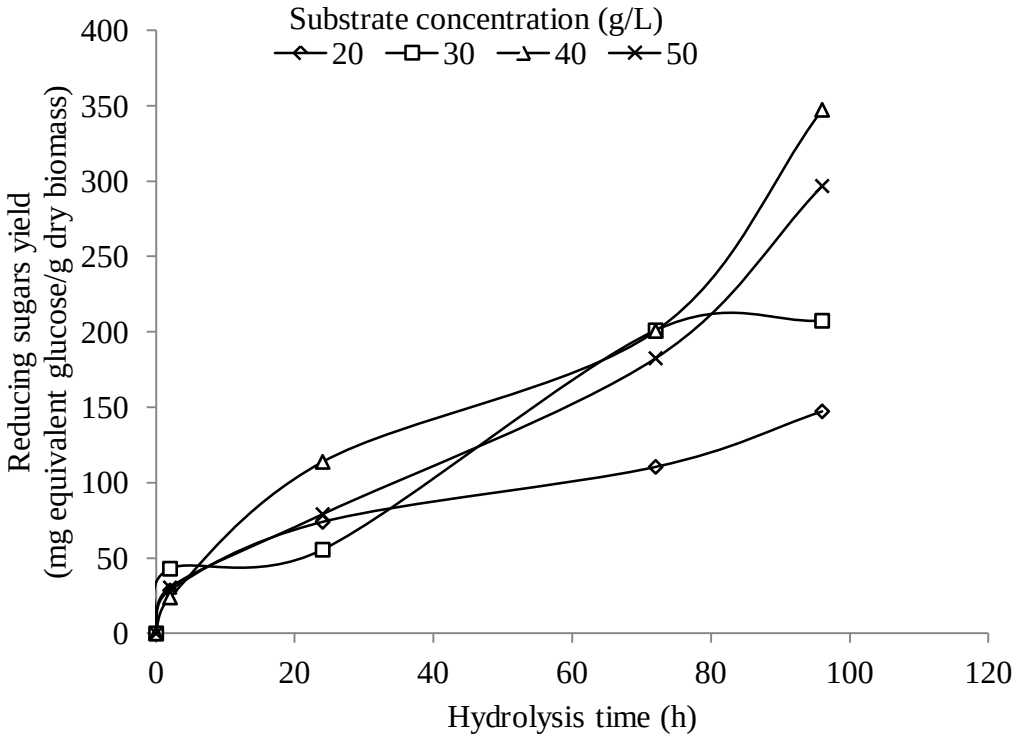


Figure 5.35 – 4-d Effect of time and substrate concentration on sugars yield with no supplemental β-glucosidase. Pretreatment conditions: 150 ^{o}C, 1% H_2O_2, 10 bar, 45 min. Enzyme hydrolysis conditions: 20 g/L (25 FPU cellulase, 15 IU β-glucosidase), 30 g/L (37.5 FPU cellulose, 22.5 IU β-glucosidase), 40 g/L (50 FPU cellulose, 30 IU β-glucosidase), 50 g/L (56.3 FPU/g dry biomass, 37.5 IU β-glucosidase/g dry biomass). 45 ^{o}C hydrolysis temperature, pH 4.8.

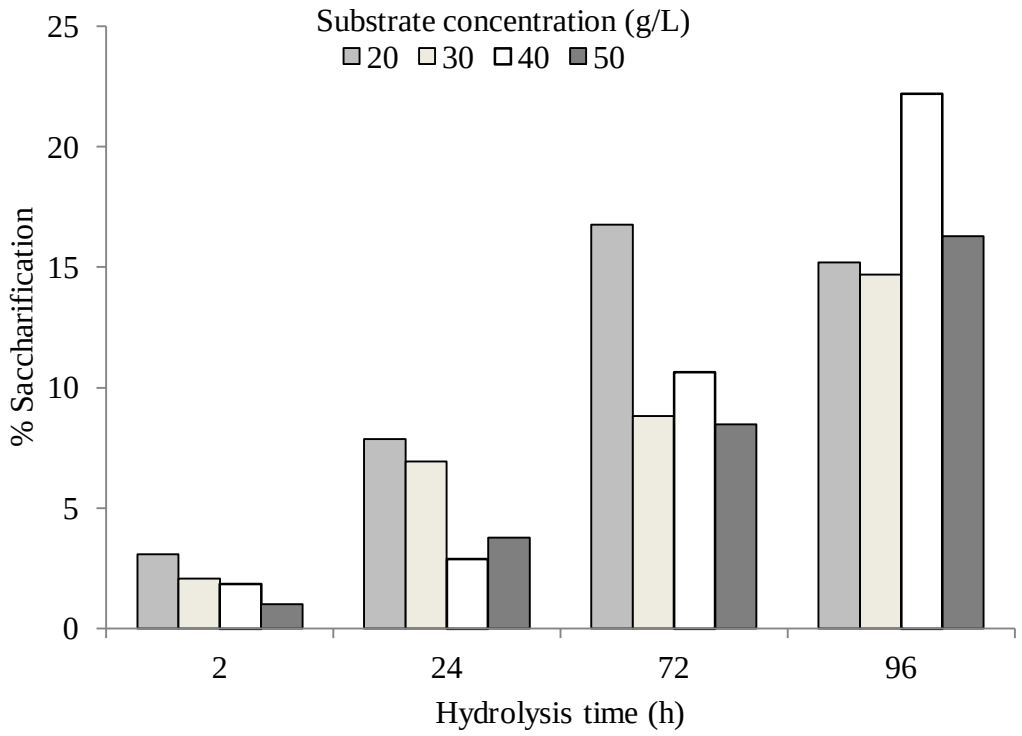


Figure 5.36 – 4-d Effect of substrate concentration on biomass conversion (% Saccharification) with supplemental β-glucosidase. Pretreatment conditions: 150 ^{o}C, 1% H_2O_2, 10 bar, 45 min. Enzyme hydrolysis conditions: 20 g/L (25 FPU cellulase, 15 IU β-glucosidase), 30 g/L (37.5 FPU cellulose, 22.5 IU β-glucosidase), 40 g/L (50 FPU cellulose, 30 IU β-glucosidase), 50 g/L (56.3 FPU/g dry biomass, 37.5 IU β-glucosidase/g dry biomass). 45 ^{o}C hydrolysis temperature, pH 4.8.

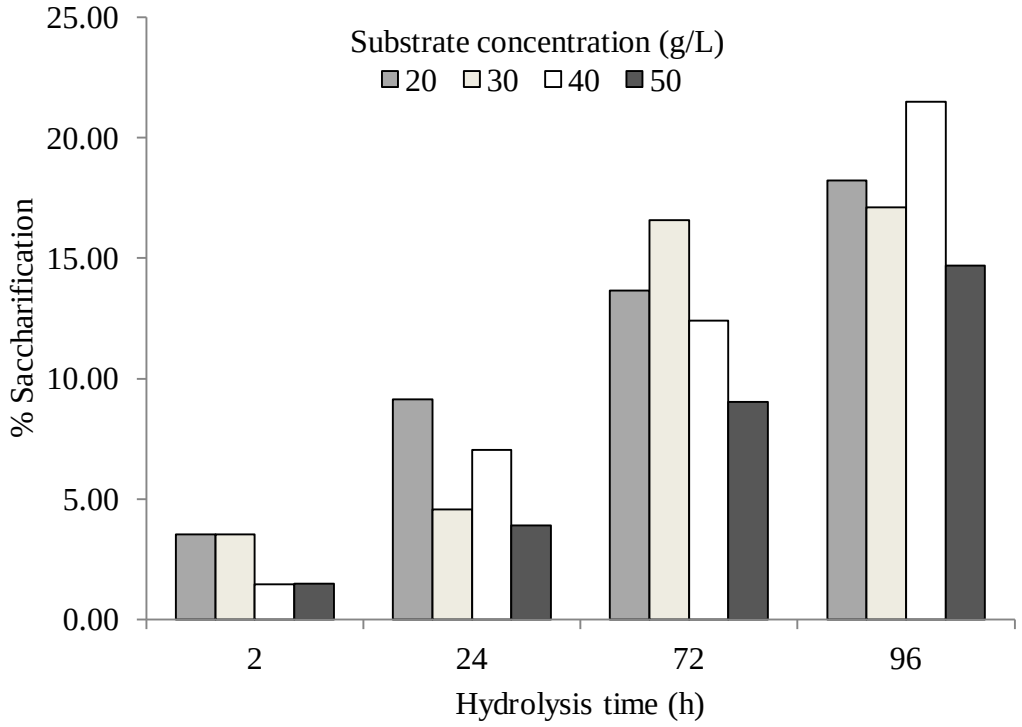


Figure 5.37 – 4-d Effect of substrate concentration on biomass conversion (% Saccharification) with no supplemental β-glucosidase. Pretreatment conditions: 150 °C, 1% H_2O_2, 10 bar, 45 min. Enzyme hydrolysis conditions: 2.0 g/L (2.5 FPU cellulase, 1.5 IU β-glucosidase), 30 g/L (37.5 FPU cellulose, 22.5 IU β-glucosidase), 40 g/L (50 FPU cellulose, 30 IU β-glucosidase), 50 g/L (56.3 FPU/g dry biomass, 37.5 IU β-glucosidase/g dry biomass). 45 °C hydrolysis temperature, pH 4.8.

The optimum temperature and pH is not only a function of the raw material and the enzyme source, but is also highly dependent on the hydrolysis time (Martín *et al*., 1988; Krishna *et al*., 1997). Enzymatic hydrolysis at 45 ^{o}C also favoured greater yields of reducing sugars up to 96 h and probably more days compared to hydrolysis at 50 ^{o}C and 72 h. Reducing sugars yields and % Saccharification were much more lower for the 120 ^{o}C, 1%H_2O_2, 30 min pretreatment (Table 4.12). However, reducing sugars yield increased without β-glucosidase supplement during the enzymatic hydrolysis (Figures 5.38 and 5.39).

5.9 Enzyme loading studies

It is important to determine whether there are significant yield benefits from loadings higher than 25 FPU/g dry biomass or whether cellulose loadings less than 25 FPU/g dry biomass are sufficient. Sugar yields with lower enzyme loadings (1, 3, 5, 7, 10 and 15 FPU/g dry biomass) exist in literature (Kaar and Holtzapple, 2000; Chang *et al*., 2001). After lime pretreatments of corn stover, Kaar and Holtzapple (2000) concluded that with 10 FPU/g dry biomass enzyme loading at 40 ^{o}C incubation temperature and 100 h period, the optimal reducing sugar yield was about 610 mg equivalent glucose/g dry biomass. Palonen *et al*., (2004) investigated the wet air oxidation pretreated (at 200 ^{o}C) softwood enzymatic hydrolysis to reducing sugar using two cellulase mixtures (Celluclast and Multifect) with loadings corresponding to 5, 10, 30 FPU/g dry biomass. They concluded that using the highest enzyme load of 30 FPU/g dry biomass, under hydrolysis conditions of 40 ^{o}C, 20 g/L substrate concentration, and 24 h, maximum sugar yield of 257 mg/g dry biomass (55% conversion of polysaccharide) was achieved. The low sugar yield was partly caused by the low lignin removal during pretreatment (between 24–42 % of lignin in the softwood was removed). The enzyme loading results are illustrated in Figure 5.40 (150 ^{o}C, 1%H_2O_2, 10 bar air pressure, and 45 min optimized conditions) and in Figure 5.41 (120 ^{o}C 1%H_2O_2, and 30 min conditions); these figures contain some important features. First and foremost, higher reducing sugars yields were obtained at higher enzyme loadings (for example 25 FPU to 50 FPU/g dry biomass) in 4-d hydrolysis time.

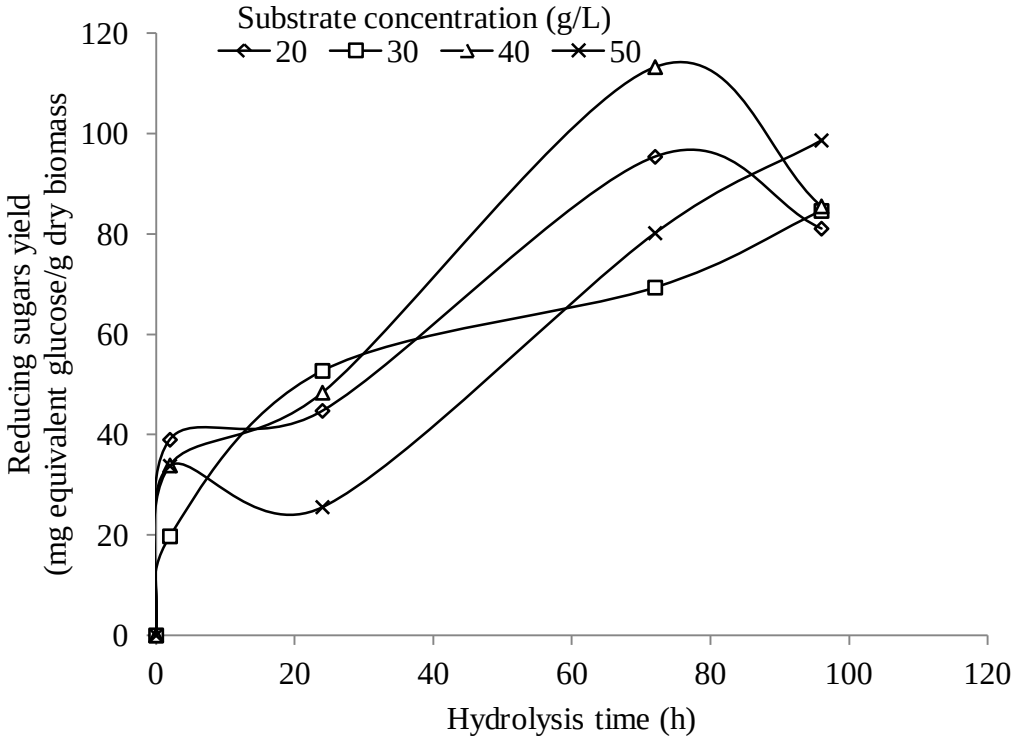


Figure 5.38 – 4-d Effect of time and substrate concentration on sugars yield with supplemental β-glucosidase. Pretreatment conditions: 120 ^{o}C, 1% H_2O_2, and 30 min. Enzyme hydrolysis conditions: 20 g/L (25 FPU cellulase, 15 IU β-glucosidase), 30 g/L (37.5 FPU cellulose, 22.5 IU β-glucosidase), 40 g/L (50 FPU cellulose, 30 IU β-glucosidase), 50 g/L (56.3 FPU/g dry biomass, 37.5 IU β-glucosidase/g dry biomass). 45 ^{o}C hydrolysis temperature, pH 4.8.

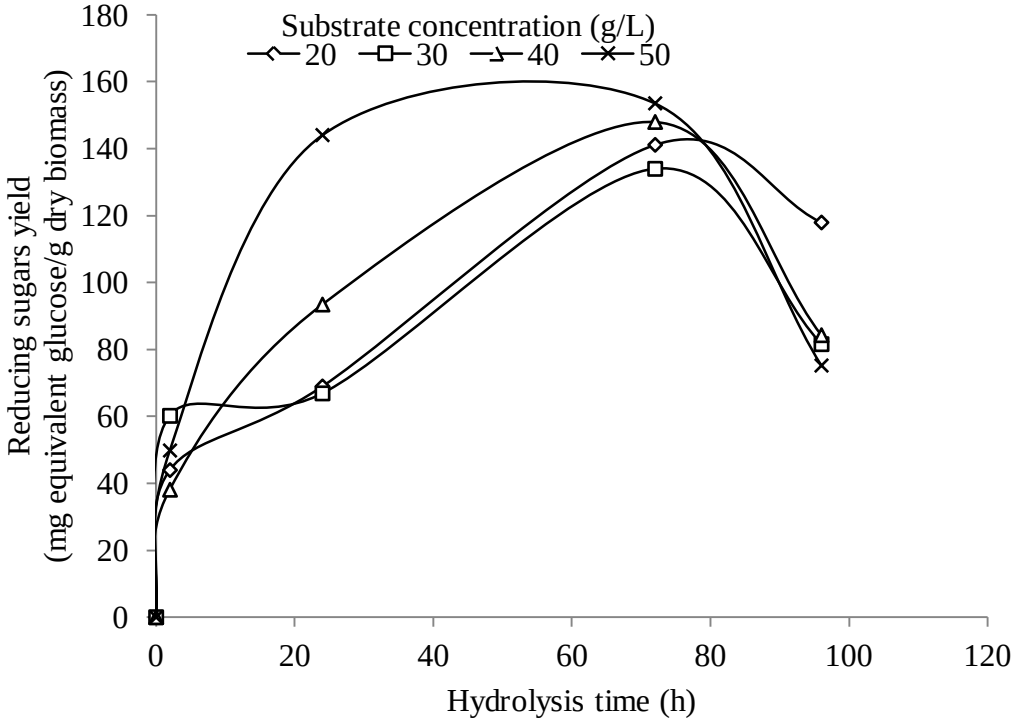


Figure 5.39 – 4-d Effect of time and substrate concentration on sugars yield with no supplemental β-glucosidase. Pretreatment conditions: 120 °C, 1% H_2O_2, and 30 min. Enzyme hydrolysis conditions: 20 g/L (25 FPU cellulase, 15 IU β-glucosidase), 30 g/L (37.5 FPU cellulose, 22.5 IU β-glucosidase), 40 g/L (50 FPU cellulose, 30 IU β-glucosidase), 50 g/L (56.3 FPU/g dry biomass, 37.5 IU β-glucosidase/g dry biomass). 45 °C hydrolysis temperature, pH 4.8.

The Figures (5.34 – 5.37) showed that 25 FPU/g dry biomass loading should be appropriate for the two pretreatment conditions considered. The 4-d reducing sugars yield at 50 FPU/g dry biomass for the 150 ^{o}C, 1%H_2O_2, 10 bar air pressure, and 30 min pretreatment condition was 365.62 mg equivalent glucose/g dry biomass while at 25 FPU/g dry biomass the yield was 335.35 mg equivalent glucose/g dry biomass. This was a difference of 8.3%, which can be considered minimal considering the economics of the process. For the 120 ^{o}C 1%H_2O_2, and 30 min pretreatment conditions, following the same trend, sugar yield at 25 FPU/g dry biomass was 11.4% less than sugar yield at 50 FPU/g dry biomass enzyme loading. It follows therefore that beyond 25 FPU/g dry biomass enzyme loading, the enzymatic hydrolysis becomes uneconomical as reducing sugars yields virtually did not change significantly. Cellulase loadings greater than 25 FPU/g dry biomass may have caused the cellulose sites to be saturated by the enzymes. Therefore, a cellulase loading of 25 FPU/g dry biomass is sufficient from a practical viewpoint because it represents the "shoulder" of the curve.

Also, we can conclude from these results that at high temperature of 150 ^{o}C 1%H_2O_2, longer time of 45 min, and 10 bar air pressure addition and at 25 FPU/g dry biomass enzymatic loading, reducing sugars yield (335.35 mg equivalent glucose/g dry biomass) increased than at 120 ^{o}C, 1%H_2O_2, and short time duration of 30 min (164.88 mg equivalent glucose/g dry biomass). Therefore, higher temperature, air pressure addition, and 1% H_2O_2 during pretreatment increased the reducing sugars yield. Compared to published works on enzymatic hydrolysis of woody materials, the optimal enzyme loading under the specified conditions in this study was higher (25 FPU/ g dry biomass) than some of the values in literature (Kaar and Holtzapple, 2000; Chang *et al*., 2001. On the other hand, the performance at 150 ^{o}C, 1% H_2O_2, 10 bar, and 45 min pretreatment and subsequent enzymatic hydrolysis was higher than that reported by Palonen *et al*., (2004). The raw materials used and operating conditions in these studies must have contributed to these discrepancies.

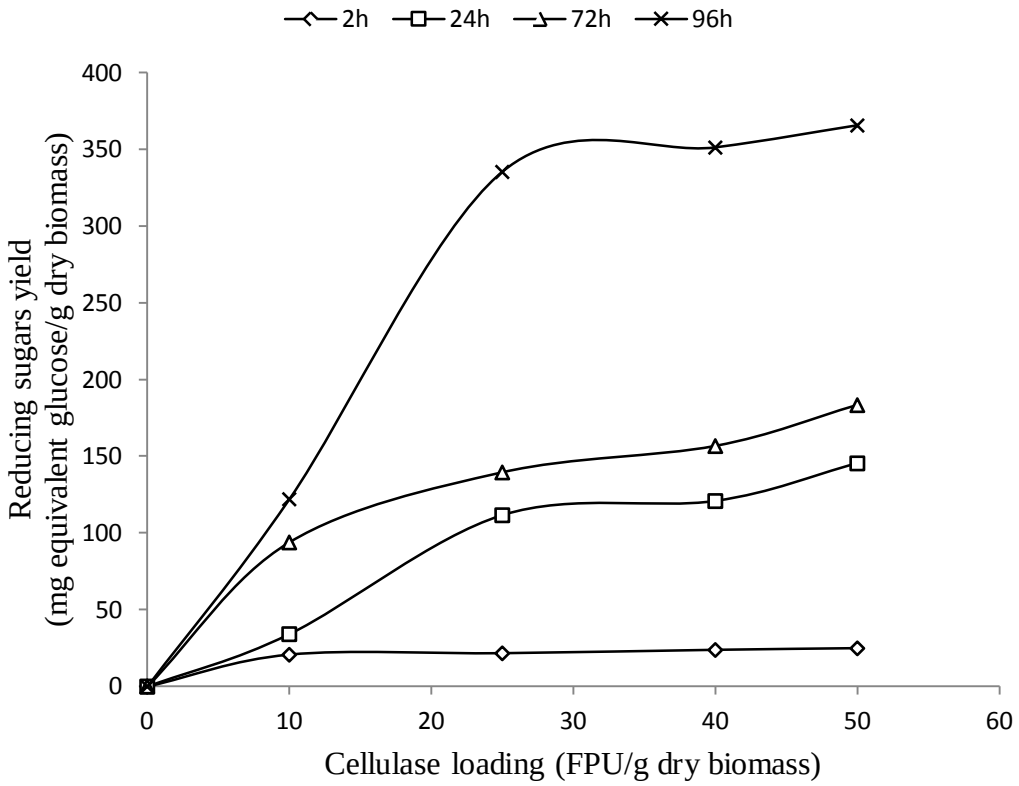


Figure 5.40 – 4-d Effect of enzyme loading on sugar yields. Pretreatment conditions: 150 ^{o}C, 1% H_2O_2, 10 bar, 45 min. Enzyme hydrolysis conditions: 5 UI β-glucosidase/g dry biomass, 45 ^{o}C hydrolysis temperature, pH 4.8, 40 g/L substrate concentration.

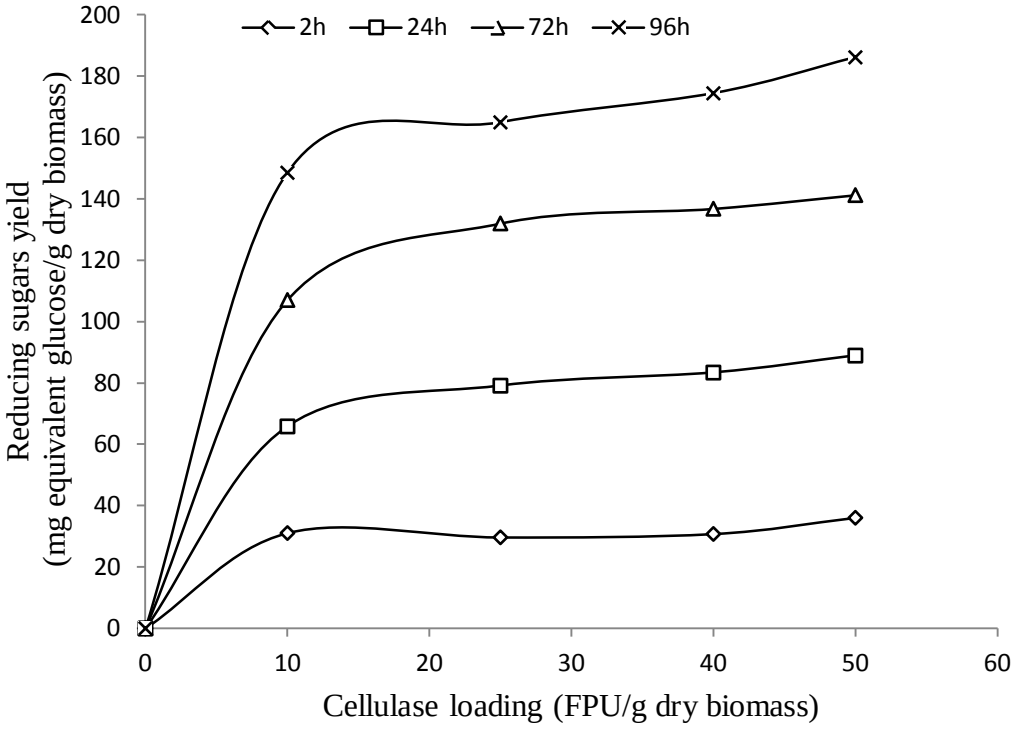


Figure 5.41 – 4-d Effect of enzyme loading on sugar yields. Pretreatment conditions: 120 ^{o}C, 1% H_2O_2, and 30 min. Enzyme hydrolysis conditions: 5 UI β-glucosidase/g dry biomass, 45 ^{o}C hydrolysis temperature, pH 4.8, 40 g/L substrate concentration.

5.10 Hydrolysis studies of untreated and washed only biomass

It can be noted from Figure 5.42 that it was necessary to treat the raw material before enzymatic saccharification. Pretreatment is said to cause a disruption in the lignocellulosic matrix thereby making the enzymes more accessible to substrates. In this study, sugar yields of the pretreated sawdust were significantly higher than untreated sawdust. Figure 5.42 shows treated biomass reducing sugars concentration of 358.45 mg equivalent glucose/g dry biomass to untreated material of 17.73 mg equivalent glucose/g dry biomass. This is a 20-fold increase in reducing sugars produced from the treated to the untreated biomass. The maximum amount of reducing sugar yield in this study under the conditions specified was 42.4% lower than that reported for poplar wood (Chang *et al*., 2001). The large difference in value may have been caused by raw material composition, pretreatment conditions, efficiency of the different enzymes used, enzyme concentration, and reaction period.

Enzymatic digestibility of shea tree sawdust was boosted by pretreatment. Oxidative lime pretreated sawdust enzyme hydrolysis was also enhanced with high pretreatment temperature (150 ^{o}C) and a combination of air and hydrogen peroxide addition. Higher temperature was more favourable because of more delignification, which resulted in more extensive enzymatic hydrolysis. However, around 170 ^{o}C and above polysaccharide degradation began to occur. 4-d enzymatic hydrolysis period with lower hydrolysis temperature (45 ^{o}C) produced higher sugar yields than 3-d at 50 ^{o}C hydrolysis temperature. The digestibility of the oxidative lime pretreated biomass depended on the cellulase as well as a little of β-glucosidase loadings. Moderate cellulase loading (25 FPU/g dry biomass) and withou t β-glucosidase supplements gave comparable sugar yields to cellulase loading with β-glucosidase supplements. 40 g/L substrate concentration was considered as optimum substrate loading. Below this substrate loading, sugar yields decreased and above it the sugar yields also decreased.

5.11 Simultaneous saccharification and fermentation

After 96 h fermentation, the quantity of ethanol obtained (g/L) from each of pretreatment conditions at 2% effective cellulose loading was 9.71 g/L for pretreatment at 150^{o}C, 1%H_2O_2, 10 bar air pressure, and 45 min. Pretreatment at 120 ^{o}C, 1%H_2O_2, 30 min produced 7.35 g/L. At increased effective cellulose loading of 3%, ethanol obtained did not significantly increase for the two pretreatment conditions. The percent theoretical ethanol yields (based on cellulose conversion) for the two pretreatment conditions were higher at 2% effective cellulose loading than at 3% effective cellulose loading.

Ethanol concentration tended to be higher at 3% substrate loading, but at this loading, the % theoretical ethanol yields were much lower than that of 2% substrate loading. This implies that more of the cellulose was converted at 2% loading than at 3% substrate loading. The high ethanol yield at 3% loading showed that more reducing sugar was produced by enzymatic hydrolysis and were probably quickly assimilated by yeast for cell growth and ethanol production than at 2% loading.

Cellulose conversion at 2% substrate loading should be more appropriate for the SSF under the conditions considered. However, more of the substrate will be needed for the fermentation process.

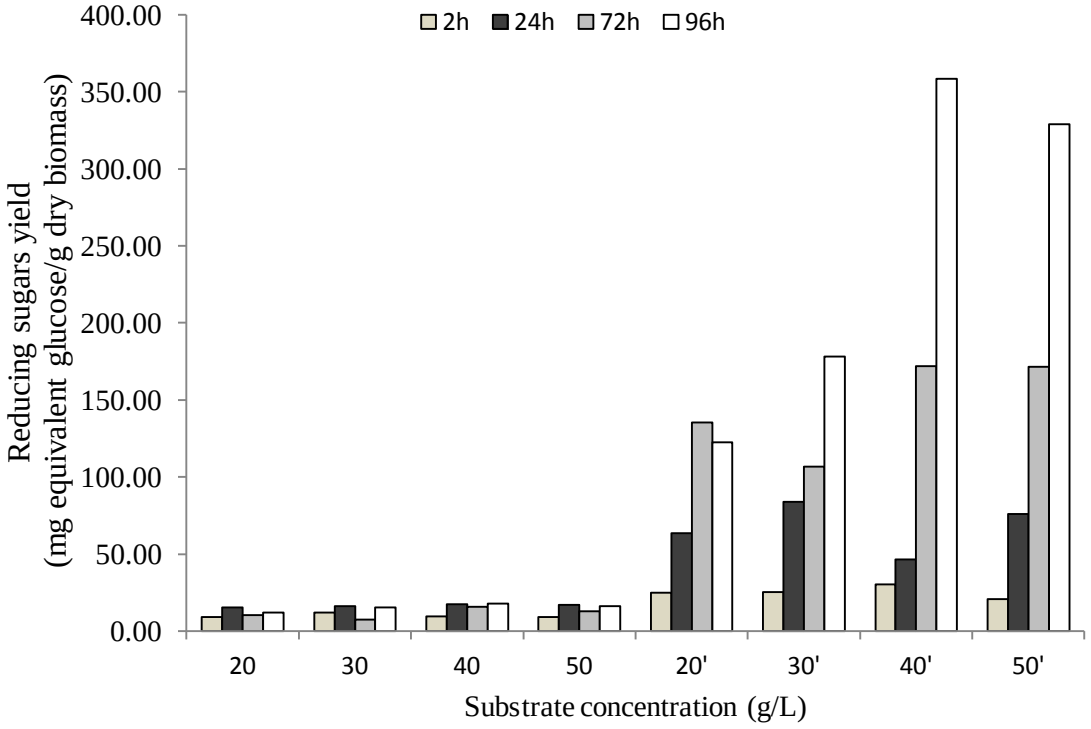


Figure 5.42 – 4-d Effect of time and substrate concentration on sugars yield for untreated and treated biomass. Concentrations in prime notation indicate enzymatic hydrolysis of pretreated samples. Pretreatment conditions: 150 ^{o}C, 1% H_2O_2, 10 bar, 45 min. Enzyme hydrolysis conditions: 20 g/L (25 FPU cellulase, 15 IU β-glucosidase), 30 g/L (37.5 FPU cellulose, 22.5 IU β-glucosidase), 40 g/L (50 FPU cellulose, 30 IU β-glucosidase), 50 g/L (56.3 FPU/g dry biomass, 37.5 IU β-glucosidase/g dry biomass). 45 ^{o}C hydrolysis temperature, pH 4.8.

CHAPTER SIX

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The effects of short-term oxidative lime pretreatment of sawdust (a wood residue) were investigated in this study. The pretreatments were evaluated using design of experiments (DOE) method. The step-by-step studies showed that time, temperature, and the combination of air and hydrogen peroxide as oxidizing agents had great impact on enzymatic saccharification.

Generally, the pretreatments showed more of hemicellulose degradation (solubilization), and less of lignin removal. However, cellulose was most preserved in the solid fractions. The pretreatment yield of lignin was 27.34 g lignin remaining/100 g lignin in raw biomass (30.79% lignin removal), and was accomplished for 170 ^{o}C, 10 bar air pressure, 1%H_2O_2, and 10 min. The corresponding pretreatment yield of cellulose was 59.34 g cellulose/100 g cellulose in raw biomass, and pretreatment yield of hemicellulose was 8.01 g hemicellulose remaining/100 g hemicellulose in raw biomass (70.15% hemicellulose solubilization). Pretreatment yields for the central composite design of experiment at 150 ^{o}C, 10 bar air pressure, 1%H_2O_2, 45 min were comparable to the higher temperature condition (170 ^{o}C). The pretreatment yield of lignin under this condition was 28.54 lignin remaining/100g lignin in raw biomass (23.85% lignin removal). Also, pretreatment yield of cellulose was 59.17 g cellulose/100g cellulose in raw biomass and pretreatment yield of hemicellulose was 7.28 g hemicellulose remaining /100g hemicelluloses in raw biomass (71.40% hemicellulose solubilization).

Lime pretreatment showed that 0.1 g $Ca(OH)_2$/g dry biomass loading was sufficient to cause appreciable delignification.

The 4-d enzymatic digestibility (saccharification) assessed with enzyme loadings (25, 37.5, 50, 56.3 FPU/mL), substrate concentrations (20, 30, 40, 50 g/L), enzymatic hydrolysis temperature of 45 ^{o}C on the treated materials showed that pretreatment conditions 150 ^{o}C, 1%H_2O_2, 10 bar air pressure, and 45 min as the most effective with the overall reducing sugars yield of 358.45 mg equivalent glucose/g dry biomass

compared to untreated wood residue of 17.73 mg equivalent glucose/g dry biomass. This was a 12-fold increase from the untreated to the treated material. This showed pretreatment step as an important part of the fuel ethanol production process. Simultaneous saccharification and fermentation method showed the woody biomass convertibility to ethanol.

Material balance studies on the pretreated solid fractions showed that lime pretreatment at high temperatures such as greater than 170 ^{o}C caused degradation of the polysaccharide. Also, pretreatments investigated below 120 ^{o}C without air addition did not cause appreciable disruption to the lignocellulosic complex.

6.2 Recommendations

As a result of these investigations, the short-term lime pretreatment on the wood residue showed promising effects, but it is also important to include in future studies the mass balances of the polysaccharides degraded/or solubilized in the pretreatment liquid fraction and to study the total reducing sugar yield of both the solid and liquid fractions; if the overall yield will be higher.

In addition, the following studies can be performed:

1. Operate the alkaline peroxide assisted wet air oxidation (APAWAO) pretreatments between 120 ^{o}C to 170 ^{o}C and time duration of between 20 min to 50 min. Optimum conditions for pretreatment can then be established.
2. Evaluate the enzymatic digestibility at 25 FPU/g dry biomass loading, varying the substrate concentrations with and without β-glucosidase supplements.
3. Separate enzymatic hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) methods can be investigated separately at 2% pretreated biomass loading and results compared. Conditions can be defined for hydrolysis and fermentation methods to realized high yields of ethanol.
4. Herbaceous lignocellulosic biomass can also be used as feedstock for pretreatment. The efficiency of this process can be compared to that of the woody biomass.

REFERENCES

Ademark, P., Varga, A., Medve, J., Harjunpaa, V., Drakenberg, T., Tjerneld, F. & Stalbrand, H. (1998). Softwood hemicellulose-degrading enzymes from Aspergillus niger: purification and properties of a beta-mannanase. *Journal of Biotechnology, 63,* 199–210.

Adney, B., & Baker, J. (2008). Measurement of Cellulase Activities. Laboratory analytical procedure (LAP). Golden, CO: National Renewable Energy Laboratory; 2008. NREL Report No.: TP-510-42628. Contract No.: DE-AC36-99-G010337. Sponsored by the U.S. Department of Energy.

Agnemo, R., & Gellerstedt, G. (1979). The reactions of lignin with alkaline hydrogen peroxide. Part II. Factors influencing the decomposition of phenolic structures. *Acta Chemical Scandinavica, B33,* 337–342.

Ahring, B.K., Licht, D., Schmidt, A.S., Sommer, P., & Thomsen, A.B. (1999). Production of ethanol from wet oxidized wheat straw by *Thermoanaerobacter mathranii*. *Bioresource Technology, 68*(1), 3–9.

Bailey, C.W., & Dence, C.W. (1969). Reactions of alkaline hydrogen peroxide with softwood lignin model compounds, spruce milled-groundwood lignin and spruce groundwood. *Tappi Journal, 52*(3), 491–500.

Bailey, J.E. & Ollis, D.F. (1986). *Biochemical Engineering Fundamentals* (2nd ed.). p. 40. McGraw Hill.

Bailey, B.K. (1996). Handbook on Bioethanol. In: C. E. Wyman (Ed.), *Production and Utilization* (pp. 37–60). Washington: Taylor and Francis.

Banerjee. S., Sen, R., Pandey, R.A., Chakrabarti, T., Satpute, D., Giri, B.S., Mudliar, S.N. (2009). Evaluation of wet air oxidation as a pretreatment strategy for bioethanol

production from rice husk and process optimization. *Biomass Bioenergy*, *33*(12), 1680–1686.

Bennet, C. (1971). Spectrophotometric acid dichromate method for the determination of ethyl alcohol. *American Journal of Medical Technology, 37,* 217–220.

Bisaria, V.S. & Ghose, T.K. (1981). Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. *Enzyme Microbiology Technology 1981, 3*(2), 90–104.

Bjerre, A.B., Olesen, A.B., & Fernqvist, T. (1996). Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Biotechnology Bioengineering, 49,* 568–577.

Blasi, C.D., Signorelli, G., Di Russo, C., & Rea, G. (1999). Product distribution from pyrolysis of wood and agricultural residues. *Industrial Engineering and Chemistry Research*, *38*(6), 2216–2224.

Blasig, J.D., Holtzapple, M.T., Dale, B.E., Engler, C.R., & Byers, F.M. (1992). Volatile fatty acid fermentation of ammonia fiber explosion (AFEX)-treated bagasse and newspaper by rumen microorganisms. *Resources Conservation and Recycling, 7*(1–4), 95–114.

Cara, C., Ruiz, E., Ballesteros, I., Negro, M.J., & Castro, E. (2006). Enhanced enzymatic hydrolysis of olive tree wood by steam explosion and alkaline peroxide delignification. *Process Biochemistry, 41*(2), 423–429.

Cecelski, B., Dunkerley, J., & Ramsay, W. (1979). Household energy and the poor in the third world. Resources for the future. Washington: RFF press.

Chacha, N., Toven, K., Mtui, G., Katima, J., & Mrema, G. (2011). Steam pretreatment of pine (*pinus patula*) wood residue for the production of reducing sugars. *Cellulose Chemical Technology, 45*(7-8), 495–501.

Chang, V.S., Burr, B., Holtzapple, M.T. (1997). Lime pretreatment of switchgrass. *Applied Biochemistry and Biotechnology, 63-65*(1), 3–19.

Chang, V.S., Nagwani, M., & Holtzapple, M.T. (1998). Lime pretreatment of crop residues bagasse and wheat straw. *Applied Biochemistry and Biotechnology, 74*, 135–159.

Chang, V.S. (1999). *Lime pretreatment of lignocellulosic biomass* (Ph.D. dissertation, Texas A&M University, College Station, Texas, U.S.A).

Chang, V.S., Holtzapple, M.T. (2000). Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry Biotechnology, 84–86*(1-9), 5–37.

Chang, V.S., Nagwani, M., Kim, C., & Holtzapple, M.T. (2001). Oxidative lime pretreatment of high-lignin biomass. *Applied Biochemistry and Biotechnology, 94*, 1–28.

Chen, Y., Shaoma–Shivappa, R.R., Keshwani, D., & Chen, C. (2007). Potential of agricultural residues and hay for bioethanol production. *Applied Biochemistry and Biotechnology, 142*, 276–290.

Chosdu, R., Hilmy, N., Erlinda, T.B., & Abbas, B. (1993). Radiation and chemical pretreatment of cellulosic waste. *Radiation Physical Chemistry, 42*, 695–698.

Chum, H.L., Johnson, D.K., Black, S.K., & Overend, R.P. (1990). Pretreatment-catalyst effects and the combined severity parameter. *Applied Biochemistry and Biotechnology, 24-25*(1), 1–14.

CIA world factbook. (2005). cited 2010 Aug., 28. Retrieved from http://www.nationmaster.com/graph/ene.

Converse, A.O., Ooshima, H., & Burns, D. (1990). Kinetics of enzymatic hydrolysis of lignocellulosic materials based on surface area of cellulose accessible to enzyme and enzyme adsorption on lignin and cellulose. *Applied Biochemistry and Biotechnology, 24-25*(1), 67−73.

Debellefontaine, H., Chakchouk, M., Foussard, J.N., Tissot, D., & Striolo, P. (1996). Treatment of organic aqeous wastes. Wet air oxidation and wet peroxide oxidation. *Environmental Pollution, 92*(2), 155–164.

DeGroot, B.D., van Dam J.E.G., & van't Riet K. (1995). Alkaline pulping of hemp woody core: kinetic modeling of lignin, xylan, and cellulose extraction and degradation. *Holzforschung*, *49*(4), 332–342.

Dolk, M., Yan, J.F., & McCarthy, J.L. (1989). Lignin 25. Kinetics of delignification of western Hemlock in flow-through reactors under alkaline conditions. *Holzforschung*, *43*(2), 91–98.

Ehrman, T. (1994). Standard test methods for moisture, total solids, and total dissolved solids in biomass slurry and liquid process samples. LAP 012. Golden, CO: National Renewable Energy Laboratory.

Eriksson, Ö., Goring, D.A.I., & Lindgren, B.O. (1980). Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. *Wood Science and Technology, 14*, 267–279.

Fan, L.T., Gharpuray, M.M., & Lee, Y-H. (1987). *Cellulose Hydrolysis* (p. 57). Berlin: Springer-Verlang.

Food and Agricultural Organisation (1992). Corporate document repository. Forest resource situation assessment of Nigeria, produced by the Forestry department, adapted from World bank. Federal Republic of Nigeria: Forestry sector review. Confidential Report No.: 10744-UNI, cited 2012 Mar. 30. Retrieved from http//:www.fao.org/docrep/004/ab578e/AB57E06/html.

Food and Agricultural Organisation (2010). A potential renewable energy development and utilization of biomass energy: cited 2010 Aug. 30. Retrieved from http://www.fao.org/docrep/t4470E/t4470eOn.html.

Forney, L.J., Reddy, C.A., Tien, M., & Anst, S.D. (1982). The involvement of hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white rot fungus *phanerochaete chrysosporium*. *Journal of Biology and Chemistry, 257*(19), 11455–11462.

Ghose, T.K. (1987). Measurement of Cellulase Activities. *Pure and Applied Chemistry, 59*, 257–268.

Gierer, J., & Norén, I. (1980). On the course of delignification during kraft pulping. *Holzforschung*, *34*(6), 197–200.

Gierer, J. (1997). Formation and Involvement of superoxide (O_2/HO_2) and hydroxyl (HO) radicals in TCF bleaching processes: A review. *Holzforschung, 51*, 34.

Gossett, J.M., Stuckey, D.C., Owen, W.F., & McCarty, P.L. (1982). Heat treatment and anaerobic digestion of refuse. *Journal of the Environmental Engineering Division, 108*(3), 437–454.

Gould, J.M. (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. *Biotechnology and Bioengineering*, *26*, 46–52.

Gould, J.M. (1985). Studies on the mechanism of alkaline peroxide delignification of agricultural residues. *Biotechnology and Bioengineering, 27,* 225–231.

Granda, C.B. (2004). *Sugarcane juice extraction and preservation, and long-term lime pretreatment of bagasse* (Ph.D. dissertation, Texas A&M University, College Station, Texas, U.S.A.).

Gratzl, J.S. (1992). The chemical principles of pulp bleaching with oxygen, hydrogen peroxide and ozone – a short review. *Papier, 46*(10), 1–8.

Gregg, D., Saddler, J.N. (1996). A techno-economic assessment of the pretreatment and fractionation steps of a biomass-to-ethanol process. *Applied Biochemistry and Biotechnology, 57-58*(1), 711–727.

Hahn-Hägerdal, B. (1996). Ethanolic fermentation of lignocellulose hydrolysates: a minireview. *Applied Biochemistry and Biotechnology, 57–58*(1), 195–199.

Harmsen, P.F.H., Huijgen, W.J.J, López Bermúdez, L.M., & Bakker, R.R.C. (2010). *Literature review of physical and chemical pretreatment processes for lignocellulosic biomass.* ECN: Energy research centre of the Netherlands. Food and biobased research, 1–49.

Hausman, M. (1999). *A mechanistic study of the degradation of lignin model compounds with oxygen species* (Ph.D. thesis. University of Maine, Maine).

Hendricks, A.T.W.M., & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology, 100*, 10–18.

Hileman, B. (1999). Case grows for climate change. *Chemical and Engineering News, 77*, 16-23.

Holtzapple, M., Cognata, M., Shu, Y., & Hendrickson, C. (1990). Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. *Biotechnology and Bioengineering, 36*(3), 275–287.

Holtzapple, M.T., Jun, J-H., Ashok, G., Patibandla, S.L., & Dale, B.E. (1991). The ammonia freeze explosion (AFEX) process. A practical lignocellulose pretreatment. *Applied Biochemistry and Biotechnology, 28–29*(1), 59–74.

Holtzapple, M.T., Ross, M.K., Chang, N.S., Chang, V.S., Adelson, S.K, & Brazel, C. (1997). Biomass conversion to mixed alcohol fuels using the MixAlco process. In B.C. Saha BC & J. Woodward (Eds.), *Fuels and Chemicals from Biomass* (pp. 130–142). Washington, U.S.A.: American Chemical Society.

Holtzapple, M.T., Davison R.R., Kaar, W., Chang, N.S., & Loescher, M.E. (1999). Biomass conversion to mixed alcohol fuels using MixAlco process. *Applied Biochemistry and Biotechnology, 79*(1-3), 609–631.

Huang, X., & Penner, M.H. (1991). Apparent substrate inhibition of the *Trichoderma reesei* cellulase system. *Journal of Agriculture and Food Chemistry, 39,* 2096–2100.

Hui, L., Nag-Jong, K., Min, J., Jong Won, K., & Ho, N.C. (2009). Simultaneous saccharification and fermentation of lignocellulosic residues pretreated with phosphoric acid-acetone for bio-ethanol production. *Bioresource Technology, 100*, 3245–3251.

Ingram, L.O., & Doran, J.B. (1995). Conversion of cellulosic materials to ethanol. *FEMS Microbiology Reviews, 16,* 235–241.

Ishizawa, C.I., Jeoh, T., Adney, W.S., Himmel, M.E., Johnson, D.K., & Davis, M.F. Can delignification decrease cellulose digestibility in acid pretreated corn stover. *Cellulose, 16,* 677–696.

Itoh, H., Wada, M., Honda, Y., Kuwahara, M., & Watanabe, T. (2003). Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. *Journal of Biotechnology, 103,* 273–280.

Iyer, P.V., Wu, Z-W., Kim, S.B., Lee, Y.Y. (1996). Ammonia recycled percolation process for pretreatment of herbaceous biomass. *Applied Biochemistry and Biotechnology, 57-58,* 121–132.

Johansson, E., & Ljunggen, S. (1994). The kinetics of Lignin reaction during oxygen delignification, Part 4. The reactivates of different lignin model compounds and the influence of metal ions on the rate of degradation. *Journal of Wood Chemistry and Technology, 14*(4), 507–525.

Johansson, L.J., Palmqvist, E., Nilvebrant, N-O., & Hahn-Hagerdal, B. (1998). Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. *Applied Microbiology and Biotechnology, 49,* 691–697.

Jorgensen, H., Kristensen, J.B., & Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities. *Biofuels Bioproduct Biorefining, 1*, 119–134.

Kaar, W.E., & Holtzapple, M.T. (2000). Using lime pretreatment to facilitate the enzyme hydrolysis of corn stover. *Biomass and Bioenergy, 18,* 189–199.

Kheshgi, H.S., Prince, R.C., Marland, G. (2000). The potential of biomass fuels in the context of global climate change: focus on transportation fuels. *Annual Review of Energy and the Environment, 25*, 199–244.

Kim, S.H. (2004). *Lime pretreatment and enzymatic hydrolysis of corn stover*. (Ph.D. dissertation, Texas A&M University, College Station, Texas).

Kim, S., & Holtzapple, M.T.(2005). Lime pretreatment and enzymatic hydrolysis of corn stover. *Bioresource Technology, 96*, 1994–2006.

Klemn, D., Heublein, B., Fink, H.P., & Bohn, A. (2005). Cellulose: fascinating biopolymer and sustainable raw material. *Angewandte Chemie International Edition, 44*(22), 3358–3393.

Klemn, D., Philipp, B., Heinze, T., Heinze, U., & Wagenknecht, W. (1998). *Comprehensive cellulose chemistry. Functionalization of cellulose* (vol. 2 ed.). Weinhein, Germany: Wiley-VCH.

Klinke, H.B., Ahring, B.K., Schmidt, A.S., & Thomsen, A,B. (2002). Characterisation of degradation products from alkaline wet oxidation of wheat straw. *Bioresource Technology, 82*(1), 15–26.

Klinke, H.B., Thomsen, A.B., & Ahring, B.K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Applied Microbiology and Biotechnology, 66*(1), 10–26.

Krishna, S.H., Prabhakar, Y., & Rao, R.J. (1997). Saccharification studies of lignocellulosic biomass from *Antigonum leptopus* linn. *Indian Journal of Pharmaceutical Sciences, 59*(1), 39–42.

Kryzanowski, T. (1998). From wood waste to ethanol fuel [updated 1998; cited 2006 Jan. 25]. Available from http://www.forestnet.com/.

Kuznetsov, B.N., Kuznetsova, S.A., Danilov, V.G., Kozlov, I.A., Taraban'ko, V.E., Ivanchenko, N.M., & Alexandrova, N.B. (2002). New catalytic processes for a sustainable chemistry of cellulose production from wood biomass. *Catalysis Today, 75*(1-4), 211–217.

Kuhad, R.C., Sharma, K.K., & Gupta, R,. (2009). Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. *Bioresource Technology, 100,* 1214–1220.

Lachenal, D., de Choudens, C., & Monzie, P. (1980). *Tappi Journal, 63*(4), 119–122.

Ladisch, M.R., Flickinger, M.C., & Tsao, G.T.(1979). Fuel and chemicals from biomass. *Energy, 4*(2), 263–275.

Larsson, S., Cassland, P., & Jonsson, L.J. (2001). Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocelluloses hydrolysates by heterogonous expression of laccase. *Applied Environmental Microbiology, 67*(3), 1163–1170.

Lawoko, M. (2005). *Lignin Polysaccharide Networks in Softwood and Chemical Pulps: characterization, structure, and reactivity* (Ph.D. thesis, Royal Institute of Technology, Stockholm).

Lee, J. (1996). Biological conversion of lignocellulosic biomass to ethanol. *Journal of Biotechnology, 56*(1), 1–24.

Li, S., Xu, S., Liu, S., Yang, C., & Lu, Q. (2004). Fast pyrolysis of biomass in free-fall reactor for hydrogen-rich gas. *Fuel Process Technology, 85*(8–10), 1201–1211.

Lin, L., Yan, R., Liu, Y., & Jiang, W. (2010). In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: cellulose, hemicellulose, and lignin. *Bioresource Technology, 101*(21), 8217–8223.

Loesche, M.E. (1996). *Volatile fatty acid fermentation of biomass and kinetic modeling using the CPDM method* (Ph.D. dissertation, Texas A&M University, College Station, Texas, U.S.A).

Mahro, B., Timm, M. (2007). Potential of bio-waste from the food industry as a biomass resource. *Engineering in Life Sciences, 7*(5), 457–468.

Martín, C., Negro, M.J., Alfonsel, M, & Sáez, R. (1988). Enzymatic hydrolysis of lignocellulosic biomass from *Onopordum nervosum*. *Biotechnology and Bioengineering, 32*, 341–344.

Martín, C., Klinke, H.B., & Thomsen, A.B. (2007). Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugar cane bagasse. *Enzyme and Microbiology Technology, 40*(3), 426–432.

McGinnis, G.D., Wilson, W.W., & Mullen, C.E. (1983). Biomass pretreated with water and high pressure oxygen. The wet oxidation process. *Industrial Engineering Chemistry Product Research and Development, 22*(2), 352–357.

Mes-Hartree, M., Hogan, C.M., & Saddler, J.N. (1980). Recycle of enzymes and substrate following enzymatic hydrolysis of steam pretreated aspen wood. *Biotechnology and Bioengineering, 30*, 558–564.

Mod, R.R., Ory, R.L., Morris, N.M., & Normand, F.L. (1981). Chemical properties and interactions of rice hemicellulose with trace minerals *in vitro*. *Journal of Agriculture and Food Chemistry, 29*, 449–454.

Momoh, M. (1997). Wood Conversion to Energy 1: The potentials and constraints of wood energy utilization in Africa. *Nigerian Journal of Renewable Energy, 5*(1–2), 142–142.

Mosier, N., Wyman, C., Dale, B., Elander, R., & Lee, Y.Y. (2005). Holtzapple M, Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*(6), 673–686.

Miller, G.L. (1959). *Analytical Chemistry, 31*(3), 426–428.

Millet, M.A., Baker, A.J., & Scatter, L.D. (1976). Physical and chemical pretreatment for enhancing cellulose saccharification. *Biotechnology and Bioengineering Symposium, 6*, 125–153.

Montgomery, D.C. (1991). *Design and analysis of experiments* (3rd ed.). New York: Wiley.

Neely, W.C. (1984). Factors affecting the pretreatment of biomass with gaseous ozone. *Biotechnology and Bioengineering, 26*(1), 59–65.

Ortega, N., Busto, M.D., & Perez-Mateos M. (2001). Kinetics of cellulose saccharification by *Trichoderma reesei* cellulases. *International Biodeterioration Biodegradation, 47*(1), 7–14.

Palonen, H., Thomsen, A.B., Tenkanen, M., Schmidt, A.S., & Viikari, L. (2004). Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. *Applied Biochemistry and Biotechnology, 117*(1), 1–17.

Panagiotou, G. & Olsson, L. (2007). Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. *Biotechnology and Bioengineering, 96*(2), 250–258.

Pettersen, R.C. (1984). The chemical composition of wood. In R.M. Rowell (ed.). *The chemistry of solid wood* (pp. 57–126). Washington, U.S.A.: American Chemical Society.

Qi, B.C., Aldrich, C., Lorenzen, L, & Wolfaardt, G.W. (2005). Acidogenic fermentation of lignocellulosic substrate with activated sludge. *Chemical Engineering Communication, 192*(9), 1221–1242.

Reczey, K., Szengyel, Zs., Eklund, R., & Zacchi, G. (1995). Cellulase production by *T. reesei*. *Bioresource Technology, 57*(1), 25–30.

Roig, A., Cayuela, M.L., & Sánchez-Monedero, M.A. (2006). An overview on olive mill wastes and their valorization methods. *Waste Management, 26*(9), 960–969.

Rubin, E.M. (2008). Genomics of cellulosic biofuels. *Nature, 454*(14), 841–845.

Saha, B.C. & Cotta, M.A. (2007). Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol. *Enzyme and Microbial Technology, 4*, 528–532.

Sánchez, O.J. & Cardona, C.A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology, 99*(13), 5270–5295.

Schmidt, A.S. & Thomsen, A.B. (1998). Optimization of wet oxidation pretreatment of wheat straw. *Bioresource Technology, 64*(2), 139–151.

Schmidt, A.S., Puls, J., & Bjerre. A.B. (1996). Comparison of wet oxidation and steaming for solubilization of the hemicellulose fraction in wheat straw and birchwood. In P. Chartier, G.L. Ferrero, U.M. Henius, S. Hultberg, J. Sachau, & M. Wiiblad (eds.). *Biomass for Energy and the Environment : Proceedings of the 9th European Bioenergy Conference* (pp. 1510–1515). Oxford, England: Pergamon.

Sewalt, V.J.H., Glasser, W.G., & Beauchemin, K.A. (1997). Lignin impact on fiber degradation. 3. Reversal inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. *Journal of Agriculture and Food Chemistry*, *45*(5), 1823–1828.

Shafiel, M., Karimi, K., & Taherzadeh, MJ. (2010). Pretreatment of spruce and oak by N. methylmorphiline-N-oxide (NMMO) for efficient conversion of their cellulose to ethanol. *Bioresource Technology*, *100*, 4914–4918.

Shea Tree. (2011). Cited Nov. 23. Retrieved from http://ucheanudu.tripod.com/id 18. html.

Silanikove, N. (1994). Effect of CaO-or NaOH-hydrogen peroxide treatments on the composition and in-vitro digestibility of cotton straw. *Bioresource Technology*, *48*, 71–73.

Sjöström, E. (1981). *Wood Chemistry. Fundamentals and Applications*. New York, U.S.A: Academic Press.

Sjöström, E. (1993). *Wood Chemistry. Fundamentals and Application* (2nd ed.). San Diego, U.S.A: Academic Press.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). Determination of Ash in Biomass: laboratory analytical procedure (LAP). Golden,

CO: National Renewable Energy Laboratory. NREL Report No.: TP-510-42622. Contract No.: DE-AC36-99-G010337. Sponsored by the U.S. Department of Energy.

Sluiter, A, Hames, B., Ruiz, R., Scarlata. C., Sluiter, J., & Templeton, D. (2008). Determination of structural carbohydrates and lignin in biomass: laboratory analytical procedure (LAP). Golden, CO: National Renewable Energy Laboratory. NREL Report No.: TP-510-42618. Contract No.: DE-AC36-99-G010337. Sponsored by the U.S. Department of Energy.

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Technology, 83*(1):1–11.

Tarkow, H., & Feist, W.C. (1969). Mechanism for improving the digestibility of lignocellulosic materials with dilute alkali and liquid NH_3. Advance Chemistry Series 95 (pp. 197–218). Washington, U.S.A: American Chemical Society.

Teixeira, L.C., Linden, J.C., & Schroeder, H.A. (1999). Alkaline and peracetic acid pretreatments of biomass for ethanol production. *Applied Biochemistry and Biotechnology, 77*(1-3), 19–34.

Tengborg, C., Galbe, M., & Zacchi, G. (2001). Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steam-pretreated softwood. *Biotechnology Progress, 17*(1), 110–117.

Teramoto, Y., Lee, S., & Endo, T (2009). Cost reduction and feedstock diversity for sulfuric acid-free ethanol cooking of lignocellulosic biomass as a pretreatment to enzymatic saccharification. *Bioresource Technology, 100*(20), 4783–4789.

Umar, I.H., Iloeje, O.C., & Bal, E.J. (2000). Reviews of renewable energy technologies in Nigeria. *Nigerian Journal of Renewable Energy*, *8*(1–2), 99–109.

United States Environmental Protection Agency. (2006). National service centre for environmental publications (NSCEP). The U.S. Inventory of Greenhouse Gas Emissions and Sinks: 1990–2004 (Apr. 15; cited 2012 Aug 02). Retrieved from http://www.epa.gov/globalwarming/publications/emission.

Varga, E., Schmidt, A.S., Réczey, K., & Thomsen, A.B. (2003). Pretreatment of corn stover using wet oxidation to enhance enzymatic digestibility. *Applied Biochemistry and Biotechnology, 104*, 37–50.

Vlasenko, E.Y., Ding, H., Labavitch, J.M., Shoemaker, S.P. (1997). Enzymatic hydrolysis of pretreated rice straw. *Bioresource Technolology, 59*, 109–119.

Weil, J.R., Sarikaya, A., Rau, S,L,, Goebz, J., Lasisch, C.M., Brwer, M., Hendrickson. R., Tadisch, M.R. (1998). Pretreatment of corn fiber by pressure cooking in water. *Applied Biochemistry and Biotechnology, 73*, 1–17.

Westermark, U., Lidbrandt, O., & Eriksson, I. (1988). Lignin distribution in spruce (*Picea abies*) determined by mercurization with SEM-EDXA technique. *Wood Science Technology, 22*(3), 243–250.

Williams, A.G., & Morrison, I.M. (1982). Studies on the production of saccharinic acids by the alkaline treatment of young grass and their effectiveness as substrates for mixed rumen microorganisms *in vitro*. *Journal of the Science of Food and Agriculture, 33*(1), 21–29.

Wong, K., Deverell, K., Mackie, K., Clark, T., & Donaldson, L. (1988). The relationship between fiber porosity and cellulose digestibility in steam exploded *Pinus radiata*. *Biotechnology and Bioengineering, 31*, 447−456.

Xiao, C., Bolton, R., & Pan, W.L. (2007). Lignin from rice straw kraft pulping: Effects on soil aggregation and chemical properties. *Bioresource Technology, 98*(7), 1482–1488.

Yan, L., & Shuya, T. (2006). Ethanol fermentation from biomass resources: Current state and prospects. *Applied Microbiology and Biotechnology, 69*(6), 627–642.

Zheng, Y., Pan, Z., Zhang, R., Labavitch, J.M., Wang, D., & Teter, S.A. (2007). Evaluation of different biomass materials as feedstock for fermentable sugar production. *Applied Biochemistry and Biotechnology, 137–140*(1-12), 423–435.

Zhu, L. (2005). *Fundamental study of structural features affecting enzymatic hydrolysis of lignocellulosic biomass* (Ph.D. dissertation, Texas A&M University, College Station, Texas, U.S.A).

Zhu, J.Y., Pan, X.J., Wang, G.S., & Gleisner, R. (2009). Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine. *Bioresource Technology, 100*(8), 2411–2418.

Zhu, J.Y., & Pan, X.J. (2010). Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation. *Bioresource Technology, 101*, 4992–5002.

APPENDIX A

DETERMINATION OF PARTICLE SIZE DISTRIBUTION OF RAW BIOMASS

Table A-1. Specification of sieves: Mesh numbers and their corresponding dimensions

British Standard Sieves (BSS)	Tyler Equivalent	US(approx.)	Opening size		
			(mm)	Standard(mm)	Inches
5	-	-	3.35	4.00	0.187
-	6	6	3.35	3.36	0.132
10	10	-	1.68	2.00	0.0787
-	-	12	1.68	1.68	0.0661
14	14	-	1.20	1.41	0.0555
-	-	16	1.20	1.19	0.0469
18	-	-	0.853	1.00	0.0394
-	20	20	0.853	0.841	0.0331
60	60	60	0.251	0.251	0.0098
-	80	80	0.178	0.177	0.0070
85	-	-	0.178	-	-
100	100	100	0.152	0.149	0.0059
200	200	200	0.075	0.074	0.0029
-	270	270	0.053	0.053	0.0021
300	-	-	0.053	-	0.0021

The raw sawdust samples were collected as sawn particles from wood milling station. They were stacked in polythene bags kept under room conditions until they were ready for use.

Procedure

1. Weigh 50 g of raw biomass using an electronic weighing balance.
2. Pour unscreened particles into the BSS 200 mesh sieve.
3. Sieve manually by vigorously shaking the container continuously till bigger fractions can no longer pass through the holes again.
4. Carefully remove the container on the bottom side.
5. Weigh the sieved sample and store into plastic bottles.
6. Transfer the left over on the sieve plate of higher mesh into the sieve of lower mesh number.

7. Repeat Steps 3–6 until mesh No. 14.
8. Take other 3 sets of 50g of raw biomass and repeat steps 1 to 7.
9. Record the weights of each fractions in all the four sets with their average values.
10. Combine +20 and +80 fractions and mix carefully by hand for 45 minutes.
11. Store the biomass samples appropriately in rubber bottles and cover them securely for future use.

Calculate

1. The weight fractions : It can be represented with this formula for fraction retained on BSS 14 (+14);

$$\text{Weight: Fraction (g/50g biomass)}_{+14} = \frac{W_{+14}}{W_{+14} + W_{+20} + W_{+80} + W_{+200}} \qquad \text{A-1}$$

This represents weight fraction on the BSS +14 mesh size. The subsequent fractions are calculated for other mesh sizes. BSS +200 had no particle weight, appreciable fines did not pass through BSS +80.

2. Percent weight fraction, % Wt. fraction :

$$\text{Weight fraction (g/50g)} \times 100\% = \text{Wt\%} \qquad \text{A-2}$$

APPENDIX B

EXTRACTIVES IN BIOMASS

This procedure is based on gravimetric analysis method to quantify extractives. By acetone extraction, lipophilic wood components, such as fatty acids, resins, fatty alcohol, sterols and glycerides were extracted. In addition low molecular phenolic compounds like lagans, were also extracted.

Apparatus

Analytical (Electronic) balance readable to 0.1 mg (Shimadzu, type AY 220).

Convection oven set to 105 ^{o}C ± 5 °C for drying (Biotechnics, India) .

Apparatus for Soxhlet extraction.

- Glass Soxhlet extraction tube (Borosilicate glass, 200 mL, JSGW with bottom neck (24/29) into the 1000 mL capacity flask .
- Heating mantles, suitable for 1000 mL boiling flasks (Biotechnics, India).
- Condenser neck into a 50/42 neck siphon tube.
- A source of chilled water (or other cooling system).
- Single thickness cotton cellulose thimbles/cellulose filter paper.

Reagent

Acetone, HPLC grade (Fisher scientific, India)

Materials

Desiccator containing desiccant

Boiling flask, round bottom, 1000 mL capacity (Neck B24)

Volumetric flask of appropriate volume

Procedure

1. Determine the moisture content of the sample (NREL standard procedure "Standard method for determination of total solids in biomass" and drying glassware in a 105(± 5) ^{o}C drying oven for a minimum of 4 h.
2. Cool in a desiccator.

3. Weigh a labeled cellulose extraction thimble and add 5 g of sample (W_o, g) to it and record the weight to the nearest 0.1 mg.
4. Measure out accurately with volumetric flasks 300 mL acetone into the 1000 mL round bottom boiling flask.
5. Assemble the Soxhlet apparatus and insert the thimble into the Soxhlet tube.
6. Set the heating mantle such that residence times for the boiling and rising stages are equal to 70 ^{o}C and 25 min respectively for a 4 h run period.
7. When reflux time is complete, turn off the heating mantle and allow the glassware to cool to room temperature.
8. Carefully remove the thimble and air-dry both the thimble and sample for few hours.
9. Dry the thimble and sample in a convection oven (105 ± 5 ^{o}C) until constant weight.
10. Cool in a desiccator and weight the final dried sample (W_1, g) to the nearest 0.1 mg.
11. The extractives expressed as percent weight by weight of dry biomass, W_E, is calculated as given below:

$$W_E = \frac{W_o - W_1}{W_0} \text{ X } 100\% \qquad \text{B-1}$$

APPENDIX C

DETERMINATION OF HEMICELLULOSE IN BIOMASS

Apparatus

Vaccum pump: Rocker 400, Taiwan.

pH meter: Eutech Instrument.

Water bath: Thermostatic (Biotechnics, India).

Analytical (Electronic) balance readable to 0.1 mg (Shimadzu, type AY 220).

Convection oven set to 105 $\pm$ 5 °C for drying (Biotechnics, India).

Filtering crucibles (G4 porosity)

Vaccum filtration set up

Material

pH paper (pH 1 – 12)

Erlenmeyer flasks, 250 mL

Distilled water

0.5 M NaOH (Fisher scientific, India)

Desiccator containing desiccant

Volumetric flasks

Procedure

1. Weigh 1 g of dried biomass (W_2, g) after the extractive analysis and carefully transferred into a 250 mL Erlenmeyer flask.
2. Measure out accurately with volumetric flasks 150 mL of 0.5 M NaOH into the Erlenmeyer flask.
3. Boil the mixture in a water bath for 3.5 h using distilled water.
4. Dry and weight the filtration crucible for minimum of 4 hours.
5. After the 3.5 h boiling of mixture, cool to room temperature.
6. Filter the slurry with the vaccum filtration set up until pH of solution approaches 7.
7. Dry the residue to a constant weight at 105 ± 5 ^{o}C .
8. Cool in a desiccator and weigh the residue (W_3, g).

9. The difference between the sample weight before and after this treatment is the hemicellulose percent weight by weight,% (w/w), W_H :

$$W_H = \frac{W_2 - W_3}{W_2} \text{ X } 100\% \qquad \text{C-1}$$

APPENDIX D

DETERMINATION OF ASH IN BIOMASS

The purpose is to measure the amount of inorganic material in biomass, either structural or extractable, as part of the total composition.

Apparatus

Muffle furnace (Tempo instruments and equipment, Mumbai, India, model Dtc. 0196).

Analytical (Electronic) balance readable to 0.1 mg (Shimadzu, type AY 220)

Materials

Ashing crucibles

Desiccator

Procedure

1. Determine the moisture content of the sample (NREL standard procedure "Standard method for determination of total solids in biomass") at the time the sample is weighed.
2. Label the appropriate number of ashing crucibles with a porcelain marker and place them in the muffle furnace at 575 ± 25 ^{o}C for a minimum of 4 h.
3. Cool in a desiccator for 30 min and dry to constant weight.
4. Weigh 0.5 to 2.0 g of sample to the nearest 0.1mg. Record the sample weight.
5. Place the crucible and sample in the muffle furnace at 575 ± 25 ^{o}C for 6 h.
6. Carefully remove the crucible from the furnace directly into the desiccators and cool for 30 min. Record the weight.
7. Ash to constant weight at 575 ± 25 ^{o}C.

The weight percent Ash is calculated from

$$\%(w/w)\ \text{Ash} = \frac{W_{CA} - W_C}{W_{DB}} \times 100\% \qquad \text{D-1}$$

W_{CA} = Weight of the crucible plus ash

W_C = Weight of crucible

W_{DB} = Weight of dry sample (corrected of moisture)

APPENDIX E

DETERMINATION OF LIGNIN (ACID-INSOLUBLE AND – ACID SOLUBLE CONTENTS IN BIOMASS)

This procedure is based on the NREL standard procedure (Sluiter *et al*,2008) "Determination of structural carbohydrates and lignin in biomass": laboratory analytical procedure (LAP). Specifically the gravimetric analysis of the acid insoluble lignin (AIL) content for this procedure was utilized. The extractive free biomass was used to run the procedure.

Apparatus

Muffle furnace (Tempo instruments and equipment, Mumbai, India, model Dtc. 0196).

Analytical (Electronic) balance readable to 0.1 mg (Shimadzu, type AY 220).

Convection oven set to 105 $\pm$ 5 °C for drying (Biotechnics, India).

Filtering crucibles (G2 porosity).

Vaccum filtration set up.

Autoclave (121 ^{o}C): Precision electronics instrument and component, Mumbai, India, Model P.F.04.

UV-Visible Spectrophotometer (Schimadzu, model UV-1800)

10-1000 µL micropipette

Materials

72% w/w H_2SO_4

Glass test tubes, 16 x 100 mm

Sampling vials with top seal to fit

Procedure

1. Determine the moisture content of the sample (NREL standard procedure "Standard method for determination of total solids in biomass").
2. Weigh 0.3 $\pm$ 0.01 g of the extracted dried sample and place into labeled 16 x 100 mm test tubes. Record the weight to the nearest 0.1 mg.
3. Add 3.00 $\pm$ 0.01 mL of 72% H_2SO_4 to each tube using a micropipette.

4. Keep the samples at room temperature for 2 h with gentle mixing with a vortex shaker every 30 min.
5. Add 84.00 $\pm$ 0.01 mL of distilled water to each test tube after the 2 h acid hydrolysis step bringing the total volume to 87 mL.
6. Autoclave the sealed samples for 1 h at 121 ^{o}C.
7. After that, allow the hydrolyzates to slowly cool to room temperature before removing the caps.
8. Vaccum filter the autoclave hydrolysis solution through the prepared G2 filtering crucibles.
9. Capture the filtrate in a filtering flask.
10. Transfer an aliquot, say 20 mL, into plastic tubes for acid soluble lignin (ASL) analysis.
11. Use 50 mL of hot distilled water to quantitatively transfer all remaining solids out of the pressure bottles into the filtering crucible.
12. Dry the crucible and acid insoluble residue at 105 $\pm$ 5 °C until a constant weight is achieved (a minimum of 4 h).
13. Remove the samples from the oven and cool in a desiccators.
14. Record the weight of the crucible and dry residue to the nearest 0.1 mg.
15. Place the crucibles and residue in a muffle furnace at 575 $\pm$ 25 °C for a minimum of 6 h to correct for acid-insoluble ash.
16. Carefully remove the ashed crucible and contents from the furnace directly into a desiccators and cool for 30 min.
17. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight.
18. Perform the acid soluble lignin analysis within 6 h hydrolysis on a UV-Visible spectrophotometer using the aliquot obtained after vaccum filtration in step 10.
19. Measure the absorbance of the sample at 320 nm on the UV-Visible spectrophotometer.
20. Dilute sample to bring absorbance into the range 0.2–1.0, and note the dilution.
21. The weight of lignin (% Klason lignin) will be reported by percent on a dry weight basis below:

$$\% \text{ Klason lignin (AIL)} = \frac{W_1 - W_2}{W_i X \frac{\%T_f}{100\%}} \times 100\% \qquad \text{E-1}$$

W_1 = weight of crucible + acid insoluble residue

W_2 = weight of crucible + ash

W_i = initial sample weight

T_f = solid content in the initial sample

22. Calculation for the percentage of acid soluble lignin on 105 ^{o}C dry weight basis:

$$\% \text{ Acid soluble lignin} = \frac{UV_{abs} \,.\, 87 \,.\, DF}{A \,.\, W_i} \qquad \text{E-2}$$

UV_{abs} = Average UV-Vis absorbance of the sample at 320nm

DF = Dilution factor

W_i = initial dry sample weight

A = absorptivity value of 30 L/(g.cm)

The value 87 stands for volume of the filtrate in mL

APPENDIX F

RAW MATERIAL PRETREATMENT PROCEDURES

Alkaline wet air oxidation pretreatment

1. With the aid of the air compressor allow air into the base portion of reactor stand, dislodge the reactor system by detaching the heating jacket from the reactor system.
2. Transfer a mixture of 30 g dry weight of the raw biomass, 1 g of calcium hydroxide and 450 mL of water to the reactor. Use 50 mL of water to rinse down the remnants of raw material into the reactor.
3. With the aid of the air compressor put back the reactor vessel to be in contact with the bomb head and securely tighten the nuts to keep reactor in place.
4. Cover up the reactor with the heating jacket with the aid of the air compressor.
5. Open the gas inlet valve on the bomb head to allow inflow of air from the gas cylinder stationed very close to the reactor. Monitor the air pressure (5 bars and 10 bars air pressure) through the CalGrafix software installed on the computer.
6. Close the gas inlet valve to the reactor as soon as the required pressure is attained.
7. Set the stirrer revolution at 200 revolution per minute with the aid of the CalGrafix software.
8. Put the reactor into operation by pressing the ON buttons on both the power and process controllers.
9. Maintain constant flow of cooling water from a source (tap water).
10. Finally with the CalGrafix software set the reaction temperature. The reaction should start as soon as the temperature is set on the computer.
11. With the aid of a stop watch monitor reaction time as appropriate.
12. After the pretreatment time elapsed, set the temperature back to zero.
13. Allow the reactor to cool down to room temperature (this can take up to 1 h depending on the operating pretreatment temperature).

14. Release the pressure in the reactor by opening the gas outlet valve on the bomb head.
15. With the air of the air compressor, dismantle the vessel and carefully empty the slurry into a 1000 mL beaker.

2^3 CCD Alkaline peroxide oxidation pretreatment

1. With the aid of the air compressor, dislodge the reactor system by detaching the heating jacket from the reactor system.
2. Transfer a mixture of 30 g dry weight of the raw biomass and the appropriate weight of calcium hydroxide required to make pH 11.5 of the 500 mL solution (No air pressure is to be applied here). Mix 450 mL of the solution with the biomass initially and use the remaining 50 mL to rinse down the reminants of raw material into the reactor. NOTE: 30% (w/v) H_2O_2 was made into different concentrations %(v/v) of 0.84, 1.00, 1.25, 1.50, 1.66% and adjusted to pH 11.5 with 9.0, 13.7, 17.5, 20.3, 30.0 g lime loadings respectively.
3. For the alkaline peroxide assisted wet air oxidation (APAWAO) pretreatment process, allow appropriate air pressure into the reactor along with the hydrogen peroxide before setting the reaction temperature.
4. Repeat steps 3–13 for WAO pretreatment.
5. With the air of the air compressor, dismantle the vessel and carefully empty the slurry into a 1000 mL beaker.

2^2 CCD Alkaline peroxide oxidation pretreatment

1. With the aid of the air compressor, dislodge the reactor system by detaching the heating jacket from the reactor system.
2. Transfer a mixture of 25 g dry weight of the raw biomass and the appropriate weight of calcium hydroxide required to make pH 11.5 of the 500 mL (1%) hydrogen peroxide solution (No air pressure is to be applied here). Mix 450 mL

with the biomass initially and use the remaining 50 mL of solution to rinse down the raw material still left in the container into the reactor.

3. Repeat steps 3–13 for WAO pretreatment.
4. With the air of the air compressor, dismantle the vessel and carefully empty the slurry into a 1000 mL beaker.

APPENDIX G

DETERMINATION OF LIME UNREACTED AFTER PRETREATMENT

For this analysis, pretreated samples from 2^3 CCD and 2^2 CCD alkaline peroxide oxidation pretreatments were used. The procedure has a dual purpose: to determine the amounts of lime in the biomass slurry left after pretreatment by pH titration using HCl and neutralize the sample to render it suitable for further analytical procedures.

Apparatus and Materials

Magnetic stirrer

Burette, 50 mL

5 M Hydrochloric acid (HCl)

pH meter (model APX175E/C)

Procedure

1. Carefully transfer the contents from the reactor into a 1 L beaker.
2. Set up titration apparatus (burette, clamp, magnetic stirrer, and the calibrated pH meter)
3. Place the beaker with the sample on a magnetic stirrer and drop a magnetic bar into the beaker. Use a burette clamp to place the burette over the beaker. Fill the burette with a standard solution of hydrochloric acid and record the starting volume (V_1).
4. Using a well-calibrated pH meter, titrate the solution in the beaker slowly under constant stirring until the pH reaches 6.80 to 7.00.
5. Provide enough time ($\geq$ 30 min) to ensure the pH of the slurry is stabilized.
6. Record the volume left in the burette (V_2).
7. Calculate the amount of $Ca(OH)_2$ left after pretreatment as follows:

$$W_{Ca(OH)_2} = \frac{1 \text{ mol } Ca(OH)_2}{2 \text{ mol HCl}} \text{ X } \frac{M_{HCl} \cdot (V_1 - V_2)}{1000} \text{ X } Mw_{Ca(OH)_2} \qquad \text{G-1}$$

Where, $W_{Ca(OH)_2}$ = The amount of lime, $Ca(OH)_2$, unreacted (g)

M_{HCl} = Molarity of HCl solution

V_1-V_2 = Total volume of HCL solution to titrate the biomass slurry (mL)

$Mw_{Ca(OH)_2}$ = Molecular weight of $Ca(OH)_2$, 74.092 g/mol

APPENDIX H

DETERMINATION OF INSOLUBLE SOLIDS IN PRETREATED BIOMASS MATERIAL

Pretreated biomass samples are composed of water-soluble and water insoluble components. These solid and liquid fractions are analyzed separately. This procedure therefore determines the percent insoluble solids in a sample of hydrolyzate slurry (The liquid and solid material in a sample resulting from biomass pretreatment). The percent insoluble solids is used to combine the liquid and solid compositions of the pretreated biomass in the mass balance determination. The procedure is intended to determine the percentage of water insoluble solids in a pretreated biomass sample after all soluble components have been extracted with aggressive water washing. Filtration step was adopted for the procedure.

Apparatus

Stainless steel Buchner funnel with appropriate porosity.

1000 mL vacuum flask.

Filtration set-up including vacuum source and vacuum adapters for Buchner funnels.

Analytical balance readable to 0.1 mg.

Convection ovens with temperature control to 105 ^{o}C ± 3 ^{o}C.

Desiccator.

Porcelain crucibles.

Teflon coated spatulas.

Measuring cylinder

Materials

pH paper (range 1-14).

Distilled water.

Procedure

1. Weigh the stainless steel Buchner funnel filter cup and record to the nearest 0.1mg on analytical balance. Tare the Buchner funnel.

2. Gradually pour the neutralized slurry into the Buchner funnel and allow vacuum filtration to proceed. Allow filtration to continues for 5 minutes for liquid fraction to drain out of the solid.
3. Pour the liquid fraction into a measuring cylinder and note the volume. Store the liquid fraction in a refrigerator for analysis later.
4. Wash the solid fraction continuously until pH is at least 6–7.
5. Let process sit under vacuum for 5 minutes to remove excess water. Remove filter cup and place on paper towel to allow water accumulation on filter cup bottom to escape.
6. Weigh filter cup (Buchner funnel) and sample. Record weight. Subtract the weight of the filter cup to get the wet weight of the washed sample.
7. Weigh a crucible already dried in the oven at 105 ^{o}C ± 3 ^{o}C and record its weight to 0.1mg. Tare the crucible.
8. Add about 3 g wet weight from the entire wet biomass to the tared dry crucible and record weight to 0.1mg.
9. Store the remaining wet biomass in the freezer (-20 ^{o}C) for extractive, ash, cellulose, hemicelluloses, lignin analyses, enzymatic hydrolysis and simultaneous saccharification and fermentation steps.
10. The percent total insoluble solids of the washed material is calculated based on the whole sample weight.

Calculation:

$$\% \text{ Insoluble solid} = \frac{\text{Dry weight of the washed sample}}{\text{Wet weight of washed sample}} \times 100\% \qquad \ldots\text{H-1}$$

APPENDIX I

DETERMINATION OF CELLULASE ENZYME ACTIVITY AND DEFINITION OF IUPAC-FPU

Determination of cellulase activity in a *Trichoderma reesei* enzyme preparation using the methods outlined by IUPAC and as described by NREL standard procedure No.: 06, "Measurements of cellulose activities". All enzyme dilutions were made in citrate buffer, pH 4.8, and 0.05 M.

Make a working enzyme stock solution diluted with buffer solution 1:20 (that is 1 mL of enzyme plus 19 mL of buffer solution). From the stock solution make about 5 different dilutions say to a working solution of 20 mL. All cellulose enzyme dilutions that were used to determine cellulose activity are given in the table below.

Table I-1. Cellulase enzymes concentrations.

	Dilution	Citrate buffer (mL)	1:20 Enzyme (mL)	Concentration[a]
1	1:4	15	5	0.0125
2	1:5	16	4	0.0100
3	1:6.67	17	3	0.0075
4	1:10	18	2	0.0050
5	1:20	19	1	0.0025

[a]The term "Concentration" is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture. For example 1:4 dilution of the 1:20 working stock of enzyme will have a concentration of 0.0125.

Apparatus

Water bath capable of maintaining 50 ^{o}C ± 0.1 ^{o}C.

Spectrophotometer suitable for measuring absorbance at 540 nm

Reagents and Materials

DNS Reagent:

Mix:

Distilled water 1416 mL

3,5 Dinitrosalicylic acid 10.6 g

Sodium hydroxide 19.8 g

Dissolve above, then add:

Rochelle salts (sodium potassium tartrate) 306 g

Phenol (melt at 50 ^{o}C) 7.6 mL

Sodium metabisulfite 8.3 g

Titrate 3 mL sample with 0.1 N HCl to the phenolphthalein endpoint. It should take 5-6 mL of HCl. Add NaOH if required (2 g = 1 mL 0.1 M HCl).

Citrate buffer

For *Trichoderma reesei*, cellulase assays are carried out in 0.05 M citrate buffer pH 4.8.

Citric acid monohydrate	210 g
Distilled water	750 mL
NaOH - add until pH equals 4.3	50 to 60 g

Dilute to 1 L and check pH. If necessary add NaOH until the pH is 4.5. When the 1 M stock citrate buffer stock is diluted with water to 50 mM the pH should be 4.8. After diluting the citrate buffer check and adjust the pH if necessary to pH 4.8.

Procedure for the Filter Paper Assay for Saccharifying Cellulase

1. Make the citrate buffer.
2. Make the DNS reagent.
3. Enzyme assay tubes.
    - 3.1 Place a rolled filter paper strip (50 mg Whatman No. 1, 1.0 x 6.0 cm) each into a 13 x 100 test tube.
    - 3.2 Add 1 mL 0.05 M sodium citrate of pH 4.8 to the tube (the buffer should saturate the paper).
    - 3.3 Equilibrate tubes with buffer and substrate at 50 ^{o}C.
    - 3.4 Add 0.5 mL enzyme diluted appropriately in citrate buffer. At least 2 dilutions must be made of each enzyme sample.
    - 3.5 Incubate at 50 ^{o}C for exactly 60 min.

3.6 At end of incubation period, remove each assay tube from the 50 ^{o}C bath and stop the the enzyme reaction by immediately adding 3 mL DNS reagent and mixing.

4. Reagent blank (Spectro zero)

4.1 Add 1.5 mL citrate buffer + 3 mL DNS reagent into a test tube.

5. Enzyme control

5.1 Add 1 mL citrate buffer + 0.5 mL enzyme dilution (prepare a separate control for each dilution tested) and 3 mL DNS reagen into a test tube.

6. Substrate control

6.1 1.5 mL citrate buffer + Filter paper strip and 3 mL DNS reagent.

7. Glucose standards

7.1 Make a working solution stock of 10 mg/ml. Dilutions are made from the working stock solution in the following manner as shown in the table below.

Table I-2. Absorbance values for glucose standards (Data for Figure 4.2)

	Glucose stock (mL)	Citrate buffer (mL)	Dilution	Concentration	Absorbance at 540 nm
1	1.0	0.5	1:1.5	3.35 mg/0.5 mL	0.139
2	1.0	1.0	1:2	2.50 mg/0.5 mL	0.304
3	1.0	2.0	1:3	1.65 mg/0.5 mL	0.376
4	1.0	4.0	1:5	1.0 mg/0.5 mL	0.502

7.2 Make glucose standards from these dilutions by adding 0.5 mL of each of the dilutions to 1 mL of citrate buffer in the 13 x 100 mm test tube. Add 3 mL DNS reagent. Boil and measure against spectro zero.

8. Blanks, controls, and glucose standards should be incubated at 50 ^{o}C along with the enzyme assay tubes, and then stopped at the end of 60 min by addition of 3 mL DNS reagent and mix.

Colour development

After the end of 60 min;

9. Boil all tubes for 5 min in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent.
10. All samples, controls, blanks, and glucose standards should be boiled together.
11. After boiling transfer to a cold ice-water bath.
12. Let the tubes sit until all the pulp has settled, or centrifuge briefly
13. Dilute all tubes in water (0.2 mL of colour developed reaction mixture plus 2.5 mL of water in a spectrophotometer cuvette works well).
14. Determine colur formation by measuring absorbance against the reagent blank (spectro zero) at 540 nm.
15. With the dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0.

Definition of FPU

FPU stands for “filter paper units” per milliliter of original (undiluted) enzyme solution and are the units to measure enzyme activity using Whatman No. 1 filter paper strips as substrate. For quantitative results the enzyme preparations must be compared on the basis of significant and equal conversion. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC. As a result, the assay procedure therefore involves finding a dilution of the original enzyme stock such that a 0.5 mL aliquot of the dilution will catalyze 4% conversion in 60 minutes (or, in practical terms finding two dilutions that bracket the 4%-conversion point so closely that the required dilution can be obtained, with reasonable accuracy, by interpolation) and then calculating the activity (in FPU/mL) of the original stock from the dilution required. According to Ghose (1987), the required calculations are based on the International Unit (IU), more specifically:

1 IU = 0.1 μmol min^{-1} of substrate converted

= 1 μmol min^{-1} of "glucose" (reducing sugars as glucose) formed during the hydrolysis reaction

= 0.18 mg min^{-1} when product is glucose.

The absolute amount of glucose released in the FPU assay at the critical dilution is 2.0 mg (equivalent to 2/0.18 μmol). This amount of glucose was produced by 0.5 ml enzyme in 60 min, i.e., in the FPU reaction:

$$2 \text{ mg glucose} = 2/(0.18 \; x \; 0.5 \; x \; 60) \text{ μmol } min^{-1} = 0.37 \text{ μmol } min^{-1} \text{ mL(IU } mL^{-1})$$

Therefore, the estimated amount of enzyme (=critical enzyme concentration = mL mL^{-1}) which releases 2.0 mg glucose in the FPU reaction contains 0.37 units, and:

$$\text{FPU} \equiv \frac{0.37}{\text{Enzyme concentration to release 2.0 mg glucose}} \quad \text{units } mL^{-1}$$

This last statement is the defining equation for FPU. The procedure was meant to determine the denominator (the enzyme concentration equivalent to releasing 2.0 mg glucose from the filter paper).

APPENDIX J

ENZYMATIC HYDROLYSIS

Materials for the enzymatic hydrolysis step were the untreated washed, and the treated washed sawdust. The untreated washed material was used as the control for comparing the enzymatic digestibility of the treated samples.

Procedure

1. Transfer appropriate wet weight of biomass equivalent to the dry biomass weight calculated from the material balance after determination of the moisture content during pretreatment (if the dry biomass content is 30.38%(w/w) from the material balance after pretreatment, for a 2%(w/w) dry biomass loading and a total volume of mixture of 10 mL, 0.66 g lime pretreated wet biomass was weighed into the culture tube used for the enzymatic hydrolysis). The wet biomass needed for each hydrolysis considered in the study depended on the dry biomass loadings. The dry biomass loading was based on the total carbohydrate composition of the substrates (not on the cellulose content).
2. For a 0.66 g wet biomass loading, add 5 mL citrate buffer, and 0.04 mL tetracycline into the tube.
3. Measure the current pH of the mixture and adjust to 4.8 if necessary.
4. Add the remaining volume of distilled water in the tube to make the final volume of reaction to be 10 mL.
5. Place the tube inside the 130 r/min shaking air bath at 50 ^{o}C for 1 h.
6. Take out the heated tube from the shaker and start the enzyme hydrolysis reaction by adding 0.087 mL of the cellulase enzyme (this volume corresponded to 25 FPU/g dry biomass for 2%(w/w) dry solid loading if the activity of enzyme is 57.8 FPU/mL) and 0.25 mL of β-glucosidase for 12.5 UI/g dry biomass loading (enzyme activity of 10 UI/mg). The final volume becomes 10 mL.

7. Vigorously shake the tube to get a homogenous mixture, immediately open the cap of the tube, take 0.5 mL sample, and transfer it to a glass tube (This point on the time axis is considered as time zero). Use a micropipette tip (cut the end of the tip to make around 5-mm internal diameter hole) to take the sample. After taking the sample, tightly cap and incubate in the shaker at 130 r/min and 50 ^{o}C.
8. Boil the samples for 15 min to denature the enzymes. Cool samples in cold water bath for 10 min and transfer samples to conical tubes (2 mL capacity).
9. Centrifuge the sample at 4,000 rev/min for 5 min to separate the liquid and solid parts.
10. Transfer the liquid part into the tube and store it in the freezer.
11. Perform steps 2 to 10 as a function time, temperature, substrate concentrations, and enzyme loadings.
12. Perform DNS assay on liquid part to measure the concentrations of reducing sugars.

APPENDIX K
REDUCING SUGAR MEASUREMENT

Dinitrosalicylic Acid (DNS) Assay

Reducing sugar was measured using the dinitrosalicylic acid (DNS) assay (Miller, 1959). A glucose standard was prepared from glucose (Fisher Scientific, India), thus the reducing sugars were measured as "equivalent glucose."

Preparation of DNS Reagents

1. Dissolve 10.6 g of 3,5-dinitrosalicylic acid crystals and 19.8 g of NaOH in 1,416 mL of distilled water.
2. Add 306 g of sodium-potassium tartrate (Rochelle salts).
3. Melt phenol crystals under a fume hood at 50 ^{o}C using a water bath. Add 7.6 mL of the dissolved phenol to the mixture.
4. Add 8.3 g of sodium meta-bisulfate ($Na_2S_2O_4$).
5. Add NaOH to adjust the pH to 12.6, if required.

DNS Reagent Calibration

1. Using 20 mg/mL glucose stock solution, prepare 1 mL of sample in test tubes according to Table K-1.
2. Place 0.5 mL of each sample into test tubes.
3. Dispense 1.5 mL of DNS reagent into each test tube with a micropippette.
4. Place the caps on the tubes and vortex.
5. Vigorously boil samples in a water bath for 15 min.
6. Cool the test tubes for a few minutes. Add 8 mL of distilled water and vortex.
8. Zero the spectrophotometer (UV-1800 Schimadzu, Japan) at 550 nm with distilled water. (The spectrophotometer should be stabilized by turning it on for at least 1 h before using).
9. Measure the absorbance and prepare a calibration curve.

Measurement of Reducing Sugar Concentration of Sample

1. Centrifuge samples at 4,000 rpm (IEC centrifuge, model PR-2) for 5 minutes.
2. Dilute the samples into test tubes such that the sugar concentration lies between 0.2 to 1.0 mg/mL. Vortex the diluted samples.
3. Place 0.5 mL of each diluted sample into test tubes.
4. Repeat Step 3 to 8 used to prepare the calibration curve.
5. Calculate sugar concentration from the absorbance of the samples using the calibration curve.
5. Calculate the reducing sugar yield by the following formula:

$$Y = S \times D \times V/W \qquad \ldots K\text{-}1$$

where Y = reducing sugar yield (mg equivalent glucose/g dry biomass)

S = sugar concentration in diluted sample (mg equivalent glucose/mL)

D = dilution factor

V = working volume (mL)

W = weight of dry biomass (g)

Table K-1. Preparation of Glucose Standard Solutions for DNS Assay

Glucose Concentration (mg/mL)	2 mg/mL Glucose Stock Solution (mL)	Distilled Water (mL)
0.2	0.1	0.9
0.4	0.2	0.8
0.6	0.3	0.7
0.8	0.4	0.6
1.0	0.5	0.5

APPENDIX L

CALCULATIONS

1. (a) 2^3 CCD APO wet pretreated samples needed:

2% Cellulose loading

Cellulose content: 59.17 g/100g dry biomass

Total dry solid: 27.43 g/100g

Equivalent cellulose loading : 1.0 g (i.e. 2% of 50 g total fermentation mixture)

$$\text{Wet biomass needed} = \frac{1.0\ (g)}{0.2743\ (g/g) \times 0.5917\ (g/g)}$$

= 6.16 g wet biomass.

(b) Cellulase enzyme (mL) needed at 25 FPU/g cellulose loading:

1.0 g cellulose loading will require

$$= \frac{1.0\ g \times 25\ FPU}{1\ g\ cellulose}$$

= 25 FPU

For cellulase activity of 57.8 FPU/mL, volume of cellulase enzyme needed

= 0.4325 mL

3% Cellulose loading

$$\text{Wet biomass needed} = \frac{1.5\ (g)}{0.2743\ (g/g) \times 0.5917\ (g/g)}$$

= 9.24 g wet biomass

(c) Cellulase enzyme (mL) needed at 25 FPU/g cellulose loading:

1.5 g cellulose loading will require

$$= \frac{1.5\ g \times 25\ FPU}{1\ g\ cellulose}$$

= 37.5 FPU

For cellulase activity of 57.8 FPU/mL, volume of cellulase enzyme needed

= 0.6488 mL

2. (a) 2^2 CCD APO wet pretreated samples needed:

2% Cellulose loading

Cellulose content: 58.52 g/100g dry biomass

Total dry solid: 34.03 g/100g

Equivalent cellulose loading : 1.0 g (i.e. 2% of 50 g of total fermentation mixture)

$$\text{Wet biomass needed} = \frac{1.0\ (g)}{0.3403\ (g/g) \times 0.5852\ (g/g)}$$

= 5.02 g wet biomass.

3% Cellulose loading

$$\text{Wet biomass needed} = \frac{1.5\ (g)}{0.3403\ (g/g) \times 0.5852\ (g/g)}$$

= 7.53 g wet biomass

Same volumes of the cellulase enzyme are required as calculated above.

APPENDIX M

DETERMINATION OF FERMENTABILITY OF PRETREATED SOLIDS

The procedure is based on the NREL standard procedure "SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation Laboratory Analytical Procedure. NREL/TP-510-42630. 2008 Jan.".

Apparatus

Autoclave.

pH meter: Eutech Instrument.

Laminar flow hood.

Analytical (Electronic) balance readable to 0.1 mg (Shimadzu, type AY 220).

Convection oven set to 105 $\pm$ 5 °C for drying (Biotechnics, India).

Centrifuge (IEC, model PR-2)

A shaker incubator (32 $\pm$ 2 ^{o}C and 130 r/min)

A bubble trap (A rubber stopper with a U-tube glass filled with water)

Sterile sampling pipettes

UV-Visible Spectrophotometer for reading optical density at 600 nm (Shimadzu, model UV-1800)

Material

MYGP medium (liquid) for seed culture

YP medium (liquid) for fermentation

Erlenmeyer flasks, 250 mL

Distilled water

0.05 M citrate buffer pH 4.8

Frozen stock culture of *Saccharomyces cerevisiae*

Liquid cellulose enzyme with activity of 57.8 FPU/mL

Procedure

1. Measure the desired wet sample weight for 2 and 3% biomass loadings.
2. Prepare the control (to determine how much ethanol is made from just the enzyme and medium) having all conditions except the pretreated sample.

3. Autoclave only correct amount of biomass samples with required distilled water, water trap without water yet. Autoclave the YP medium.
4. Weigh the assembly in (3) before autoclaving.
5. Then autoclave at 121 ^{o}C for 30 minutes.
6. Cool to room temperature after autoclaving and re-weigh; if there is any loss in weight make up with distilled water as loss mL of sterile water.
7. In a laminar flow hood aseptically add to the flasks:
    - Water loss from autoclaving.
    - YP and citrate buffer 0.05 M (pH 4.8) at 5 mL each.
    - 10X concentrated inoculum to achieve OD of 0.5 at 600 nm, 10%(v/v) of total working volume of fermentation (in this case 5 mL inoculum was added).
    - Add the required volume of the cellulase enzyme.
    - Mix contents well. Use a sterile sampling pipette and take 5 mL sample of the slurry.
8. Store in capped tubes and chill on ice.
9. Take 0.5 mL for pH sample.
10. Centrifuge, collect and filter the supernatant from the rest of the sample.
11. Analyze for glucose and ethanol (through dichromate assays).
12. Freeze supernatant in glass vials if analysis will be done later.
13. Add water to the water traps (the traps are used to maintain anaerobic conditions) and incubate the flasks in a shaker at 130 rev/min at 32 $\pm$ 2 ^{o}C.
14. Sample at 96 hours.
15. Autoclave all materials used before disposal.

APPENDIX N

ETHANOL ANALYSIS

The ethanol concentrations of SSF samples were measured using the dichromate assays.

Dichromate assay preparation and calibration (Adopted from Bennet, 1971)

1. Dissolve 7.5 g of potassium dichromate in 70 mL 5 M H_2SO_4.
2. Adjust the volume to 250 mL with distilled water.
3. Place 300 µL of ethanol solutions (using pure ethanol i.e. 200 proof) as prepared from Table N-1 into small plastic caps based on a total volume of 100 mL.
4. Place the plastics into beakers containing 3 mL of acid dichromate.
5. Tightly seal the set up and keep at room temperature for 30 min.
6. Measure the absorbance at 590 nm.
7. Prepare the calibration curve.

Table N-1. Preparation of Ethanol Standard Solutions

Ethanol Concentration (g/L)	Pure Ethanol (g)
1	0.1
5	0.5
10	1.0
25	2.5

Measurement of ethanol concentration of fermented samples

1. At the end of 96 h, sample out 5 mL of mixture with a sterilized syringe.
2. Centrifuge samples at 4,500 rpm (IEC centrifuge, model PR-2) for 5 minutes.
3. Place 300 µL of liquid sample into small plastic caps.
4. Place the plastics into beakers containing 3 mL of acid dichromate.
5. Tightly seal the set up and keep at room temperature for 30 min.
6. Measure the absorbance at 590 nm.
7. Calculate ethanol concentration from the absorbance of the samples using the calibration curve.

APPENDIX O

EXPERIMENTAL DATA

Table O-1. Specific lime consumption (g $Ca(OH)_2$/g dry raw biomass) for 2^3 CCD APO pretreatment.

Run order	Temperature (^oC)	Time (min)	H_2O_2 (%)	Lime loading (g)	5N HCl used (mL)	Unreacted lime (g)	Reacted lime (g)	g $Ca(OH)_2$/g dry raw biomass
1	135	30	0.84	9.0056	38.40	7.1128	1.8872	0.0629
2	110.51	30	1.25	17.5111	83.90	15.5408	1.9592	0.0653
3	159.50	30	1.25	17.5087	75.40	13.9663	3.5337	0.1178
4	135	46.33	1.25	17.5240	76.10	14.0960	3.4040	0.1135
5	135	30	1.66	30.0564	141.70	26.2471	3.7529	0.1251
6	135	30	1.25	17.5340	82.70	15.3185	2.1815	0.0727
7	135	13.67	1.25	17.5234	77.10	14.2812	3.2188	0.1073
8	120	40	1.00	13.7102	58.90	10.9100	2.7900	0.0930
9	120	20	1.50	20.3102	93.20	17.2634	3.0466	0.1016
10	120	20	1.00	13.7120	64.60	11.9659	1.7341	0.0578
11	135	30	1.25	17.5102	79.20	14.6702	2.8298	0.0943
12	120	40	1.50	20.3112	91.40	16.9300	3.3800	0.1127
13	150	20	1.50	20.3108	91.50	16.9485	3.3615	0.1120
14	150	40	1.50	20.3100	90.50	16.7633	3.5467	0.1182
15	150	40	1.00	13.7008	59.60	11.0397	2.6603	0.0887
16	150	20	1.00	13.7004	61.00	11.2990	2.4010	0.0800
17	135	30	1.25	17.3005	79.2	14.6702	2.6303	0.0877
18	135	30	1.25	17.7999	80.18	14.8524	2.9475	0.0983
19	135	30	1.25	16.7004	77.79	14.4090	2.2914	0.0764
20	135	30	1.25	17.4907	79.87	14.7943	2.6964	0.0899

Data for Figure 5.1.

Table O-2. Specific lime consumption (g $Ca(OH)_2$/g raw biomass) for 2^2 CCD APO.

Run order	Temperature (^oC)	Time (min)	Lime loading (g)	5N HCl used (mL)	Unreacted lime (g)	Reacted lime (g)	g $Ca(OH)_2$/g dry raw biomass
1	90	15	3.17	13.00	2.4080	0.7620	0.0254
2	90	30	3.17	12.70	2.3524	0.8176	0.0273
3	120	15	4.15	16.10	2.9822	1.1678	0.0389
4	120	30	3.69	13.60	2.5191	1.1709	0.0390
5	105	22.5	3.46	14.20	2.6303	0.8297	0.0277
6	105	22.5	3.42	13.00	2.4080	1.0120	0.0337
7	83.79	22.5	3.96	17.00	3.1489	0.8111	0.0270
8	126.21	22.5	3.87	14.50	2.6858	1.1842	0.0395
9	105	11.89	3.60	14.40	2.6673	0.9327	0.0311
10	105	33.11	3.31	13.10	2.4265	0.8835	0.0294
11	105	22.5	2.59	10.00	1.8523	0.7377	0.0246
12	105	22.5	2.66	10.30	1.9079	0.7521	0.0251
13	105	22.5	2.57	10.20	1.8893	0.6807	0.0177

Data for Figure 5.2.

Table O-3. Pretreatment yield (%) of holocellulose for 2^3 full factorial design of WAO.

	Cellulose content[a]	Hemicellulose content[a]	Lignin content[a]	Holocellulose	Carbohydrate ratio (C/H)	
Raw biomass	45.86	20.31	29.9	66.17	2.26	
Pretreatments			Lignin removal[a]			% Yield[b] (Holocellulose)
1	56.47	8.08	3.00	64.55	6.99	97.55
2	56.86	9.91	10.97	66.77	5.74	100.91
3	56.31	10.57	24.06	66.88	5.33	101.07
4	49.07	13.28	19.24	62.35	3.70	94.23
5	51.53	9.08	11.63	60.61	5.68	91.60
6	51.84	11.18	20.06	63.02	4.64	95.24
7	45.94	13.88	22.99	59.82	3.31	90.40
8	42.75	17.11	19.09	59.86	2.50	90.46

Data for Figure 5.3.
[a]g component/100g dry biomass. [b](g holocellulose recovered/g holocellulose in raw biomass) x 100%.

Table O-4. Pretreatment yield (%) of holocellulose for 2^3 CCD APO.

	Cellulose content[a]	Hemicellulose content[a]	Lignin content[a]	Holocellulose	Carbohydrate ratio (C/H)	
Raw biomass	45.86	20.31	29.90	66.17	2.26	
Pretreatments			Lignin removal[a]			% Yield[b] (Holocellulose)
1	53.71	8.59	4.12	62.30	6.25	94.15
2	55.49	8.98	12.78	64.47	6.18	97.43
3	52.71	9.79	12.23	62.50	5.38	94.45
4	53.81	9.83	11.73	63.64	5.47	96.18
5	53.57	10.13	8.72	63.70	5.29	96.27
6	51.47	9.98	5.77	61.45	5.16	92.87
7	54.48	9.83	8.91	64.31	5.54	97.19
8	54.07	8.01	9.61	62.08	6.75	93.82
9	46.85	8.98	3.71	55.83	5.22	84.37
10	42.96	9.83	4.84	52.79	4.37	79.78
11	53.58	9.12	11.14	62.70	5.88	94.76
12	48.95	8.60	10.81	57.55	5.69	86.97
13	53.10	9.29	11.79	62.39	5.72	94.29
14	54.05	7.78	12.27	61.83	6.95	93.44
15	52.59	6.88	8.12	59.47	7.64	89.87
16	51.44	7.35	8.09	58.79	7.00	88.85
17	53.14	9.17	8.36	62.31	5.79	94.17
18	52.82	8.98	10.17	61.80	5.88	93.40
19	51.75	9.20	5.32	60.95	5.63	92.11
20	53.15	9.75	12.37	62.90	5.45	95.06

Data for Figure 5.4.
[a] %(w/w). [b](g holocellulose recovered/g holocellulose in raw biomass) x 100%.

Table O-5. Pretreatment yield of holocellulose for 2^2 CCD of APO pretreatment.

	Cellulose content[a]	Hemicellulose content[a]	Lignin content[a]	Holocellulose	Carbohydrate ratio (C/H)	
Raw biomass	45.86	20.31	29.90	66.17	2.26	
Pretreatments			Lignin removal[a]			% Yield[b] (Holocellulose)
1	59.03	6.90	14.09	65.93	8.56	99.64
2	58.28	7.47	7.14	65.75	7.80	99.37
3	60.06	9.04	13.43	69.10	6.64	104.43
4	60.64	8.44	14.66	69.08	7.18	104.40
5	55.59	9.03	9.17	64.62	6.16	97.66
6	58.20	8.63	14.29	66.83	6.74	101.00
7	58.59	8.63	16.84	67.22	6.79	101.59
8	59.18	8.78	12.83	67.96	6.74	102.71
9	56.69	9.15	12.65	65.84	6.20	99.50
10	56.24	8.92	4.96	65.16	6.30	98.47
11	61.12	7.23	18.00	68.35	8.45	103.29
12	58.03	8.58	13.59	66.61	6.76	100.66
13	58.98	6.77	6.27	65.75	8.71	99.37

Data for Figure 5.5.

[a] %(w/w). [b](g holocellulose recovered/g holocellulose in raw biomass) x 100%.

Table O-6. Lignin removal and lime consumption variation with pretreatment conditions (2^3 CCD APO)

Run Order	Temperature (^oC)	Time (min)	H_2O_2 (%)	Lime consumed (g/g raw biomass)	Lignin removal %(w/w)
1	135	30	0.84	0.0629	4.12
2	110.5	30	1.25	0.0653	12.78
3	159.5	30	1.25	0.1178	12.23
4	135	46.33	1.25	0.1135	11.73
5	135	30	1.66	0.1251	8.72
6	135	30	1.25	0.0727	5.77
7	135	13.67	1.25	0.1073	8.91
8	120	40	1.00	0.0930	9.61
9	120	20	1.50	0.1016	3.71
10	120	20	1.00	0.0578	4.84
11	135	30	1.25	0.0943	11.14
12	120	40	1.50	0.1127	10.81
13	150	20	1.50	0.1120	11.79
14	150	40	1.50	0.1182	12.27
15	150	40	1.00	0.0887	8.12
16	150	20	1.00	0.0800	8.09
17	135	30	1.25	0.0877	8.36
18	135	30	1.25	0.0983	10.17
19	135	30	1.25	0.0764	5.32
20	135	30	1.25	0.0899	12.37

Data for Figures 5.6 and 5.7.

Table O-7. Composition of solid and liquid fractions of WAO treated sawdust.

		Solid Fraction, %(w/w)						Liquid Fraction	
		Dry matter yield (g)	Extractives	Cellulose	Hemicellulose	Lignin	Ash	RS[a] (g/L)	pH
Run Order	Raw biomass	100	1.89	45.86	20.31	29.90	2.04		12.73
(I)	170 °C, 5 bar 10 min	92.11	2.53	56.47(113.42)[b]	8.08(36.64)[b]	31.49(97.00)[b]	1.43	6.61	7.64
(II)	170 °C, 5 bar 20 min	90.98	2.76	56.86(112.80)	9.91(44.39)	29.26(89.03)	1.21	10.22	7.58
(III)	170 °C, 10 bar 10 min	82.42	3.57	56.31(101.20)	10.57(42.89)	27.55(75.94)	2.00	6.11	7.65
(IV)	170 °C, 10 bar 20 min	78.73	5.58	49.07(84.24)	13.28(51.48)	30.67(80.76)	1.40	13.53	6.88
(V)	195 °C, 5 bar 10 min	79.80	5.18	51.53(89.66)	9.08(35.68)	33.14(88.37)	1.07	18.24	6.46
(VI)	195 °C, 5 bar 20 min	74.74	3.38	51.84(84.49)	11.18(41.14)	31.98(79.94)	1.62	17.23	6.67
(VII)	195 °C,10 bar 10 min	73.66	6.46	45.94(73.79)	13.88(50.34)	31.26(77.01)	2.46	24.45	6.72
(VIII)	195 °C, 10 bar 20 min	74.69	6.12	42.75(69.62)	17.11(62.92)	32.39(80.91)	1.63	19.04	6.24

[a]Reducing sugars. [b]recovery of the components is shown in parentheses.

Table O-8. Chemical composition of raw and pretreated sawdust, % (w/w) of the 2^3 CCD APO pretreatment.

	Dry matter yield (g)	Extractives	Cellulose[a]	Hemicellulose[a]	Lignin[a]	Ash
Raw biomass	100	1.89	45.86	20.31	29.90	2.04
Run Order						
1	90.58	4.17	53.71(106.08)	8.59(38.31)	31.65(95.88)	1.88
2	90.30	5.11	55.49(109.26)	8.98(39.93)	28.88(87.22)	1.62
3	85.96	4.86	52.71(98.80)	9.79(41.43)	30.53(87.77)	2.11
4	87.83	4.51	53.81(103.06)	9.83(42.51)	30.05(88.27)	1.80
5	90.28	4.37	53.57(105.46)	10.13(45.03)	30.23(91.28)	1.70
6	90.02	5.41	51.47(101.03)	9.98(44.23)	31.30(94.23)	1.84
7	91.46	4.00	54.48(108.65)	9.83(42.27)	29.78(91.09)	1.91
8	88.96	5.82	54.07(104.88)	8.01(35.08)	30.38(90.39)	1.72
9	94.46	11.70	46.85(96.50)	8.98(41.77)	30.48(96.29)	1.99
10	92.71	14.06	42.96(86.85)	9.83(44.87)	30.69(95.16)	2.46
11	89.43	5.17	53.58(104.48)	9.12(40.16)	29.71(88.86)	2.42
12	87.18	9.43	48.95(93.05)	8.60(36.92)	30.59(89.19)	2.43
13	88.62	5.13	53.10(102.61)	9.29(40.54)	29.76(88.21)	2.72
14	85.95	5.35	54.05(101.30)	7.78(32.92)	30.52(87.73)	2.12
15	87.27	7.67	52.59(100.08)	6.88(29.56)	31.48(91.88)	1.38
16	87.41	7.85	51.44(98.05)	7.35(31.63)	31.44(91.91)	1.92
17	91.89	5.89	53.14(106.48)	9.17(41.49)	29.82(91.64)	1.98
18	88.67	5.78	52.82(102.13)	8.98(39.21)	30.29(89.83)	2.13
19	91.14	5.98	51.75(102.85)	9.20(41.74)	31.06(94.68)	2.01
20	87.98	5.11	53.15(101.97)	9.75(42.24)	29.78(87.63)	2.21
21	91.87	3.84	54.62(109.42)	9.07(41.03)	30.74(94.45)	1.73
22	89.54	4.31	57.01(111.31)	8.57(37.78)	28.09(84.12)	2.02

Table O-8 Continued

	Dry matter Yield (g)	Extractives	Cellulose	Hemicellulose	Lignin	Ash
Raw biomass	100	1.89	45.86	20.31	29.90	2.04
Run order						
23	84.36	5.46	52.46(96.50)	9.26(38.46)	30.84(87.01)	1.98
24	86.34	4.83	53.79(101.27)	9.76(41.49)	29.72(85.82)	1.90
25	89.45	5.37	50.67(98.42)	11.03(48.58)	31.01(92.77)	1.92
26	89.08	5.89	52.46(101.90)	8.94(39.21)	30.92(91.81)	1.79
27	90.82	4.23	54.45(107.83)	9.09(40.65)	30.18(91.67)	2.05
28	89.46	5.02	54.16(105.65)	9.31(41.00)	29.58(88.50)	1.93
29	93.89	9.70	48.86(100.03)	8.48(39.20)	30.88(96.97)	2.08
30	91.86	12.68	45.05(90.24)	10.07(45.55)	29.89(91.83)	2.31
31	88.93	4.87	47.27(91.66)	10.21(44.71)	29.97(89.14)	2.22
32	86.78	8.78	48.99(92.70)	9.06(38.71)	31.04(90.09)	2.13
33	89.78	5.46	52.37(102.52)	9.19(40.62)	30.15(90.53)	2.83
34	83.65	5.92	52.91(96.51)	8.28(34.10)	30.57(85.52)	2.32
35	85.78	6.98	53.36(99.81)	7.14(30.16)	30.94(88.76)	1.58
36	89.01	8.05	49.61(96.29)	9.54(41.81)	31.01(92.31)	1.79
37	88.79	6.10	50.94(98.63)	10.23(44.72)	30.85(91.61)	1.88
38	86.77	4.28	54.25(102.64)	9.44(40.33)	29.83(86.57)	2.20
39	89.47	5.48	51.35(100.18)	10.07(44.36)	30.97(92.67)	2.13
40	89.28	6.11	52.38(101.97)	8.59(38.31)	29.19(87.16)	2.17

Table O-9. Chemical composition of raw and pretreated sawdust %(w/w) of the 2^2 CCD APO pretreatments.

	Dry matter yield (g)	Extractives	Cellulose	Hemicellulose	Lignin	Ash
Raw biomass	100	1.89	45.86	20.31	29.90	2.04
Run Order						
1	85.94	2.61	59.03(110.62)	6.90(29.20)	29.89(85.91)	1.57
2	93.39	3.06	58.28(118.68)	7.47(34.35)	29.73(92.86)	1.46
3	92.78	1.41	60.06(121.51)	9.04(41.30)	27.90(86.57)	1.56
4	94.05	2.19	60.64(124.36)	8.44(39.08)	27.23(85.34)	1.50
5	93.30	4.74	55.59(113.10)	9.03(41.48)	29.11(90.83)	1.53
6	88.19	2.72	58.20(111.92)	8.63(37.47)	29.06(85.71)	1.39
7	87.28	2.65	58.59(111.51)	8.63(37.09)	28.49(83.16)	1.64
8	93.72	1.89	59.18(120.94)	8.78(40.52)	27.81(87.17)	1.94
9	93.01	4.46	56.69(114.98)	9.15(41.90)	28.08(87.35)	1.62
10	94.10	3.18	56.24(115.40)	8.92(41.33)	30.20(95.04)	1.46
11	90.09	2.48	61.12(120.07)	7.23(32.07)	27.48(82.80)	1.69
12	89.34	2.95	58.03(113.05)	8.58(37.74)	28.92(86.41)	1.52
13	94.27	3.11	58.98(121.24)	6.77(31.42)	29.73(93.73)	1.41
14	87.54	3.07	60.10(114.72)	7.22(31.12)	28.34(82.97)	1.27
15	91.59	2.56	56.76(113.36)	8.84(39.86)	30.06(92.08)	1.78
16	94.03	2.73	60.99(125.05)	7.76(35.93)	26.60(93.09)	1.92
17	95.32	1.89	60.4(125.54)	6.94(32.57)	28.79(91.78)	1.98
18	90.28	3.44	59.22(116.58)	8.23(36.58)	27.78(86.90)	1.33
19	89.07	3.02	58.85(114.30)	7.93(34.78)	28.51(84.93)	1.69
20	89.94	3.35	59.61(116.91)	7.93(35.12)	27.29(82.09)	1.82
21	91.27	2.53	59.52(118.46)	7.28(32.72)	29.01(88.55)	1.66
22	94.41	3.69	58.37(120.16)	8.74(40.63)	27.28(86.14)	1.92
23	92.95	2.28	59.25(120.09)	7.83(35.83)	28.97(90.06)	1.67
24	89.54	2.04	59.42(116.02)	8.63(38.05)	28.03(83.94)	1.88
25	88.74	2.74	59.06(114.28)	6.96(30.41)	29.32(87.02)	1.92
26	95.07	2.87	58.77(121.83)	7.98(37.35)	28.43(90.40)	1.95

Table O-10. Experimental and predicted values for solid and liquid fractions of WAO treated sawdust.

	SOLID FRACTION %(w/w)						LIQUID FRACTION			
WAO Condition	Cellulose content A[a]	Cellulose content B[b]	Lignin removal A	Lignin removal B	Hemicellulose solubilization A	Hemicellulose solubilization B	Reducing sugar (g/L) A	Reducing sugar (g/L) B	pH A	pH B
I	56.47	56.99	3.00	3.06	63.36	62.58	6.61	5.59	7.64	7.64
II	56.86	56.34	10.97	10.91	55.61	56.40	10.22	11.25	7.58	7.57
III	56.31	55.79	24.06	24.00	57.11	57.90	6.11	7.14	7.65	7.65
IV	49.07	49.59	19.24	19.30	48.52	47.74	13.53	12.50	6.88	6.88
V	51.53	51.01	11.63	11.57	64.32	65.11	18.24	19.26	6.46	6.45
VI	51.84	52.36	20.06	20.12	58.86	58.08	17.23	16.21	6.67	6.67
VII	45.94	46.46	22.99	23.05	49.66	48.88	24.45	23.42	6.72	6.72
VIII	42.75	42.23	19.09	19.03	37.08	37.87	19.04	20.06	6.24	6.23

Data for Figures 5.8–5.18.
[a]experimental values. [b]predicted values.

Table O-11. Estimated *t*-values and *p*-values for the regression coefficients for WAO pretreatments.

	t-value			*p*-value		
	Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin
Constant	99.46	69.19	284.87	0.006	0.009	0.002
X_1	-6.45	-2.34	35.87	0.098	0.257	0.018
X_2	-5.48	-7.93	86.35	0.115	0.079	0.007
X_3	-2.36	-5.47	16.70	0.256	0.115	0.038
X_1X_2	-1.63	-3.68	-14.17	0.350	0.169	0.015
X_1X_3	0.96	-0.27	3.00	0.513	0.832	0.205
X_2X_3	-2.69	-1.27	-54.61	0.226	0.425	0.012

Table O-12. Experimental and predicted values for cellulose content, hemicellulose solubilization, and lignin removal in the solid fraction of 2^3 CCD APO treated sawdust.

APO	Cellulose content		Hemicellulose solubilization		Lignin removal	
Condition	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	53.71	54.11	61.69	64.18	4.12	4.55
2	53.81	53.78	57.49	62.35	11.73	12.72
3	54.48	54.47	57.73	61.90	8.91	8.39
4	54.07	54.14	64.92	68.15	9.61	9.50
5	53.58	53.00	59.84	63.73	11.14	9.22
6	48.95	48.91	63.08	65.64	10.81	10.14
7	53.1	52.69	59.46	63.75	11.79	10.30
8	54.05	53.47	67.08	71.36	12.27	13.08
9	52.59	52.97	70.44	75.02	8.12	9.38
10	51.44	51.53	68.37	72.48	8.09	7.87
11	53.14	53.00	58.51	63.73	8.36	9.22
12	52.82	53.00	60.79	63.73	10.17	9.22
13	54.62	54.11	58.97	64.18	5.55	4.55
14	53.79	53.78	58.51	62.35	14.18	12.72
15	50.67	50.78	51.42	54.96	7.23	7.07
16	52.46	53.00	60.79	63.73	8.19	9.22
17	54.45	54.47	59.35	61.90	8.33	8.39
18	48.99	48.91	61.29	65.64	9.91	10.14
19	52.37	52.69	59.38	63.75	9.47	10.30
20	52.91	53.47	65.90	71.36	14.48	13.08
21	53.36	52.97	69.84	75.02	11.24	9.38

Data for Figures 5.19-5.27.

Table O-13. Estimated *t* and *p* values for the regression coefficients for 2^3 CCD APO treated sawdust.

	t-value			*p*-value		
Factor	Cellulose	Hemicellulose	Lignin	Cellulose	Hemi-cellulose	Lignin
Constant	2.962	11.634	0.653	0.013	0.000	0.527
X_1	0.345	-12.256	-0.915	0.737	0.000	0.380
X_2	-3.765	-4.655	-0.137	0.003	0.001	0.893
X_3	-5.402	2.442	0.902	0.003	0.033	0.386
X_1X_1	-2.294	13.558	0.913	0.042	0.000	0.381
X_2X_2	3.466	-2.355	1.361	0.005	0.038	0.201
X_3X_3	-1.544	-5.185	-3.185	0.151	0.000	0.009
X_1X_2	3.321	4.686	-0.37	0.007	0.001	0.719
X_1X_3	8.629	-0.777	1.543	0.000	0.453	0.151
X_2X_3	-0.991	3.423	0.638	0.343	0.006	0.537

R^2 value = Cellulose : 0.9549, Hemicellulose : 0.9732, Lignin : 0.8404
R^2(adjusted) = Cellulose : 0.9180, Hemicellulose : 0.9513, Lignin 0.7098

Table O-14. Analysis of Variance (ANOVA) for polynomial model obtained from experimental design (WAO pretreatments).

Cellulose content					
Source	DF	SS	MS	F	P
Main effect	3	164.63	54.88	25.74	0.144
2 - way interaction	3	23.12	7.71	3.61	0.370
Residual error	1	2.13	2.13		
Total	7	189.88			
Hemicellulose Solubilization					
Main effect	3	484.44	161.48	32.76	0.130
2 - way interaction	3	74.98	24.99	5.07	0.310
Residual error	1	4.93	4.93		
Total	7	564.35			
Lignin Removal					
Main effect	3	238.61	79.54	3007.10	0.013
2 - way interaction	3	123.96	41.32	1562.10	0.020
Residual error	1	0.03	0.03		
Total	7	564.35			

DF = Degree of Freedom. SS = Sum of squares. MS = Mean Square. F = Fisher's variance ratio. P = Probability value.

Table O-15: Analysis of Variance(ANOVA) for the polynomial models obtained from 2^3 CCD APO treated sawdust

Cellulose content						
Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	49.708	49.708	5.5231	25.87	0.0000
Linear	3	17.093	9.369	3.1230	14.63	0.0000
Square	3	12.858	6.620	2.2067	10.34	0.0020
Interaction	3	19.757	19.757	6.5860	30.84	0.0000
Residual error	11	2.348	2.348	0.2135		
Lack of fit	1	0.040	0.040	0.0400	0.17	0.6860
Pure error	10	2.308	2.308	0.2308		
Total	20	52.056				
Hemicellulose solubilization						
Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	422.500	422.500	46.9444	44.39	0.000
Linear	3	186.298	179.448	59.8161	56.56	0.000
Square	3	190.355	203.789	67.9296	64.23	0.000
Interaction	3	45.847	45.847	15.2824	14.45	0.000
Residual error	11	11.634	11.634	1.0576		
Lack of fit	1	0.128	0.128	0.1283	0.11	0.745
Pure error	10	11.506	11.506	1.1506		
Total	20	434.134				
Lignin removal						
Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	109.368	109.368	12.152	6.43	0.003
Linear	3	68.183	3.658	1.2190	0.65	0.602
Square	3	36.263	38.158	12.7194	6.74	0.008
Interaction	3	4.922	4.922	1.6408	0.87	0.486
Residual error	11	20.773	20.773	1.8884		
Lack of fit	1	0.026	0.026	0.0261	0.01	0.913
Pure error	10	20.747	20.747	2.0747		
Total	20	130.141				

Table O-16. Summary of enzymatic hydrolysis conditions at various substrate concentrations and pretreatment conditions[a]

150 °C, 1% H_2O_2, 10 bar, and 45 min pretreatment conditions				
Substrate concentration, (g/L)	20	30	40	50
Weight of dry biomass (g)	0.727	1.091	1.454	1.818
Volume of 1-M citrate buffer (mL)	5	5	5	5
Volume of 10 mg/mL tetracycline solution (mL)	0.04	0.04	0.04	0.04
Volume of distilled water (mL)	3.846	3.289	2.732	2.196
Volume of cellulase enzyme (mL)	0.087	0.131	0.174	0.196
Volume of β-glucosidase (mL)	0.30	0.45	0.60	0.75
Total volume of mixture (mL)	10	10	10	10
Cellulase loading (FPU/g dry biomass)	25	37.5	50	56.3
β-glucosidase loading (IU/g dry biomass)	0[b], 15	0, 22.5	0, 30	0, 37.5
Hydrolysis time (h)	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96
120 °C, 1% H_2O_2, and 30 min, pretreatment conditions				
Substrate concentration, (g/L)	20	30	40	50
Weight of dry biomass (g)	0.588	0.882	1.176	1.470
Volume of 1-M citrate buffer (mL)	5	5	5	5
Volume of 10 mg/mL tetracycline solution (mL)	0.04	0.04	0.04	0.04
Volume of distilled water (mL)	3.985	3.498	3.01	2.544
Volume of cellulase enzyme (mL)	0.087	0.131	0.174	0.196
Volume of β-glucosidase (mL)	0.30	0.45	0.60	0.75
Total volume of mixture (mL)	10	10	10	10
Cellulase loading (FPU/g dry biomass)	25	37.5	50	56.3
β-glucosidase loading (IU/g dry biomass)	0[b], 15	0, 22.5	0, 30	0, 37.5
Hydrolysis time (h)	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96

[a]Hydrolysis temperature = 45 °C. [b]Enzymatic hydrolysis conditions with no supplemental β-glucosidase loading

Table O-17. Summary of enzymatic hydrolysis conditions for enzyme loading studies.

150 °C 1% H_2O_2 10 bar 45 min, pretreatment conditions				
Substrate concentration, (g/L)	←	40	→	
Weight of dry biomass (g)	1.454	1.454	1.454	1.454
Volume of 1-M citrate buffer (mL)	5	5	5	5
Volume of 10 mg/mL tetracycline solution (mL)	0.04	0.04	0.04	0.04
Volume of distilled water (mL)	3.337	3.232	3.129	3.060
Volume of cellulase enzyme (mL)	0.069	0.174	0.277	0.346
Volume of β-glucosidase (mL)	0.10	0.10	0.10	0.10
Total volume of mixture (mL)	10	10	10	10
Cellulase loading (FPU/g dry biomass)	10	25	40	50
β-glucosidase loading (IU/g dry biomass)	5	5	5	5
Hydrolysis time (h)	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96
120 °C 1% H_2O_2 30 min, pretreatment conditions				
Substrate concentration, (g/L)	←	40	→	
Weight of dry biomass (g)	1.176	1.176	1.176	1.176
Volume of 1-M citrate buffer (mL)	5	5	5	5
Volume of 10 mg/mL tetracycline solution (mL)	0.04	0.04	0.04	0.04
Volume of distilled water (mL)	3.615	3.510	3.407	3.338
Volume of cellulase enzyme (mL)	0.069	0.174	0.277	0.346
Volume of β-glucosidase (mL)	0.10	0.10	0.10	0.1
Total volume of mixture (mL)	10	10	10	10
Cellulase loading (FPU/g dry biomass)	10	25	40	50
β-glucosidase loading (IU/g dry biomass)	5	5	5	5
Hydrolysis time (h)	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96	2, 24, 72, 96

Table O-18. Fermentation study conditions.

		Sample (g)	Buffer (mL)	Cellulase (mL)	Yeast inoculum (mL)	YP [a] (mL)	DW [b] (mL)
(150 ^{o}C, 1%H_2O_2, 10 bar, 45 min)[c]							
	2%	6.16	5	0.433	5	5	28.41
	3%	9.24	5	0.649	5	5	25.11
(120 ^{o}C, 1%H_2O_2, 30 min)[c]							
	2%	5.02	5	0.433	5	5	29.55
	3%	7.53	5	0.649	5	5	26.82
Control flasks							
	2%	NIL	5	0.433	5	5	34.57
	3%	NIL	5	0.649	5	5	34.34

[a]Yeast and Peptone medium. [b]Distilled water. [c]Pretreatment conditions.