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공학박사학위논문

Enhancement of Lignin Degradation and Cellulose Hydrolysis Using Peracetic Acid

과산화아세트산 처리를 통한
리그닌 분해 및 셀룰로오스 가수분해의 향상

2017년 8월

서울대학교 대학원
공과대학 화학생물공학부
이 형 래

ABSTRACT

Enhancement of Lignin Degradation and Cellulose Hydrolysis Using Peracetic Acid

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The objective of this research is to develop an integrated process based on peracetic acid (PAA) to enhance degradation of lignin and separate components of lignocellulosic biomass, thereby increasing hydrolysis of the cellulose in lignocellulosic biomass.

The single process with enzymatically-generated PAA was inefficient in lignin oxidation from Kraft lignin and lignocellulosic biomass due to low concentration of PAA. High concentration of PAA or several cycles at low concentration of PAA are required for oxidizing lignin effectively. The strategy for degradation of Kraft lignin using a combined treatment with PAA

followed by supercritical water was optimized. Optimization of combined treatment enhanced degradation of Kraft lignin and produced catechol. In addition, synergistic effect of the combined treatment led to reduction in the supercritical water reaction time from 20 to 3 min.

PAA treatment limits its use due to only lignin removal from yellow poplar (*Liriodendron tulipifera*) as lignocellulosic feedstock. Therefore, sequential processes are developed for fractionation of cellulose, hemicellulose and lignin by addition hydrothermal process to enzymatically-generated PAA treatment.

First, combined pretreatment using hot compressed water (HCW) and PAA was established for separation of components from yellow poplar. The combined pretreatment started with HCW (200 ^{o}C, 1.5 MPa 15 min), which selectively solubilized most of the xylan. And then, subsequent PAA treatment increased the amount of lignin removal to 80% while single PAA treatment removed 20% of original lignin. In addition, the combined pretreatment enhanced the purity of cellulose and the accessibility of cellulase to the cellulose resulting in more efficient cellulose hydrolysis. Next, a mild pretreatment was developed by addition of sulfuric acid to hydrothermal process for fractionation of components. The optimization of dilute acid pretreatment (140 ^{o}C, 5min) was achieved for 80% xylose recovery. And then, subsequent PAA enhanced separation of lignin. The mild pretreatment using

sequential dilute acid and PAA efficiently separated the three major components of yellow poplar. This sequential pretreatment enhanced the purity of cellulose and the enzymatic digestibility of the cellulose.

Sequential process has the advantage of efficient separation of cellulose, xylose and lignin fraction. It requires only single treatment with dilute peracetic acid to remove lignin. This indicate that requirement of peracetic acid can be decreased when preceded by hydrothermal process that modified the biomass structure. In other words, accessibility of peracetic acid to lignin can be more easily done by separation of xylan.

Pretreatment of biomass with dilute acid requires high temperatures of >140 °C to remove xylan, but does not remove lignin. Finally, one-step process was developed for achieving cellulose with high purity. The addition of PAA to dilute acid pretreatment dramatically increased removal of lignin. The optimization of one-step pretreatment (120 ^{o}C, 5 min) improved both xylan and lignin removal, and resulted in more efficient enzymatic hydrolysis by increasing relative amount of cellulose. Thus, the addition of PAA dramatically increases the effectiveness of dilute acid pretreatment of biomass and reduces the requirement of temperature and time. This one-step process achieved similar hemicellulose removal, delignification and enzymatic digestibility compared to other organosolv pretreatment such as ethanol, glycerol, ethyl acetate or THF, but it has the advantage of requiring low

temperature and short time.

Overall, integrated process based on PAA could enhance degradation of lignin and purity of cellulose resulting in more efficient cellulose hydrolysis. This process could also reduce energy consumption and cellulase requirement. This research is expected to reduce dependence on petroleum-based fuels and chemicals, and provide research direction for economic success of biorefinery utilizing all the components of lignocellulosic biomass.

Keywords: lignocellulosic biomass, Kraft lignin, yellow poplar, peracetic acid, supercritical water, hot compressed water, dilute acid, pretreatment

Student Number: 2010-21008

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List of Abbreviations

AFEX: ammonia fiber explosion

ATR: attenuated total reflection

BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide

$Ca(OH)_2$: calcium hydroxide

$Ca(NO_3)_2$: Calcium nitrate

CO_2: carbon dioxide

CrI: crystalline index

CSF: combined severity factor

DA: dilute acid

DW: deionized water

EA: ethyl acetate

E. coli: *Escherichia coli*

FA: formic acid

FE-SEM: field emission-scanning electron microscopy

FPU: filter paper unit

FT-IR: Fourier transform infrared

GC/MS: gas chromatography-mass spectrometer

GHG: greenhouse gas

GVL: γ-valerolactone

5-HMF: 5-hydroxymethylfurfural

HCl: hydrochloric acid

HCW: hot compressed water

H_2O_2: hydrogen peroxide

HPLC: high performance liquid chromatography

H_2SO_4: sulfuric acid

I_{002}: intensity of crystalline portion

I_{am}: intensity of amorphous portion

IL: ionic liquid

IPTG: isopropyl-β-D- thiogalactopyranoside

KNO_3: potassium nitrate

LHW: liquid hot water

LiP: lignin peroxidase

MIBK: methyl isobutyl ketone

MnP: manganese peroxidase

M_p: peak molecular weight

2-MTHF: 2-methyltetrahydrofuran

$NaNO_3$: sodium nitrate

NaOH: sodium hydroxide

NREL: national renewable energy laboratory

PAA: peracetic acid

PFE: *Pseudomonas fluorescens* esterase

*p*NPGU: *p*-Nitrophenyl β-D-glucopyranoside unit

PSS: polystyrene sulfonate

RI: refractive index

SCW: supercritical water

S/L: solid to liquid ratio

THF: tetrahydrofuran

TMS: trimethylsilyl

XRD: x-ray diffraction

YP: yellow poplar

Chapter 1.

Research background and objectives

Chapter 1. Research background and objectives

Depletion of fossil fuels with the increasing energy demand has increased an interest about alternative energy resources. With the growing concerns about the greenhouse gas emissions and global warming, many countries have already developed to replace fossil fuels over decades. Although alternative resources such as solar power, hydropower, geothermal power and wind power are proposed as potential candidates, they are insufficient in the efficiency and economic feasibility compared to fossil fuels.

Biofuels and materials generated from biomass are considered as one of renewable, promising energy sources. The low cost, abundance and sustainability of biomass make it a potential feedstock for fuels and high-value added chemicals [1-3]. Furthermore, the use of bioenergy from biomass is more environmentally friendly. In plant growth, biomass absorbs CO_2 which is the main cause of global warming, resulting in reduction of net CO_2 to almost zero. The carbon balance of bioenergy is close to neutral compared to petroleum-derived energy, Figure 1.1. In addition, the carbon cycles of biomass takes only a few decades which is greatly shorter than that of fossil fuels [4].

For producing biofuels form biomass, a typical two steps are required: hydrolysis of the polysaccharides to sugar and fermentation of the sugars to

fuels [5]. Lignocellulosic biomass is a heterogeneous matrix that is difficult to hydrolyze due to its recalcitrance; it is structurally indestructible and resistant to microbial/enzymatic breakdown [6-8]. Additional process is necessary to overcome the recalcitrance of biomass and isolate cellulose from biomass [9].

Integrated system converting biomass to energy is called 'biorefinery system' [10]. Although cellulosic ethanol from biomass is efficient, maximal conversion of biomass such as utilization of hemicellulose and lignin is required to be economical. Hemicellulose can be hydrolyzed easily due to its highly branched structure. C5 sugars from hemicellulose can be fermented to produce biofuels and utilized as raw material such as furfural [11]. Although lignin is second most abundant, the structure of lignin is complex, resulting in limited utilization. The aromatic monomers produced from lignin through new technologies can be used in the plastic and adhesive industry.

Peracetic acid, a strong oxidants, is used for antimicrobial, disinfectant and delignification agent [12]. For delignification of biomass, peracetic acid cleaves β-aryl ether bonds, which reduces the molecular weight of lignin, and introduces hydroxyl groups, which increases the water solubility of lignin and its fragments [13, 14]. But, concentrated peracetic acid is unstable and explosive [15]. Safety concern increase the cost of production, transportation and storage. An alternative is the *in situ* generation of peracetic acid catalyzed

by perhydrolase [16, 17] or by mixing acetic acid and hydrogen peroxide [18]. While peracetic acid is effective in selective oxidization toward lignin, high concentrations or several cycles at low concentration are required for obtaining high efficiency in degradation or removal of lignin.

In this research, peracetic acid with supercritical water was applied to Kraft lignin to investigate whether it has effects on degradation of Kraft lignin and production of aromatic compounds. Then explored how peracetic acid with hydrothermal process affect lignocellulosic material (yellow poplar). Biomass pretreatment was performed using peracetic acid with hot compressed water or dilute acid, and effect on biomass composition and enzymatic digestibility was investigated. Finally, peracetic acid is applied to develop one-step pretreatment under mild conditions.

In summary, the objectives of this study are:

1. Enhanced degradation of Kraft lignin using combinatorial treatment with enzymatically-generated peracetic acid and supercritical water
2. Improved pretreatment of yellow poplar using hot compressed water and enzymatically-generated peracetic acid
3. Mild pretreatment of yellow poplar using sequential dilute acid

and enzymatically-generated peracetic acid to enhance cellulase accessibility

4. One-step pretreatment of yellow poplar using peracetic acid to enhance enzymatic digestibility

In this study, effect of peracetic acid with supercritical water on degradation of Kraft lignin was investigated. Peracetic acid with hydrothermal process was applied to fractionation of lignocellulosic biomass, thereby increasing hydrolysis of cellulose. This study is expected to provide information and research direction for development of biorefinery system.

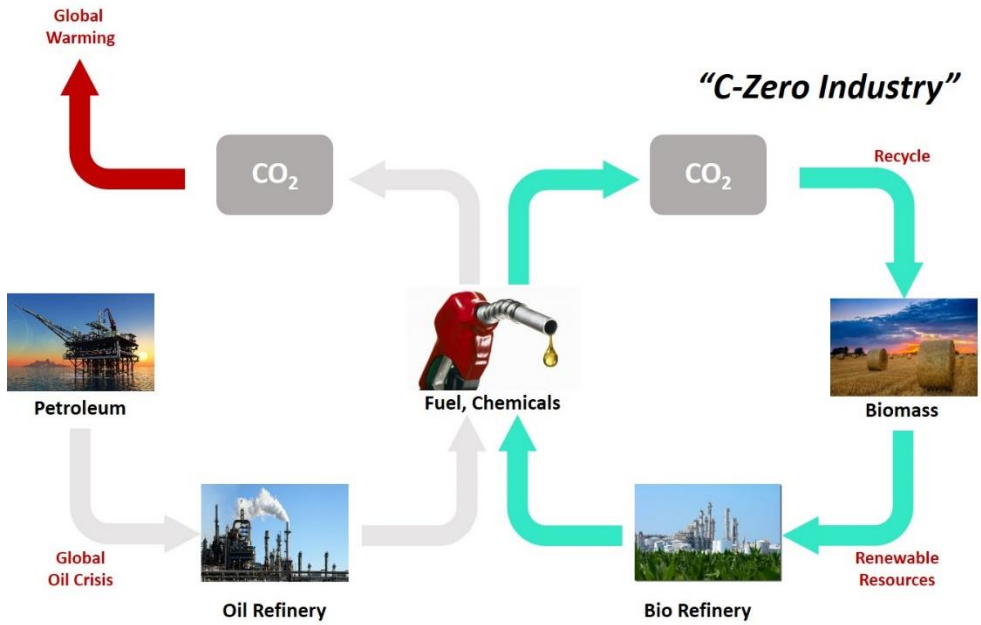

Figure 1.1 Sustainable society based on biomass.

Chapter 2.

Literature Review

Chapter 2. Literature Review

2.1 Biofuels from biomass

The production of biofuels aims to reduce greenhouse gas (GHG) and decrease oil production. The new resources such as tight oil and shale gas may postpone the depletion of fossil fuels, but there is still an ongoing issue concerning climate change and release of GHG (Figure 2.1.1). Also, countries importing fossil fuel are dependent on regions exporting fossil fuel. The increase in oil price for the last decade stimulated the interest in biofuel further (Figure 2.1.2). Biofuels can reduce the world's dependence on fossil fuels and limit the amount of GHG released into the atmosphere. While fossil fuels need 280 million years for replenishment, biomass is renewable over time ranging from one month to 80 years [4]. It is reported that the total amount of biomass in the earth is estimated at 184 billion tons per year and about 100 million tons per year of biomass is generated in Korea [19]. In addition, biofuel can use existing infrastructure unlike other alternative resources.

Biofuels are classified into different categories. Primary biofuels are used in an untreated form for heating or power production. Secondary biofuels are produced by the treatment of biomass. Secondary biofuels are also classified

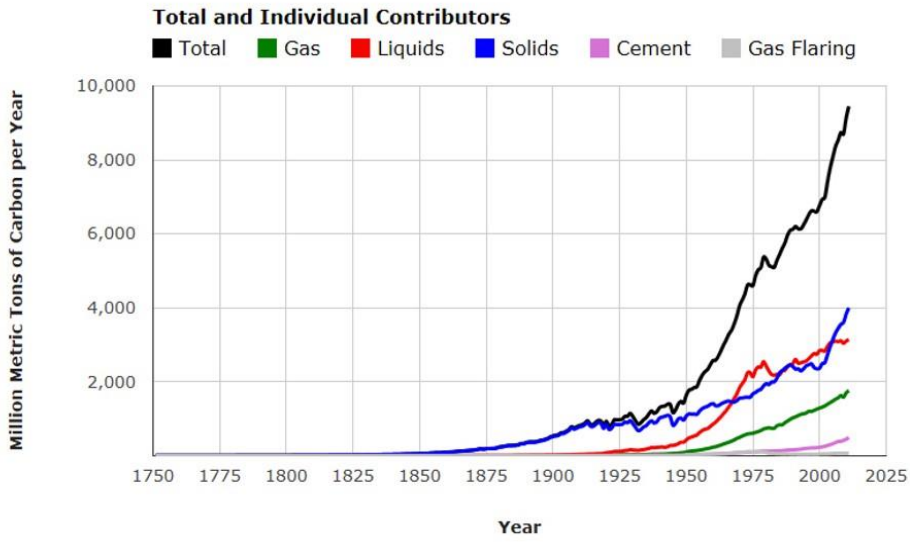

Figure 2.1.1 Growing trends of carbon emission [21].

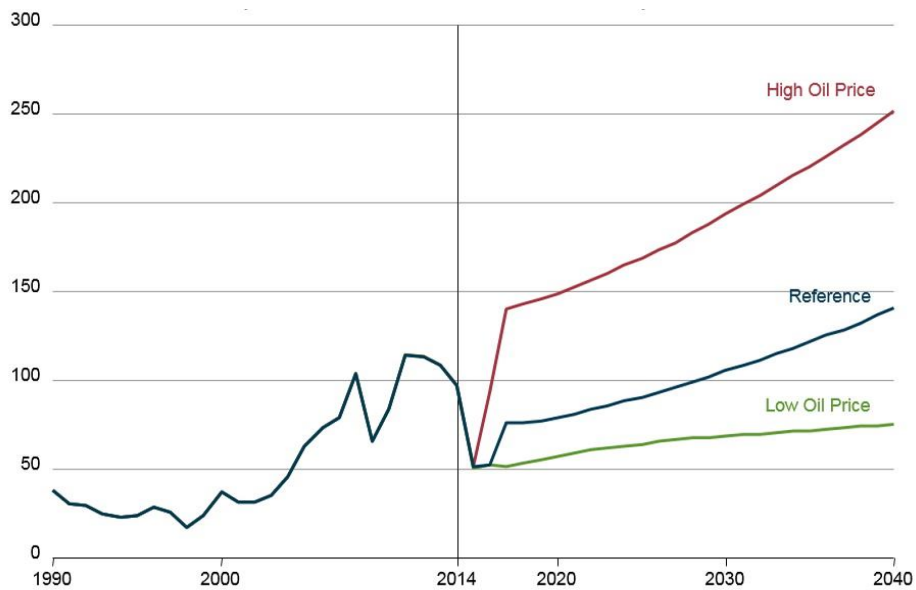

Figure 2.1.2 Trend of fuel price in U.S. (dollars per barrel) [22].

into first, second and third-generation biofuels [20]. First-generation biofuels were produced from the sugars and grains such as corn and sugarcane, or seeds and vegetable oils such as soybean, canola and palm. The first-generation biofuels were widely used as raw materials for bioethanol and biodiesel in the United States and Brazil. It has the advantage to produce biofuels due to simple and low cost process. However, production of first-generation biofuels has a problems in competition with food production and use of large-scale farmland causing damages to environment. Also, it is limited by the increasing price of sugar or vegetable oils.

Second-generation biofuels are produced from different feedstocks: either non-edible crop waste or non-edible plant biomass. The use of biomass for production of biofuels has received greater interest because of relatively low costs, high productivity, sustainable supply and non-edible resources [23]. However, an additional process is required for production of biofuels due to constitute and complex structure of biomass [24].

Third-generation biofuels are mostly related to microscopic organisms such as microbes and microalgae for oil production. Especially, algae is considered as renewable resources for next generation due to fasting-growth, tolerance to various environments conditions and high productivity. However, algae has some limitations such as lipid extraction and dewatering of biomass, and transportation and processing in the sea.

2.2 Lignocellulosic biomass

Lignocellulosic biomasses are promising resources for the production of fuels and high value-added chemicals [25]. Lignocellulosic biomass is complex material composed of intertwined cellulose, hemicellulose and lignin. The unique structure and its chemical and physical properties make biological deconstruction difficult [8, 26].

2.2.1 Cellulose

Cellulose is a linear polysaccharide composed of a linear chain of β-(1→4) linked D-glucose units, Figure 2.2.1, which is the most abundant natural polymer on the earth. Cellulose has compact crystalline structure due to the regular arrangement of molecules and formation of well-ordered hydrogen bonding networks between its hydroxyl groups. Therefore, it is generally not soluble in water or organic solvents and has resistance to acids and alkalis. Partial cellulose chains which are unorganized result in the amorphous region of cellulose. 20~300 cellulose polymer chains are formed microfibrils that then form cellulose fiber. Generally, lignocellulosic biomass consists of 40~50% of cellulose [8, 27, 28].

2.2.2 Hemicellulose

Hemicellulose is heterogeneous polysaccharide composed of pentoses (xylose and arabinose), hexoses (glucose, mannose and galactose) and sugar acids, Figure 2.2.2. Hemicellulose has branched structure with short side chains and relatively low molecular weight, existing in amorphous form [29]. These different structures are more accessible to enzymatic hydrolysis than cellulose [24, 30].

2.2.3 Lignin

Lignin is abundant polymers following by cellulose in nature and 20~30% of lignocellulose consists of lignin. Lignin is a rigid biopolymers composed of aromatic monomers linked by carbon-carbon or ether bonds, Figure 2.2.3. Lignin plays a role in structural support and mechanical rigidity in the cell wall. Lignin is an amorphous three-dimensional polymer composing of three different phenyl propionic alcohol units (*p*-coumaryl, coniferyl and sinapyl alcohol), Figure 2.2.4. Due to the variety of three units and random linkage between them, lignin structure is quite complex and differ from plant to plant. [31].

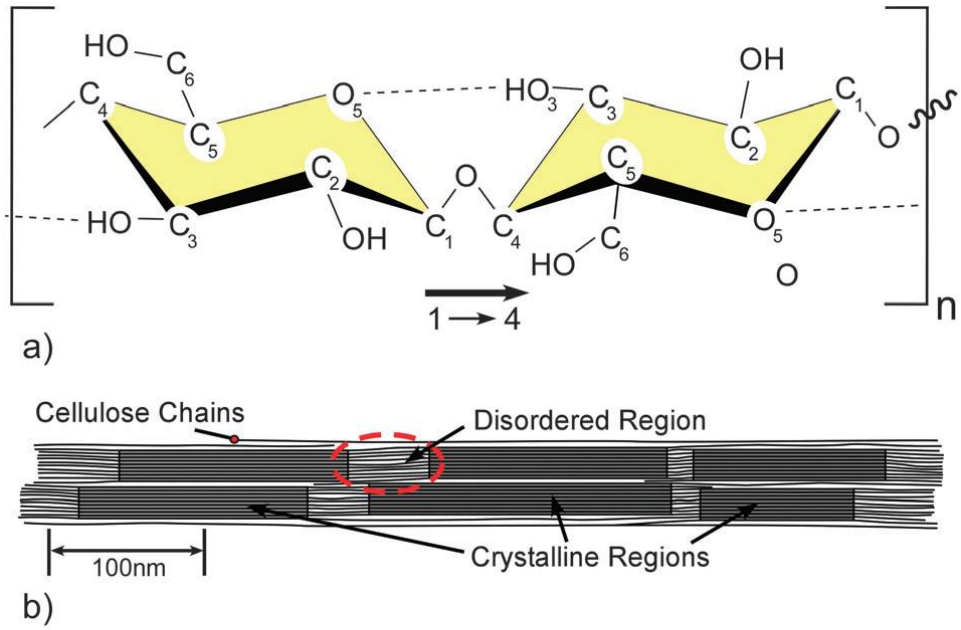

Figure 2.2.1 Schematics of (a) cellulose chain repeat unit (b) cellulose microfibril showing the crystalline and amorphous regions [32].

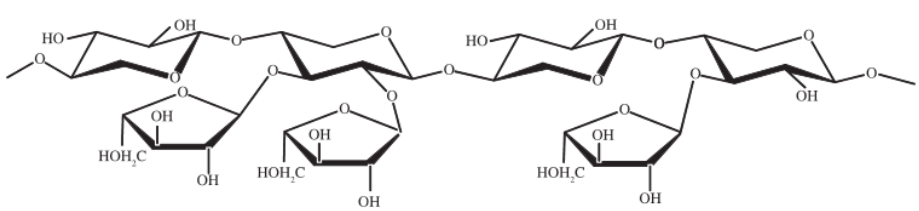

Figure 2.2.2 Structure of hemicellulose (L-arabino-D-xylane) [33].

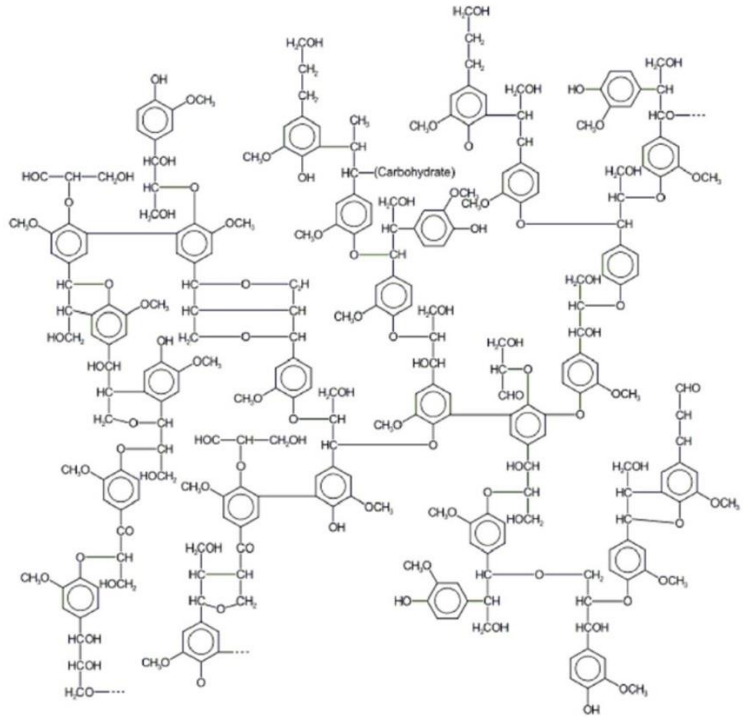

Figure 2.2.3 Structure of lignin [34].

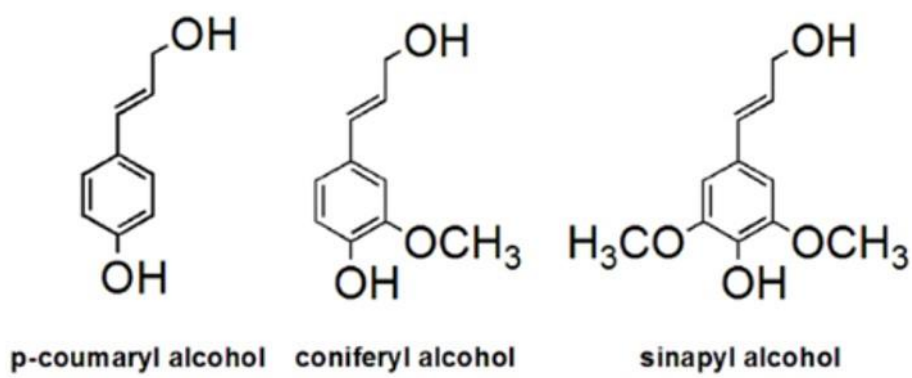

Figure 2.2.4 Three phenyl propane monomers in lignin [34].

2.2.4 Application of cellulose, hemicellulose and lignin

Because lignocellulosic biomass is a mixture of various components, additional process such as pretreatment is required for utilizing it as a fuel. But, additional process lead to economic problems. Although the competitive price of biomass-based process has increased in recent years, the development of economically feasible processes is essential. Until recently, pretreatment of lignocellulosic biomass was focused on cellulose recovery and saccharification yield for the production of bioethanol alternative to sugarcane or corn, but it has limits. Therefore, it is necessary to study the application of cellulose, hemicellulose and lignin for a higher value-added products, Table 2.2.1. Especially, utilization of material from biomass for raw material of chemical products can higher added value than using biomass for fuel.

2.3 Pretreatment of lignocellulosic biomass

The lignocellulosic biomass forms a complex interwoven structure that hinders the access of enzymes to the cellulose. Pretreatment removes hemicellulose and lignin, and enhances the accessibility of cellulose to enzymes, thereby overcoming the recalcitrance of lignocellulosic biomass, Figure 2.3.1 [9, 35]. An effective pretreatment should require low capital and

Table 2.2.1 Application of cellulose, hemicellulose and lignin

Component	Application
Cellulose	➢ Biofuel: bioethanol, biobutanol ➢ Material: cellulose fiber (Lyocell, Tencel), cellulose derivative (filter, film) ➢ Raw material: putrescine, FDCA
Hemicellulose	➢ Biofuel: bioethanol ➢ Material: prebiotics, functional sugar ➢ Raw material: furfural, levulinic acid
Lignin	➢ Fuel ➢ Material: antioxidant, carbon fiber, adhesive ➢ Raw material: catechol, vanillin

operation costs, low energy consumption, minimal loss of polysaccharides and produce cellulosic substrate that is easily hydrolyzed with low enzyme loadings [24, 36].

A variety of pretreatment methods have developed to improve the efficiency of subsequent saccharification and fermentation process for last several decades [37]. Each pretreatment can be applied differently depending on the species, constitutes and environmental, and classified into four types [37, 38]. The characteristics, advantages and disadvantages of each pretreatment will be explained on detail in the following section.

2.3.1 Biological pretreatment

Biological pretreatments have been performed to apply microorganisms to pretreat various lignocellulosic biomass. Microorganisms such as brown-, white- and soft-rot fungi degrade lignin and hemicellulose selectively. Among these fungi, white-rot fungi are the most effective for the pretreatment of lignocellulosic biomass [36, 39]. White-rot fungi mainly produced lignin-degrading enzymes such as laccases, lignin peroxidases (LiPs) and manganese peroxidases (MnPs). These enzymes had obvious effects on lignin degradation [40]. Various white-rot fungi have been conducted to pretreat different lignocellulosic biomass showing good efficiency in the delignification [40, 41].

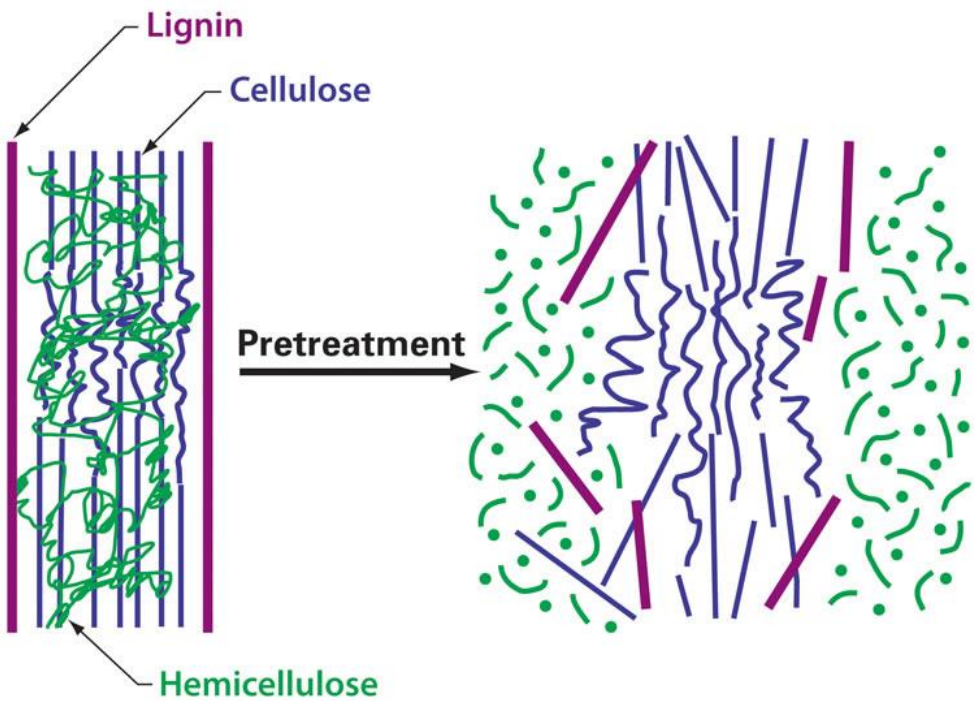

Figure 2.3.1 Schematic of roles of pretreatment on lignocellulosic material [42].

Biological pretreatment has low energy, low capital cost, mild pretreatment conditions and no chemicals requirement. Although this environmentally friendly methods has many advantages, depolymerization of lignin by fungi requires long pretreatment time, low hydrolysis yield, continuous monitoring of microorganism growth and a series of washing steps, which are less attractive for industrial application [36].

2.3.2 Physical pretreatment

Physical pretreatments generally mean mechanical comminution such as chipping, grinding or milling. The main objective of physical pretreatment is to increase the saccharification efficiency of lignocellulosic biomass by reducing their size, crystallinity of cellulose, and increasing specific surface area. Mechanical comminution can reduce size of lignocellulosic biomass ranging from μm to mm. The chipping or grinding is used for bulk lignocellulosic biomass into small fragments. After chipping or grinding, lignocellulosic biomass is milled into fine powder using various milling methods such as ball, two-roll and colloid milling [43]. Milling can significantly decrease the particle size of lignocellulosic biomass and crystallinity of cellulose, thereby improving enzymatic hydrolysis [44].

However, lignin, hemicellulose and other components remain intact. The high energy consumption is required for high enzymatic hydrolysis, which is

related to the final particle size after mechanical comminution [29].

Irradiation (gamma rays, electron beam or microwave) and extrusion methods have been conducted. These methods are not a typical physical pretreatment method, but used as assistance to other pretreatment.

2.3.3 Chemical pretreatment

2.3.3.1 Alkali pretreatment

The alkali pretreatment is mainly effective for lignin solubilization. Alkali pretreatment can be conducted under mild pretreatment conditions and generate less amount of byproducts than dilute acid pretreatment. Among various alkali reagents, NaOH and $Ca(OH)_2$ are commonly used to pretreat lignocellulosic biomass.

NaOH pretreatment can modify the structure of lignocellulosic biomass by swelling cellulose and removing lignin and hemicellulose [43]. After swelling, the surface area of cellulose is increased and the degree of polymerization and crystallinity is decreased. Removal of lignin and acetyl group from hemicellulose reduces steric hindrance of enzymes and increases accessibility of cellulose to enzymes, thereby increasing cellulose digestibility [45]. $Ca(OH)_2$, known as lime, also has been applied to pretreat lignocellulosic biomass for high enzymatic hydrolysis. Furthermore, use of $Ca(OH)_2$ is more safe than using a strong base and can be easily recovered by reaction with

CO_2 [35, 37].

However, this pretreatment remains most of xylan fraction in the solids, which is required hemicellulose hydrolytic enzymes (e.g. xylanase) during enzymatic hydrolysis step [46]. In addition, this pretreatment removes substantial amount of xylan with lignin, which is difficult to utilize xylose [47].

2.3.3.2 Acid pretreatment

Acid pretreatment has been conducted for various lignocellulosic biomass and promising method for industrial applications. Acid pretreatment is aimed to improve the accessibility of enzymes to cellulose by hydrolyzing hemicellulose component from lignocellulosic biomass. In other words, high recovery of xylose in the liquid and most of cellulose in the solid residue are expected by acid pretreatment.

The concentrated acid pretreatments can generate a large amount of wastewater and byproduct, and cause equipment corrosion, which makes less attractive [37]. The dilute acid pretreatments are the most widely studied and relatively close to commercialization. Sulfuric acid is the most commonly used and hydrochloric acid, phosphoric acid and nitric acid have also been studied [35]. Dilute acid pretreatment is performed at temperatures ranging from 140 to 200 ^{o}C with a H_2SO_4 concentration of 0.5-2.5%, providing 80-

90% hemicellulose recovery.

However, acid pretreatment require highly severe conditions to obtain high hydrolysis yield. These harsh conditions will form fermentation inhibitors such as acetic acid, 5-hydroxymethylfurfural (HMF) and furfural through further degradation of sugar in liquid, Figure 2.3.2 [48, 49]. In addition, acid pretreatment is not effective in the removal of lignin. Acid pretreatment usually produces solid residue with high amount of lignin. Additional process for lignin removal is required to further enhance the cellulose digestibility.

Organic acids such as fumaric acid, maleic acid or oxalic acid are also studied to enhance hydrolysis yield [50].

2.3.3.3 Organosolv process

The organosolv process can extract lignin from lignocellulosic biomass by using organic or aqueous organic solvents, which can increase surface area and reduce amount of lignin.

Various organic solvents such as methanol, ethanol, ethylene glycol, organic acid and acetone have been used to pretreat lignocellulosic biomass [14]. Organosolv process is usually performed with assistance of various catalysts (acids and alkalis) [52, 53]. This process results in a cellulose in the solid, a hemicellulose-based sugar in aqueous liquid and lignin fragments in organic solvent.

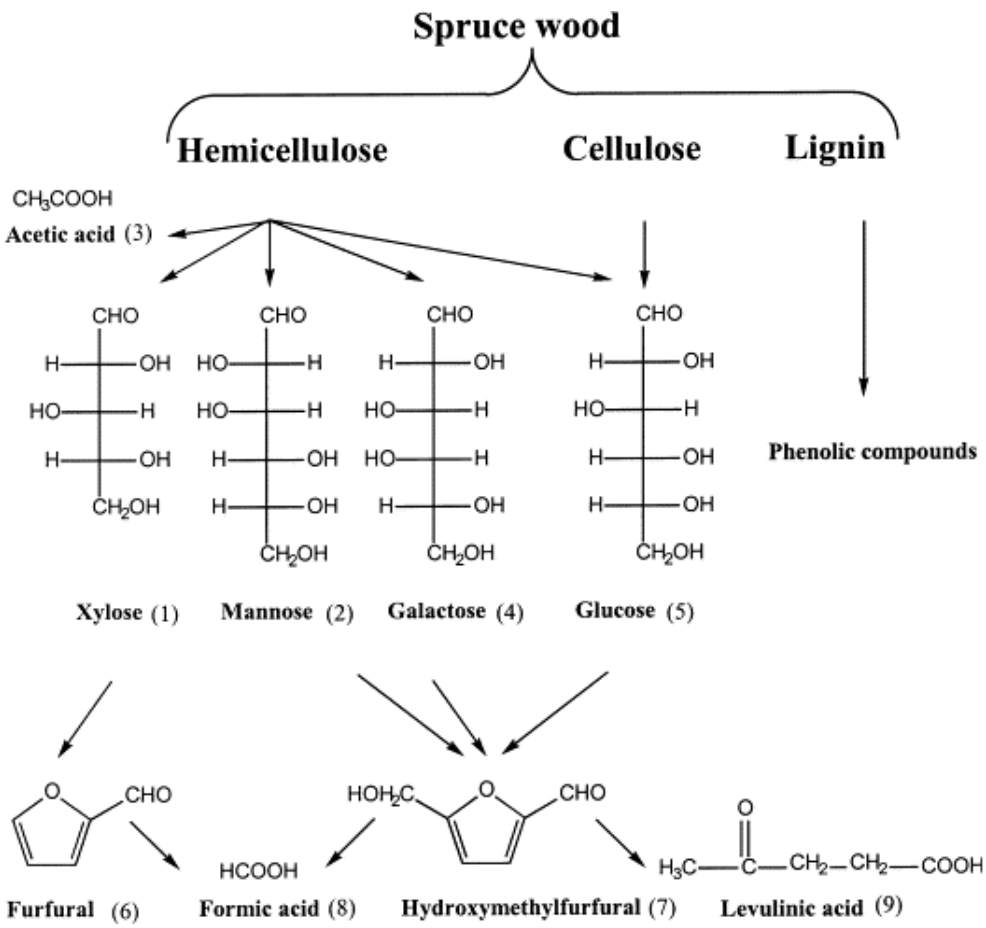

Figure 2.3.2 Formation of inhibitory compounds during hydrolysis of lignocellulosic materials [51].

In addition, the organosolv process produce the original form of lignin, which can be utilized in many fields [14, 54, 55].

However, most of the organic solvents used in organosolv pretreatment are highly volatile and toxic. The additional washing step is necessary because organic solvents have a negative effect on subsequent saccharification and fermentation processes. Furthermore, the effective recycling and recovery strategies of the organic solvent are required to reduce costs.

2.3.3.4 Ionic liquids (ILs) pretreatment

Ionic liquids (ILs) consist of large organic cations and small inorganic anions. ILs are generally present in a liquid phase at temperature below 100 ^{o}C and have a little volatility and high ionic conductivity. In addition, the physical properties of ILs can be variously controlled by combining the cations and anions [56].

The IL pretreatments have received much attention that some ILs can exhibit an excellent effect on dissolution of lignocellulosic biomass, thus making homogenous solutions. Commonly, the regenerated biomass obtained by addition of antisolvent is carbohydrate-rich solid, whereas lignin is separated in the liquid fraction. The IL pretreatments can modify the structures of lignocellulosic biomass by extracting lignin, decrease the crystallinity of cellulose and increase the accessibility of cellulases to the cellulose [57]. The

acidic IL pretreatments can obtain the oligomeric forms by hydrolysis of cellulose [58], which means that the amount of enzyme used in subsequent saccharification process can be dramatically reduced.

Despite these advantages, most ILs are very expensive. The effective recovery and recycling strategies, and continuous decrease in the price of ILs are required.

2.3.3.5 Peracetic acid pretreatment

Formic acid and acetic acid are reacted with hydrogen peroxide, producing performic acid and peracetic acid (PAA). These peracids are strong oxidants and very effective in delignification, which have been used for pretreating lignocellulosic biomass [59-63]. Performic acid has been used in Milox process for pulping, but it has low stability.

During peracetic acid pretreatment, electrophilic HO^+ ions are formed. The HO^+ ions are reacted with lignin through ring hydroxylation to form hydroquinone, resulting in forming soluble carboxylic acid. Peracetic acid also oxidizes hydroxyl group and cleaves of β-aryl ether bonds, leading to reduce molecular weight of lignin, Figure 2.3.3 [12, 13]. Peracetic acid pretreatment removed the high amount of lignin by HO^+ ions from lignocellulosic biomass [61]. In addition, peracetic acid pretreatment can increase surface area and expose the cellulose fiber, resulting in enhancing

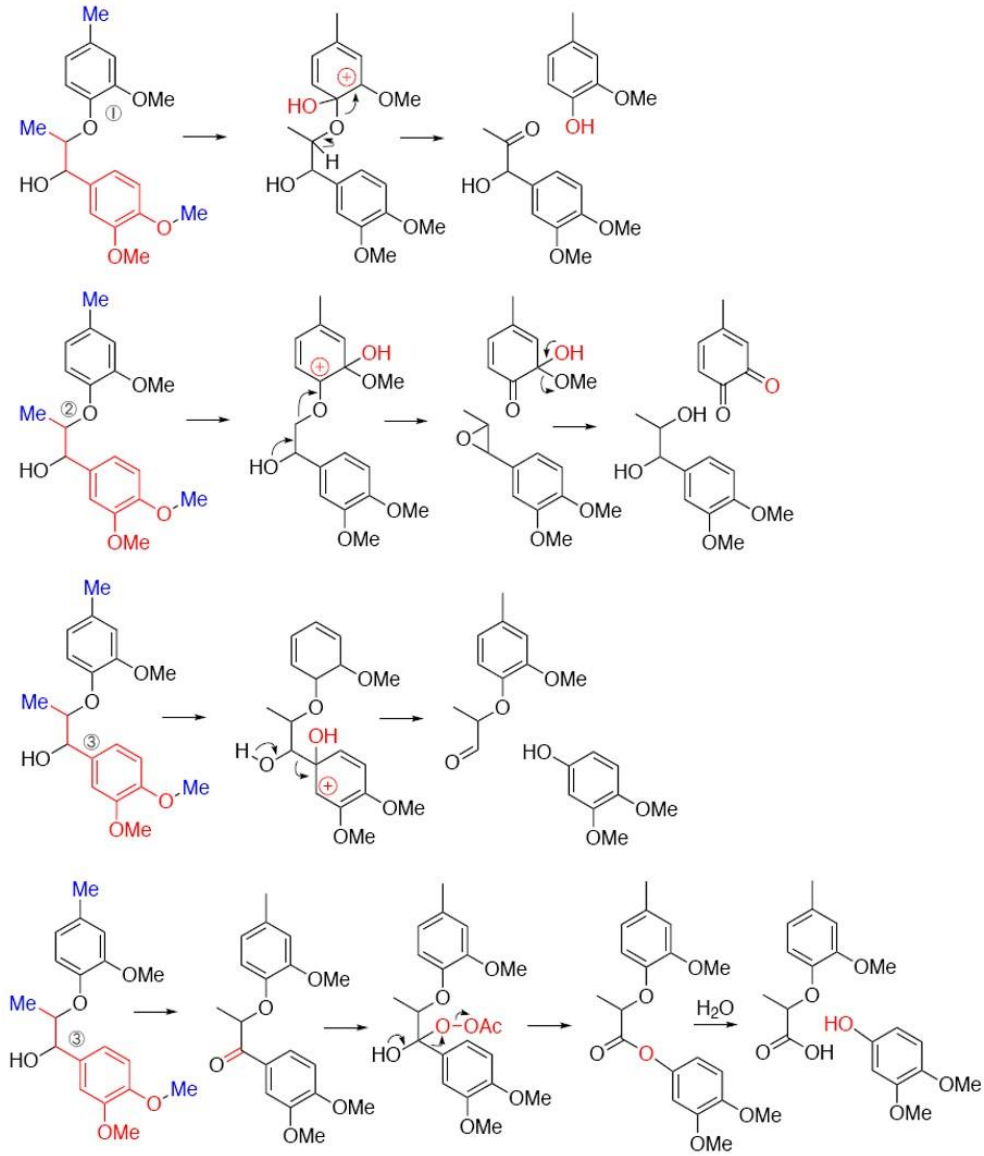

Figure 2.3.3 Lignin cleavage with peracetic acid [13].

cellulose digestibility [14]. Alkaline pretreatment followed by PAA pretreatment can also improve the enzymatic digestibility with reducing amount of PAA from 50 wt% to 15 wt% based on biomass [63].

PAA pretreatment can be performed at mild conditions, which avoids the formation of sugar degradation products (furfural and 5-hydroxymethyl furfural) that are toxic to fermentative microorganisms [64]. In addition, the loss of carbohydrate is lower due to the selectivity of PAA toward lignin [65]. There are four ways to chemical synthesis of peracetic acid, Figure 2.3.4. First, hydrogen peroxide is mixed with acetic acid. Perhydrolysis of an ester (second) or acetyl chloride (third) is thermodynamically controlled. Fourth, oxidation of acetaldehyde is performed below 0 ^{o}C to generate peracetic acid due to explosion.

Despite good performance of peracetic acid, the cost of related to the use of peracetic acid limits its practical use. Peracetic acid is 4 times expensive compared to hypochlorite. And concentrated form of PAA is unstable and explosive. Safety concerns increase the cost related production, transportation and storage of peracetic acid. [15, 66]. An alternative is the *in situ* generation of peracetic acid from hydrogen peroxide and acetate esters catalyzed by perhydrolase enzymes [16, 17], which avoids the hazards of concentrated form and may reduce costs.

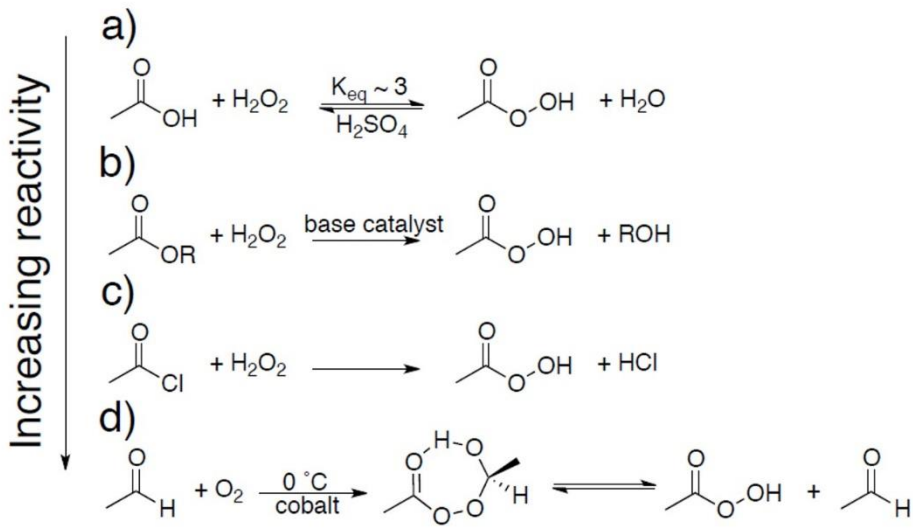

Figure 2.3.4 Chemical synthesis of peracetic acid [67].

2.3.4 Physicochemical pretreatment

2.3.4.1 Steam explosion

Steam explosion is one of the most widely used physicochemical pretreatment method. Lignocellulosic biomass is exposed to high temperature/pressure steam for a few seconds or minutes, and then the pressure is dropped in a moment. Steam explosion can simultaneously cause chemical hydrolysis by high temperature steam and physical alteration of structure by rapid release of pressure. Hemicellulose is hydrolyzed and lignin is decomposed through steam explosion [36]. Steam explosion has also been developed with the addition of catalyst. Although acid catalysts can cause equipment corrosion and increase the amount of byproducts, the hydrolysis of hemicellulose can be accelerated, thus reducing the reaction temperature. And partial hydrolysis of cellulose can improve the cellulose hydrolysis [68, 69]. Especially, steam explosion using sulfuric acid catalyst is more effective in pretreatment of conifer than non-catalytic steam explosion.

Steam explosion is advantageous to remove hemicellulose for relatively short time (less than a few minutes). However, the production of fermentation inhibitors and high temperature requirements (160-260 ^{o}C) are challenges to overcome.

2.3.4.2 Liquid hot water

Liquid hot water (LHW) pretreatment, known as autohydrolysis or hot compressed water, uses only water without additional catalysts or chemicals and does not require rapid release of pressure unlike steam explosion [37]. LHW pretreatments are commonly performed at 160-240 ^{o}C and a pressure is introduced to maintain the liquid phase of the water [70, 71]. LHW pretreatments can mainly hydrolyze hemicellulose and remove about 80% of hemicellulose depending on conditions. The mechanism of this pretreatment is similar to dilute acid pretreatment or steam explosion.

LHW pretreatment has advantages on no chemicals requirement, low equipment corrosion and removal of most hemicellulose. In addition, this pretreatment can cause high sugar recovery and relatively low fermentation inhibitors compared to steam explosion. However, the conditions with high temperature and pressure increase the energy requirements.

2.3.4.3 Ammonia fiber explosion (AFEX)

In ammonia fiber explosion (AFEX) process, the biomass is treated with liquid ammonia at high temperature and pressure. After a certain reaction time, the vaporization of ammonia occurs through instantaneous decompression, which can result in physical changes such as swelling of the cellulose fiber. While other pretreatment processes should be separated into a liquid and solid phase, only solid residues are obtained from AFEX process of lignocellulosic

biomass [37].

AFEX process can reduce the crystallinity of the cellulose and affect the binding between lignin and carbohydrates by swelling of the cellulose, thus increasing hydrolysis yield [40, 72]. In addition, AFEX process is very attractive because of its advantages such as high energy efficiency, negligible byproducts and less loss of carbohydrates. However, safety due to use of high temperature and pressure ammonia is considered, and recovery strategies of ammonia are required to reduce the operating costs [73].

2.4. Yellow poplar (*Liriodendron tulipifera*)

The Korea Forest Service recommended yellow poplar (*Liriodendron tulipifera*, *tulip tree*) for lignocellulosic biomass [74]. Yellow poplars are not true poplars, but related to magnolias. They are fasting-growing (approximately 20 years harvest cycle) hardwoods indigenous to eastern North America, but planted world-wide because they are acclimated to many environments by their extensive root system. In addition, they sequesters more amounts of carbon dioxide and accumulates a higher fraction of polysaccharides than other wood species such as beech, larch and corn stover Table 2.4.1.

Table 2.3.1 Major advantages and disadvantages with different pretreatment methods

Pretreatment method	Advantages	Disadvantages
Biological	Low-capital cost, Low energy, No chemical requirement, Environmentally friendly	Long pretreatment time, Low hydrolysis yield Continuous monitoring microorganism growth
Milling	Increasing surface are, Reducing crystallinity	High energy consumption
Alkali	Lignin solubilization, Mild condition	Hemicellulase (xylanse)
Acid	High recovery of hemicellulose	Inhibitory compounds (furfural, HMF, acetate)
Organosolv	Separation of lignin	High price of organic solvents
Ionic liquid	Reducing crystallinity	High price of ionic liquids
Peracetic acid	Lignin solubilization, Mild condition	High price, Hazardous at concentrated form
Steam explosion	Hydrolysis of hemicellulose	High equipment cost Inhibitory compounds
Liquid hot water	High recovery of hemicellulose	High equipment cost
Ammonia fiber explosion	Cellulose swelling, Increasing surface area	High equipment cost

Table 2.4.1 Chemical composition of biomass species

Biomass	Chemical composition (%)							
	Glu [a]	Xyl [b]	Gal [c]	Ara [d]	Man [e]	AIL [f]	ASL [g]	Ref.
Yellow poplar	37.9	17.0	4.9	1.9	2.0	20.7	5.6	This study
Beech	38.8	16.4	1.1	1.1	1.0	24.2	1.3	[75]
Lodgepole Pine	42.6	6.9	2.2	1.6	10.9	27.0	-	[76]
Corn stover	30.7	15.0	-	-	-	14.2	-	[77]

[a] Glucan, [b] xylan, [c] galactan, [d] arabinan, [e] mannan, [f] acid insoluble lignin, [g] acid soluble lignin.

Chapter 3.

Experimental procedures

Chapter 3. Experimental procedures

3.1 Production of recombinant *Pseudomonas fluorescens esterase*

Pseudomonas fluorescens esterase (PFE) F162L gene containing a C-terminal $(His)_6$ tag [17] was transferred to pET-28b(+) expression vector (EMD biosciences, USA). After transformation of *Escherichia coli* BL21 (DE3, Novagen, USA), cells were grown in lysogeny broth-kanamycin medium at 37 °C. The expression of PFE was induced when the cell density reached an O.D. of 0.6 by addition of IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM final concentration) and the cells were further incubated at 37 ^{o}C for 4 h. To isolate the protein, the culture was centrifuged at 8,870 g and 4 ^{o}C for 20 min, the supernatant was discarded and the cell pellet was suspended in His-binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 8.0). The cells were disrupted by sonication at 25% amplitude and 5 s pulse on/off for 3 min. The cell lysate was centrifuged (13,680 g for 30 min) and the supernatant was filtered through a 0.45 μm membrane filter. The PFE F162L protein was purified using HisTrap HP column (GE healthcare, UK) with fast protein liquid chromatography (AKTA, GE healthcare), desalted in 20 mM Tris-HCl buffer (pH 8.0) using HisTrap

Desalting column (GE healthcare), and stored at –70 °C until use.

3.2 Enzymatic generation of peracetic acid

Peracetic acid (PAA) was generated by the enzyme-catalyzed perhydrolysis of ethyl acetate (500 mM) with hydrogen peroxide (1.0 M) in sodium phosphate buffer (100 mM, pH 7.2) using 0.5 mg/mL of purified PFE [16]. Approximately 90 mM PAA was generated in 10 min, which was used directly to pretreat yellow poplar (YP) particles. The concentration of PAA was measured by the methyl tolyl sulfide assay [78].

3.3 Supercritical water treatment of Kraft lignin

Supercritical water (SCW) treatment was performed with 2.1 mL of water and 50 mg of Kraft lignin. They were placed in a stainless steel batch type reactor and then the reactor was tightly sealed. It was immersed in molten nitrate salt (a mixture of $NaNO_3$, KNO_3 and $Ca(NO_3)_2$, ratio of 24:46:30) preheated at 420 ^oC. The reactor was finally pressurized to 50 MPa and the reaction time ranged from 1 to 30 min. After the required reaction time, the reactor was removed from the bath and cooled immediately in cold water bath. All experiments were run in triplicate. After releasing gas and pressure, the reaction mixture was filtered through a P4 glass filter crucible (DURAN, Germany) and the solid residue was washed with water. The solid residue was

dried at 105 °C. A filtrate was neutralized with 1 M HCl to pH 5-6, and kept in 4 °C.

3.4 Peracetic acid treatment of Kraft lignin

The reaction mixture containing 10 mg/mL of Kraft lignin or SCW-treated lignin, 500 mM hydrogen peroxide, 600 mM ethyl acetate and 0.5 mg/mL PFE was shaken at 37 °C and 200 rpm for 24 h. After reaction, the reaction mixture was kept in 4 °C.

3.5. Analysis of degraded lignin

3.5.1 Size exclusion chromatography

The relative molecular weights and their distribution of degraded lignin was analyzed by high-performance liquid chromatography (HPLC, Surveyor Plus, Thermo Scientific, USA) equipped with TSK-GEL W3000G column (300 × 7.5mm) and photodiode array (PDA) detector. The column was eluted with 50 mM NaOH (pH 11.5, 20% acetonitrile) as mobile phase at a flow rate of 0.6 mL/min for 60 min. The polystyrene sulfonate was used as standards for molecular weight calibration.

3.5.2 Gas chromatography-mass spectrometer

The filtrate was extracted by adding ethyl acetate (Sigma, Korea) and vortexed vigorously. The mixture were centrifuged at 13,000 rpm for 5 min and upper organic layer was vacuum dried. The vacuum dried sample was dissolved with ethyl acetate for the analysis of volatile compounds by gas chromatography-mass spectrometer (GC/MS) (TRACE GC ULTRA, ITQ1100, Thermo Scientific, USA). Volatile compounds were converted to their trimethylsilyl (TMS) derivatives by incubating for 20 min at 70 ^{o}C with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The TMS-derivatives were analyzed using column of TR-5MS (30 m × 0.25 mm × 0.25 μm, Thermo Scientific) with temperature programming (50 ^{o}C for 2 min, 10 ^{o}C/min to 280 ^{o}C and hold for 2 min).

3.6 Hot compressed water pretreatment of yellow poplar

Hot compressed water (HCW) pretreatment was performed in batch reactor [79] made of stainless steel (SUS 316) with a total volume of 26.7 mL. Approximately 1.3 g of raw YP particles and deionized water (solid: liquid = 1:15, w/w) were placed in the reactor, tightly sealed and immersed in molten salt bath (a 24:46:30 mixture of $NaNO_3$, KNO_3, $Ca(NO_3)_2$) preheated to 200 ^{o}C (1.5 MPa) with horizontal shaking of the reactor for 15 min (excluding warm-up time: 2 min 30 s). The temperature and reaction time was selected as optimal condition for removal of xylan [79]. After reaction the reactor was

removed from the bath and immediately cooled to room temperature in a cold-water bath. The mixtures were filtered through DURAN P4 glass filter crucibles (DURAN, Germany) and the solid residues were washed with hot water to remove water-soluble degradation products until the filtrate was neutral. A portion of solid residue was dried at 105 ^{o}C for 24 h for characterization, while remaining solid residue was stored at 4 ^{o}C until next pretreatment and enzymatic hydrolysis.

3.7 Dilute acid pretreatment of yellow poplar

Raw YP wood particles (0.20 g) were suspended in dilute sulfuric acid (0.05 or 0.1 M; DA) and sealed in a glass tube (10 mL). The vessel heated to 120 to 160 ^{o}C in a microwave reactor (CEM Discover; CEM Corporation, USA, 300 W, 300 ^{o}C maximum temperature). The reaction reached the desired temperature within 3 min and that time marked the start of the reaction. At the end of the treatment, the glass tube was removed from the reactor and cooled to room temperature within 2 min by immersion in water. The suspension was filtered through a P4 glass filter crucible (DURAN, Germany) and the solid residues washed with distilled water until the pH of the filtrate was neutral. A portion of this washed solid residue was dried at 105 ^{o}C for analysis and the rest was stored at 4 ^{o}C for further pretreatment or enzymatic hydrolysis.

3.8 Peracetic acid pretreatment of yellow poplar

Raw YP or pretreated solid (1.0 g based on dry weight) was suspended in PAA solution (40 mL) and shaken at 60 ^{o}C for 6 h. The suspension was filtered through a P4 glass filter crucibles (DURAN) and the solid was washed with distilled water to remove PAA, hydrogen peroxide and ethyl acetate. A portion of the washed solid residue was dried in an oven at 105 ^{o}C for 24 h for analysis and another portion was stored at 4 ^{o}C for enzymatic hydrolysis.

3.9 One-step pretreatment of yellow poplar

The solution was prepared by mixing 30% hydrogen peroxide and acetic acid (1:1; v/v) at room temperature for 72h. Sulfuric acid (50 or 100 mM) was added to the solution as a catalyst. Concentration of peracetic acid in the solution was measured according to the methyl tolyl sulfide assay [78].

0.2 g of raw YP wood particles were loaded into a 10 mL glass tube followed by the addition of 4 mL mixture. The mixture contained a 1:9, 2:8 or 3:7 volume ratio of peracetic acid to water. Depending on the experiment, sulfuric acid was added to form a 20, 50 or 100 mM sulfuric acid solution. The glass tube was sealed and put into a microwave reactor (CEM Discover; CEM Corporation, USA) at the desired temperature for the designated residence time. The reaction reached the desired temperature within 3 min and started at that time. After pretreatment, the glass tube was removed from the reactor

and cooled down within 1 min by immersing quickly in water. The slurry was filtered through a P4 glass filter crucible (DURAN, Germany) and the residue was washed extensively with water. After washing, the residue was stored at 4 ^{o}C for enzymatic hydrolysis and the other fraction of residue was dried in an oven at 105 ^{o}C for compositional analysis and characterization. The filtrate was analyzed by HPLC to determine the amount of solubilized sugars and degraded compounds.

3.10 Enzymatic hydrolysis

Enzymatic hydrolysis of raw YP or pretreated solids was performed at 50 ^{o}C for 72 h in a shaking incubator at 150 rpm following NREL standard procedures [80]. A substrate amount equivalent to 0.1 g glucan (based on dry weight) was loaded into a glass vial (15 mL) containing citrate buffer (7.4-7.7 mL, 50 mM, pH 4.8, 0.2 mg/mL sodium azide as antimicrobial agent) The slurry was put into the incubator at 50 ^{o}C for 1 h before addition of enzyme. An enzyme mixture of cellulase (Celluclast 1.5L, 319 FPU/mL, Sigma, USA) and β-glucosidase (Novozyme 188, 118 *p*NPGU/mL Sigma) was added to each vial to make a total volume of 10 mL. Aliquots (200 µL) were sampled periodically to follow the progress of the hydrolysis by HPLC. Controls reactions contained the same mixtures without substrate or without enzymes. Enzymatic hydrolysis was conducted in triplicate and the average values are

reported.

3.11 Sugar and inhibitor analysis

After pretreatment and hydrolysis, sugars and inhibitors in filtrate were separated on an Aminex HPX-87H column (300 × 7.8 mm, 5 μm; Bio-Rad, USA) at 60 °C eluted with aqueous sulfuric acid (5 mM) at a flow rate of 0.6 mL/min using an HPLC (UltiMate 3000, Thermo Fisher Scientific, USA) with a refractive index (RI) detector. The calibration standards were glucose, xylose, acetic acid, furfural and 5-hydroxymethyl furfural (HMF) (Sigma).

3.12 Analysis of solid residue

3.12.1 Chemical composition analysis

Acid-insoluble lignin and carbohydrates were measured by according to NREL standard protocol [81]. Raw YP or pretreated solid (0.3 g) was suspended in sulfuric acid (72%, 3 mL) and stirred for 1 h at room temperature. The mixture was diluted to 4% sulfuric acid with distilled water, heated to 121 °C in an autoclave for 1 h, cooled to room temperature and filtered through P4 glass filter crucibles (DURAN). All the samples were measured in triplicate and the average value was used. The filtrate was used for sugar analysis and the washed solid residue was dried at 105 ^{o}C overnight to

determine acid insoluble lignin.

3.12.2 X-ray diffraction (XRD) analysis

Crystallinity of the solids was determined using a powder X-ray diffractometer (D8 Advance, Bruker, Germany). The solids were scanned from 2θ =5^{o} to 40^{o} at 40 kV and 40 mA. The crystallinity index (Crl), which indicates the relative portion of biomass crystallinity, was calculated according to [82]:

$$\text{CrI}\ (\%) = \frac{I_{002} - I_{am}}{I_{002}} \times 100\%$$

where I_{002} is the maximum intensity of the crystalline portion of biomass (crystalline cellulose) at 2θ = 22.0-22.4^{o}, and I_{am} is the minimum intensity of the amorphous portion of biomass (amorphous cellulose, hemicellulose and lignin) at 2θ = 18.7^{o}.

3.12.3 Fourier transform infrared (FT-IR) analysis

FTIR spectrophotometer (Nicolet 6700, Thermo Scientific, USA) with an ATR (Attenuated Total Reflection) accessory was used to measure the spectra using 32 scans from 4000 to 650 cm^{-1} with a resolution of 4 cm^{-1}.

3.12.4 Field emission-scanning electron microscopy (FE-SEM)

The surface morphological characteristics of the solids were observed using a field emission-scanning electron microscope (FE-SEM; AURIGA, Carl Zeiss, Germany) with 2 kV of acceleration voltage. Before observation, solid samples were placed on aluminum stubs using carbon tape and sputter-coated with platinum.

3.13 Definition of terms

3.13.1 Estimation of pretreatment efficiency

The data were estimated with the following definitions:

Solid recovery (%)

$$= \frac{Yellow\ poplar_{Pretreated}[g]}{Yellow\ poplar_{Untreated}\ [g]} \times 100$$

Xylose yield (%)

$$= \frac{Measured\ amount\ of\ xylose\ in\ prehydrolysate\ [g]}{Total\ amount\ of\ xylan\ [g]} \times 100$$

3.13.2 Estimation of enzymatic hydrolysis efficiency

Glucose yield (%)

$$= \frac{Measured\ amount\ of\ glucose\ generated\ by\ enzymatic\ hydrolysis\ [g]}{Total\ amount\ of\ glucan\ [g]}$$

$\times 100$

Chapter 4.

Enhanced degradation of Kraft lignin using combinatorial treatment with enzymatically-generated peracetic acid and supercritical water

Chapter 4. Enhanced degradation of Kraft lignin using combinatorial treatment with enzymatically-generated peracetic acid and supercritical water

4.1 Introduction

Lignocellulose, which is the most abundant biomass on Earth, is composed of cellulose, hemicellulose and lignin [29]. Among them, lignin has not received much attention because of its complicated structure and high resistance to chemical and biological degradation [83]. In common practice, lignin is viewed as a waste material or burned as a low value fuel to produce electricity and power [84, 85]. Although lignin has been used as an industrial material, only 2% of lignin is used for dispersants or binding agents [86, 87]. However, lignin is the only renewable source of aromatics not present in other components of biomass [3, 88]. When chemical or biological methods are found to degrade the lignin properly and efficiently, lignin is anticipated to replace the conventional petroleum-based aromatic feed stocks such as benzene, toluene, xylene and phenol [31].

Lignin is an amorphous three-dimensional polymer in which hydroxypheylpropane units are connected with ether and C-C bonds [89, 90]. Several chemical treatment methods such as thermochemical, hydrolytic,

reductive and oxidative processes have been developed to break down lignin to fragments by disconnecting the bonds [91]. Among them, supercritical water reaction release cellulose from lignocellulosic materials and it also has a significant effect on lignin degradation [92]. Degradation of lignin in supercritical fluids has been studied to obtain bio-oil or phenolic chemicals [93, 94]. Most supercritical water reactions operates under high temperature and pressure that do not require catalysts. Separation or extraction process of various degraded products from solvent can be easily done. In addition, it is especially advantageous for lignin degradation due to its economic, safe and rapid (e.g. less than a minute and a few seconds) process. However, achieving a supercritical condition requires a rather big mechanical power, which limits the size of the reactor for mass production [95].

Peracetic acid (PAA) is a strong oxidant used as a bleaching agent or disinfectant [96]. PAA degrades lignin more effectively than other oxidants, like hydrogen peroxide and acetic acid. Moreover, it selectively degrades lignin without degradation of other components in biomass; cellulose and hemicellulose [97]. However, PAA has some drawbacks; expensive cost and highly explosive nature at high concentration [98]. To solve these problems, the method for *in situ* generation of PAA using perhydrolase was suggested [16], instead of using commercial PAA directly.

In this research, Kraft lignin was used as a substrate material. It has a

different structure and characteristic with the native lignin, but it is the major form of the lignin produced in the pulp and paper industry [31]. The Kraft lignin was treated by lignin-degrading methods with supercritical water (SCW) and peracetic acid (PAA) to mass-produce the aromatic compounds. Therefore, the degradation efficiency of each and combined treatment were compared.

4.2 Degradation of Kraft lignin using enzymatically-generated peracetic acid

To confirm the lignin degradability of PAA, Kraft lignin was first treated with different concentrations of PAA. 10 mM PAA treatment on Kraft lignin showed almost no difference in the molecular weight as compared with the control (data not shown). However, the elution time of PAA-treated lignin was delayed with the increasing concentration of PAA in size exclusion chromatogram. When 50 mM PAA was used, the peak molecular weight (M_p) of degraded lignin decreased by approximately 50% (data not shown). With 100 mM PAA, its molecular weight was 960 g/mol based on polystyrene sulfonate (PSS) calibration, which means the M_p decreased down to about 40% of the control (data not shown). Instead of using PAA directly, PAA can be generated *in situ* from hydrogen peroxide and ethyl acetate by *Pseudomonas fluorescens* esterase (PFE) (Figure 4.1(A)). The concentration of PAA

steadily increased up to 90 mM for 20 min, but it gradually decreased afterwards. PAA was just 4 mM after 24 h due to the spontaneous decomposition (Figure 4.1(B)). These results indicate that PAA is steadily generated and reacted with lignin during 24 h. Degradation of Kraft lignin by PAA produced using PFE was similar with using 100 mM PAA (Figure 4.2). The elution time of major peak was longer than that obtained with 50 mM PAA, and it was almost the same as that obtained with 100 mM PAA. Its peak molecular weight was approximately 960 g/mol. This indicates that the molecular weight decreased down to ~38% of the control. In addition, treating PAA on Kraft lignin for 48 and 72 h did not change the molecular weight.

4.3 Optimization of supercritical water treatment

For efficient lignin degradation, variables such as temperature, pressure, and time were changed in order to optimize the supercritical water (SCW) treatment conditions. The experiment was carried out under various reaction time lengths, ranging from 1 to 30 min, with fixed maximum temperature and pressure condition (420 ^{o}C and 50 MPa). Color of SCW-treated lignin seemed to get more transparent with increasing time until 15 min (Figure 4.3(A)). In other words, brown color of Kraft lignin solution would lightened with increasing reaction time. But, no difference in color was observed after 15 min. Under different reaction time, SCW treatment for 20 min degraded lignin

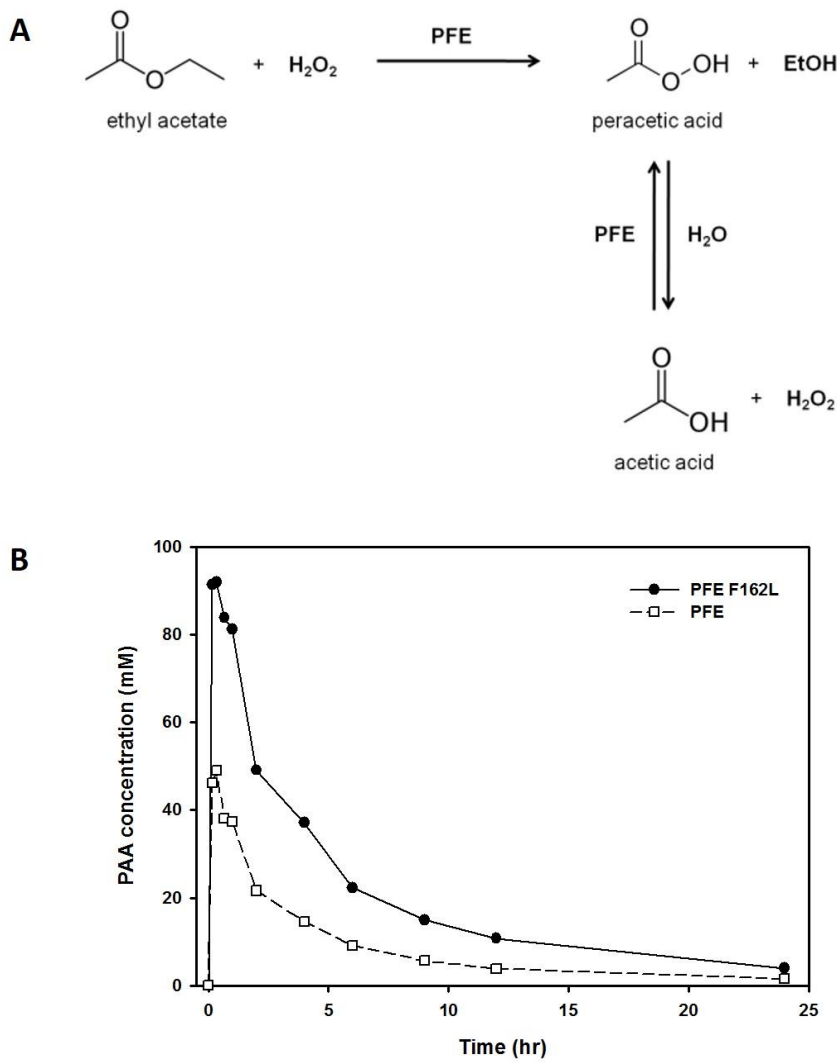

Figure 4.1 Enzyme-catalyzed generation of peracetic acid. (A) Peracetic acid is formed by perhydrolysis of ethyl acetate. Peracetic acid was hydrolyzed to acetic acid. *Pseudomonas fluorescens* esterase (PFE) catalyzes both reactions. (B) About 90 mM peracetic acid is formed in 10 min. A mutant enzyme (PFE F162L) generated about 2 times higher peracetic acid than wild-type PFE.

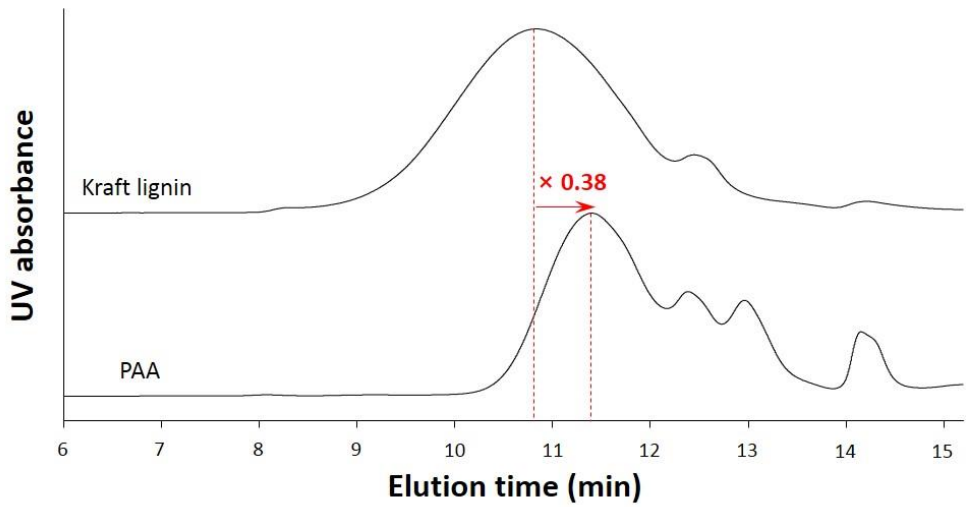

Figure 4.2 Degradation of Kraft lignin using PAA treatment. The peak molecular weight decreased down to ~38% of the control.

more efficiently than shorter reaction time (Figure 4.3(B) and Table 4.1). While M_p of lignin treated within 15 min was ~840 g/mol, the M_p value from the 20 min reaction was 640 g/mol. This indicate that the molecular weight decreased down to ~25% of the control. The M_p value from 15 min reaction was apparent result of insufficient reaction time. The degradation continued, and tended to equilibrate at 20 min. After 20 min, it was observed that the lignin degradation was almost complete. Increasing the reaction time (30 min) did not change the value of M_p further.

4.4 Enhanced degradation of Kraft lignin using SCW and PAA

After establishing optimal condition of each process, PAA treatment with SCW treatment was combined to enhance the degradation of Kraft lignin. During the SCW treatment, ethanol was added to reduce the solid residue and increase the solubility of degraded products [99, 100]. The use of alcohols for solvolysis of the ethers has been proved for lignin decomposition [101, 102]. By addition of 50% ethanol in the SCW reaction, the M_p of degraded lignin was ~840 g/mol. SCW treatment followed by PAA treatment did not have a significant change in molecular weight distribution. No change was due to the almost a near-complete degradation from the optimal SCW reaction and very low concentration of PAA for further degradation. But, synergistic effect

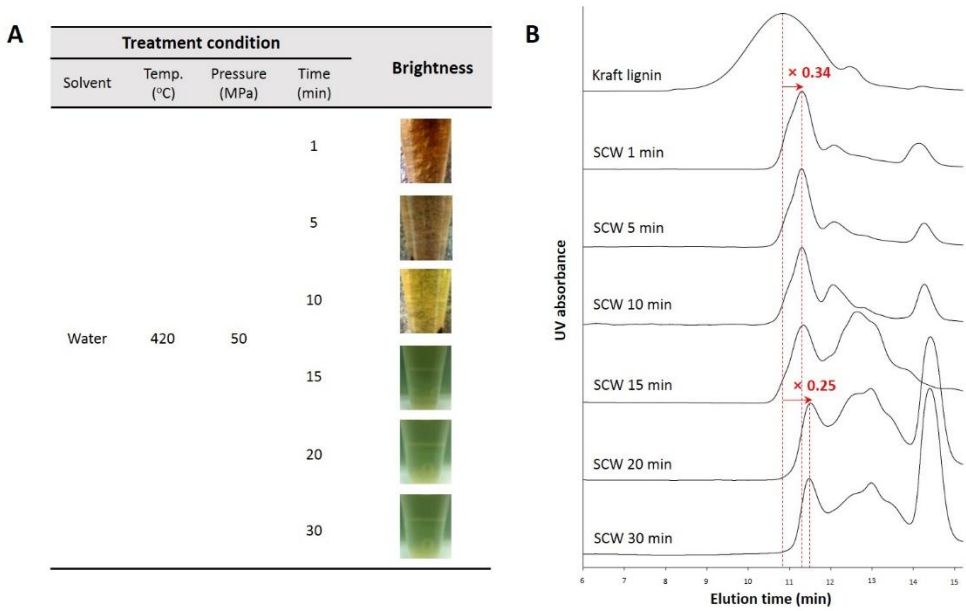

Figure 4.3 Optimization of supercritical water treatment on Kraft lignin. (A) Color change of the degraded lignin under different reaction time. Brown color of Kraft lignin solution would lightened with increasing reaction time. But, no difference in color was observed after 15 min. (B) GPC analysis of the degraded lignin under different reaction time. Supercritical water treatment for 20 min degraded lignin more efficiently than shorter reaction time. The peak molecular weight decreased down to ~25% of the control.

Table 4.1 Optimization of supercritical water treatment on Kraft lignin

Treatment condition				**Peak molecular weight (M_p) (g/mol)**
Solvent	Temp. (°C)	Pressure (MPa)	Time (min)	
				2,500
Water	420	50	1	840
			5	840
			10	840
			15	840
			20	640
			30	640

occurred when PAA and SCW were used in a reverse order (Table 4.2). As a result of combined treatment, M_p of degraded lignin was 560 g/mol, which is smaller than that of sole SCW- or PAA-treated lignin. Although low concentration of PAA cleaved some bonds in lignin, harsher SCW condition reacted with the remained lignin, which led to the enhanced degradation of Kraft lignin.

In addition, PAA followed by SCW treatment made a difference in the degraded products as compared with the sole treatment of PAA or SCW (Figure 4.4). The volatile compounds extracted from each sample with ethyl acetate were analyzed by GC. The SCW-treated lignin converted to a lot of small compounds as compared with Kraft lignin and PAA-treated lignin. SCW+PAA-treated lignin had no difference with solely SCW-treated lignin in the products. But, PAA+SCW treatment had a difference peak of products in the chromatogram. Degraded products consisted of compounds from SCW treatment and five additional compounds (Figure 4.5(A)). Using MS analysis, these additional compounds were identified as catechol, 4-methyl catechol and 3-methyl catechol, and proved by comparison with authentic compounds (Figure 4.5(B), (C)). When the subsequent SCW reaction time increased from 10 to 20 min, the amount of compounds was decreased. PAA+SCW treatment was optimized by reducing SCW reaction time to yield high amount of catechol. Regarding the amount of catechol, 3-methyl catechol and 4-methyl

catechol reached their maximum at 3 min, and the amount of these compounds decreased after 3 min (Figure 4.5(D)). It indicated that the catechols were produced in the early phase and degraded over specific time due to the harshness of supercritical water. Various kinds of phenols such as 2-methoxy phenol, 2-methoxy-5-methyl phenol and 3-ethyl phenol were also produced at 1 min. Based on the results, degradation mechanism of Kraft lignin may be changed by addition of PAA treatment prior to SCW treatment, which produced catechols from Kraft lignin.

Furthermore, the M_p of degrade lignin from PAA+SCW treatment for 3 min was 670 g/mol, which was slightly bigger than the smallest M_p from 5 min reaction time (Table 4.3). But, this PAA+SCW treatment for 3 min had a similar effect to SCW treatment for 20 min on M_p.

Therefore, the reaction time of SCW treatment in combinatorial treatment was shortened to 3 min as compared with that of sole SCW treatment, which was an expected advantage for the degradation of Kraft lignin.

Table 4.2 Enhanced degradation of Kraft lignin using two-step process

Treatment condition								Peak molecular weight (M_p) (g/mol)
1st step				2nd step				
Solvent	Temp (°C)	Pressure (MPa)	Time (min)	Solvent	Temp (°C)	Pressure (MPa)	Time (min)	
-								2,500
Water/ Ethanol	420	50	20	-				840
Water/ Ethanol	420	50	20	PAA	37	-	24 h	840
PAA	37	-	24 h	-				960
PAA	37	-	24 h	Water/ Ethanol	420	50	20	560

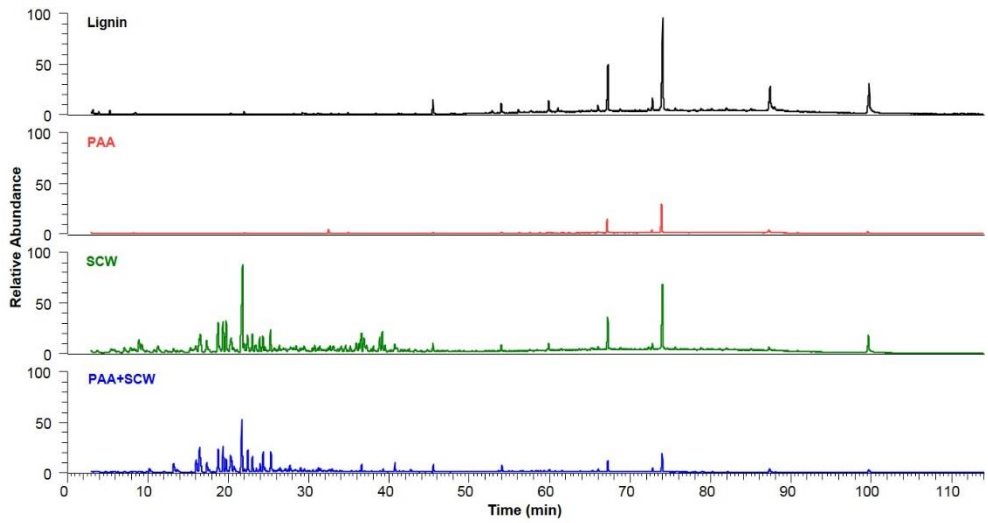

Figure 4.4 GC analysis of the degraded lignin under various reaction treatments.

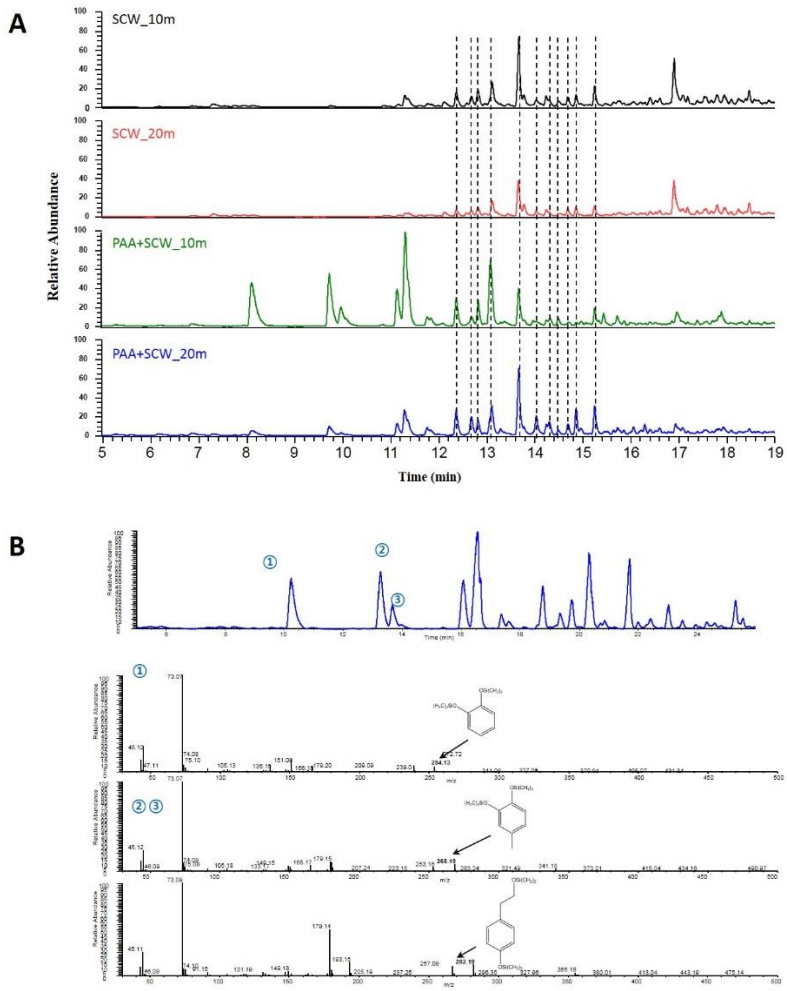

Figure 4.5 Production of catechol from Kraft lignin using combined treatment. (A) Comparison of the degraded lignin between sole-SCW treatment and combined treatment (B) MS analysis of compounds.

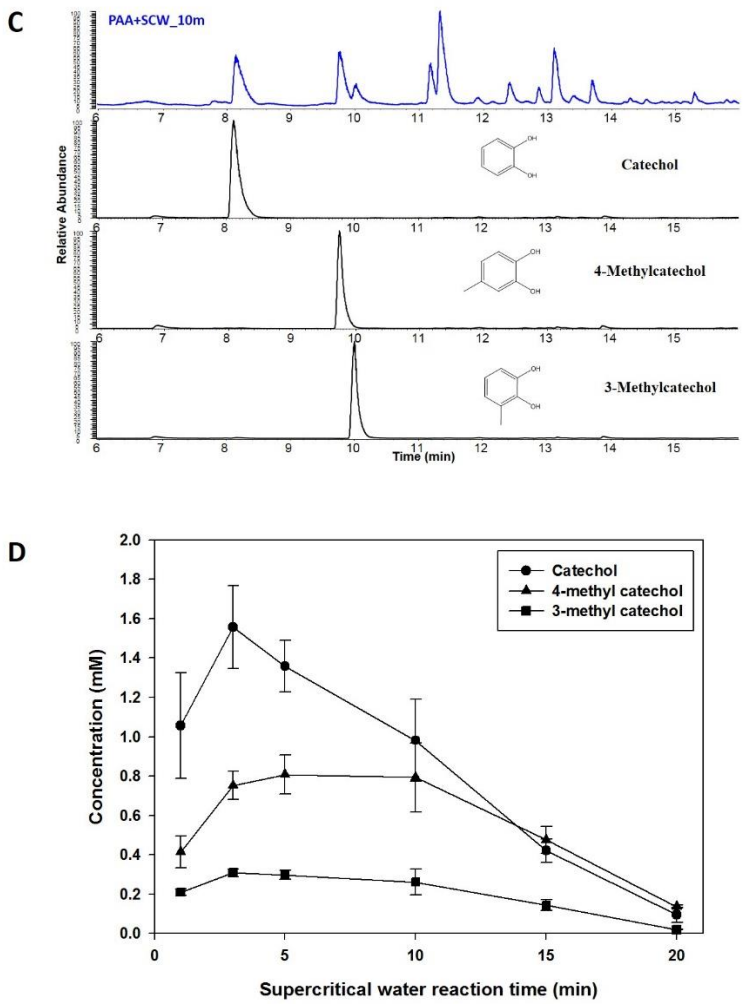

Figure 4.5 Production of catechol from Kraft lignin using combined treatment. (C) Comparison of compounds with authentic compounds (D) Dependency of catechol production on supercritical water reaction time.

Table 4.3 Optimization of supercritical water treatment on PAA-treated Kraft lignin

Treatment condition								Peak molecular weight (M_p) (g/mol)
1st step				2nd step				
Solvent	Temp (°C)	Pressure (MPa)	Time (min)	Solvent	Temp (°C)	Pressure (MPa)	Time (min)	
-								2,500
PAA	37	-	24 h	-				960
Water	420	50	20	-				640
PAA	37	-	24 h	Water/ Ethanol	420	50	1	670
							3	670
							5	560
							10	560
							20	560
							30	560

4.5 Conclusions

A hybrid method, using the peracetic acid (PAA) produced from *Pseudomonas fluorescens* esterase (PFE) with the supercritical water (SCW) treatment was used to enhance degradation of Kraft lignin and produce catechol. A mutant enzyme was able to produce more PAA than the wild-type. This resulted in reduction in the reaction time with lignin from 72 hr to 24 hr. Under fixed temperature and pressure, 420 ^{o}C and 50 MPa, 20 min reaction time was found to be the optimal time length. While PAA in the SCW-treated lignin has no effect, PAA prior to SCW treatment enhanced degradation of Kraft lignin. The peak molecular weight (M_p) of Kraft lignin, PAA- and SCW-treated lignin was 2,500, 960 and 640 g/mol, respectively. The combinatorial (PAA+SCW) treatment decreased molecular weight of lignin to ~560 g/mol. Also, the combinatorial treatment newly produced catechols; catechol, 3-methyl catechol and 4-methyl catechol from the Kraft lignin that was nonexistent in the sole SCW treatment. This synergistic effect of combinatorial treatment led to the reduction in the SCW reaction time to 3 min and produced catechols from the Kraft lignin.

Chapter 5.

Improved pretreatment of yellow poplar using hot compressed water and enzymatically-generated peracetic acid

Chapter 5. Improved pretreatment of yellow poplar biomass using hot compressed water and enzymatically-generated peracetic acid

5.1 Introduction

Replacement of fossil fuels with biomass-derived fuels is driven by the depletion of fossil fuels and by the emission of greenhouse gases from their combustion. Biomass is a complex material composed of intertwined cellulose, hemicellulose and lignin. The low cost, abundance and sustainability of biomass make it a potential feedstock for fuels and high-value chemicals [1]. Biomass may come from agricultural residues, energy crops, softwood or hardwood. The Korea Forest Service recommended yellow poplar (*Liriodendron tulipifera*, tulip tree) for lignocellulosic biomass [74]. Yellow poplars are not true poplars, but related to magnolias. They are fasting-growing (approximately 20 years harvest cycle) hardwoods indigenous to eastern North America, but planted world-wide because they acclimate to many environments and sequester larger amounts of carbon dioxide than other tree due to their extensive root system. Effective pretreatment of yellow poplar biomass is an important research goal [103, 104].

Producing biofuels from lignocellulosic biomass requires, first, deconstruction and hydrolysis of the polysaccharides to sugars and, second, fermentation of the sugars to fuels. The deconstruction of lignocellulosic biomass to fermentable sugars is inefficient because its complex structure blocks access to the oligosaccharides and the hydrophobic lignin binds the hydrolytic enzymes. This inefficiency is an obstacle to produce biofuels because it increases their cost [7]. Pretreatment of biomass breaks up the complex structure and may remove hemicellulose and lignin, thereby leaving a disrupted structure where enzymes can more efficiently hydrolyze the oligosaccharides to sugars.

Hot compressed water (HCW), also called autohydrolysis or subcritical water, is a promising pretreatment method. HCW pretreatment uses only water, which does not need to be recycled unlike methods that use organic solvents or catalysts [105, 106]. HCW pretreatment selectively hydrolyzes the hemicellulose component of biomass to monosaccharides yielding a cellulose- and lignin-rich solid residue [107]. The removed solution contains xylose and other five-carbon sugars, which can be used for fermentation [35].

One limitation of the HCW pretreatment is the limited removal of the lignin. The remaining lignin interferes with the cellulase-catalyzed hydrolysis by binding cellulases and by hindering their access to the cellulose [45]. Higher cellulase loading can overcome this interference by lignin, but higher loading

increases the cost. Adding pretreatment steps to the HCW pretreatment to remove lignin could increase the yields of glucose without requiring high cellulase loading.

Peracetic acid (PAA), a strong oxidizing reagent, selectively oxidizes and removes lignin, while leaving the polysaccharide fraction intact [96]. An acidic mixture of acetic acid and hydrogen peroxide at 80 ^{o}C forms PAA in situ and also removes lignin [18]. Peracetic acid cleaves β-aryl ether bonds, which reduces the molecular weight of lignin, and introduces hydroxyl groups, which increases the water solubility of lignin and its fragments [13, 14]. Peracetic acid is expensive [66] and concentrated forms are explosive and expensive to transport and store. An alternative is the in-situ generation of peracetic acid from hydrogen peroxide and acetate esters catalyzed by perhydrolase enzymes [16, 17].

Previous research optimized time, temperature and concentrations for biomass pretreatment with HCW [79] and with PAA [17]. In this study, optimal conditions of each one are combined to yield a synergistic increase in effectiveness, Figure 5.1. The first step hydrolyzes the xylan, while the second step removes most of the lignin. The effectiveness of combined-pretreatment was evaluated by measuring recovery yields, composition, enzymatic digestibility and changes in the structural characteristics of the pretreated solid residues.

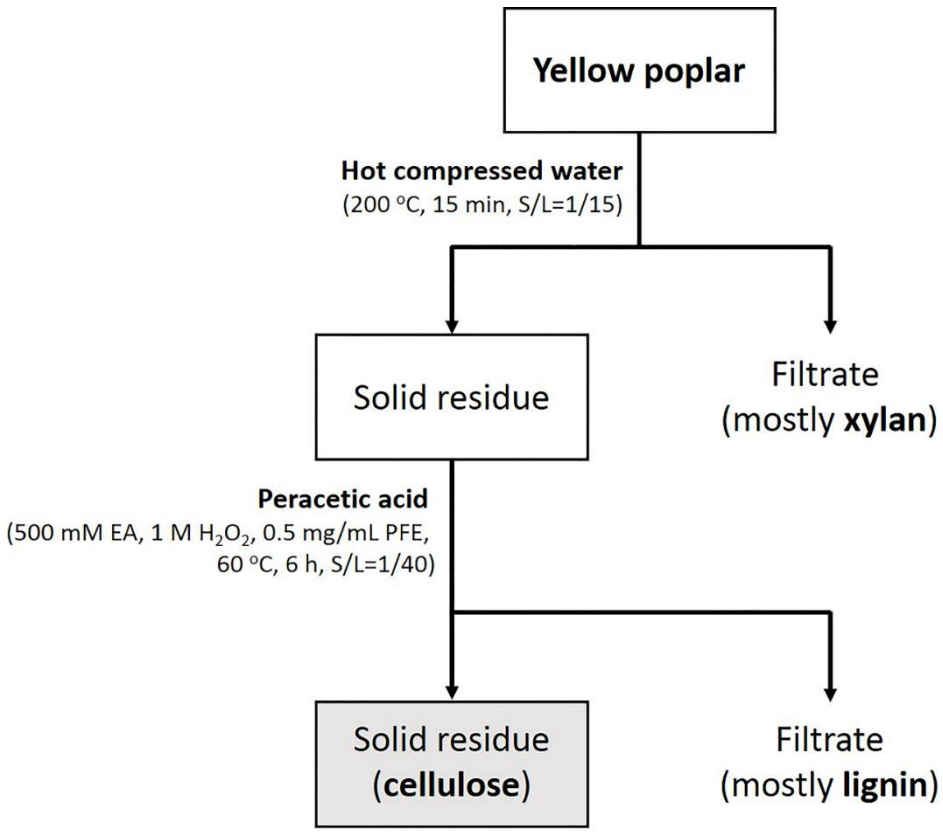

Figure 5.1 Schematic illustration of the combined pretreatment. The combined-pretreatment of yellow poplar biomass uses hot compressed water to solubilize the xylan fraction followed by peracetic acid to solubilize the lignin fraction. S/L = solid to liquid ratio; EA = ethyl acetate; PFE = *Pseudomonas fluorescens* esterase.

5.2 Composition of solids after single and combined pretreatments

Untreated yellow poplar biomass (YP) contains 39.0 wt% glucan, 18.8 wt% xylan and 21.7 wt% lignin based on dry weight. The remaining 20.5 wt% likely consists of other carbohydrates such as arabinan, galactan and mannan, but these were not measured.

Pretreatment with oxidants selectively removes lignin from biomass [108]. Pretreatment of YP with peracetic acid (PAA) removed~40% of the xylan and ~20% of the lignin, Figure 5.2. Heating YP to 60 °C for 6 h with 90 mM PAA followed by filtration and drying yielded 84.1% of the original solid mass with a composition of 40.7 wt% glucan, 13.3 wt% xylan and 20.4 wt% lignin. The Effect of H_2O_2 and ethyl acetate, starting materials for PAA, on lignin removal would be inefficient. H_2O_2 could react with lignin, but use of H_2O_2 in an alkaline condition accelerates delignification. Its activity was weakened in acid or neutral condition. While H_2O_2 pretreatment of maize stems solubilized 86.2% of original lignin at pH 11.5, the lignin removal at pH 4.4 and 9.5 was 15.9 and 17.4%, respectively [12]. The process using ethyl acetate require over 30 w/v% of ethyl acetate with ethanol and high temperature [109]. In addition, although acetic acid (byproduct) are known to be a good solvent for lignin, most of lignin was not dissolved at lower than

60 w/v% acetic acid and below 90 ºC [110]. Therefore, the delignification ability of H_2O_2, ethyl acetate and acetic acid in PAA pretreatment could be negligible.

Pretreatment of YP with compressed hot water (HCW) at 200 °C, 1.5 MPa for 15 min removed mainly the xylan component, Figure 5.2. After cooling, filtration and drying, 63.6% of the original solid mass remained. The pretreated solid contained 57 wt% glucan, 2 wt% xylan and 26 wt% lignin. This change corresponds to removal of 90% of xylan. Thus, the HCW pretreatment yielded a solid enriched in glucan and lignin as compared to untreated YP.

A combined pretreatment of YP with HCW followed by PAA (HCW+PAA) removed both the xylan and lignin components yielding a solid recovery of 45.3% containing ~75 wt% glucan, Figure 5.2. The amount of xylan removed (93.6%) was similar to the amount removed in the single pretreatment with HCW, but the amount of lignin removed increased from ~20% in the single PAA pretreatment to 76% in the combined pretreatment indicating a cooperative effect of the two treatments.

The synergistic increase in effectiveness dramatically reduced the amount of PAA needed. While PAA is a strong oxidant and effectively removes lignin, high concentrations [111] or several cycles [16, 17] at lower concentration were required. In this work, the HCW step prior to the PAA step allows

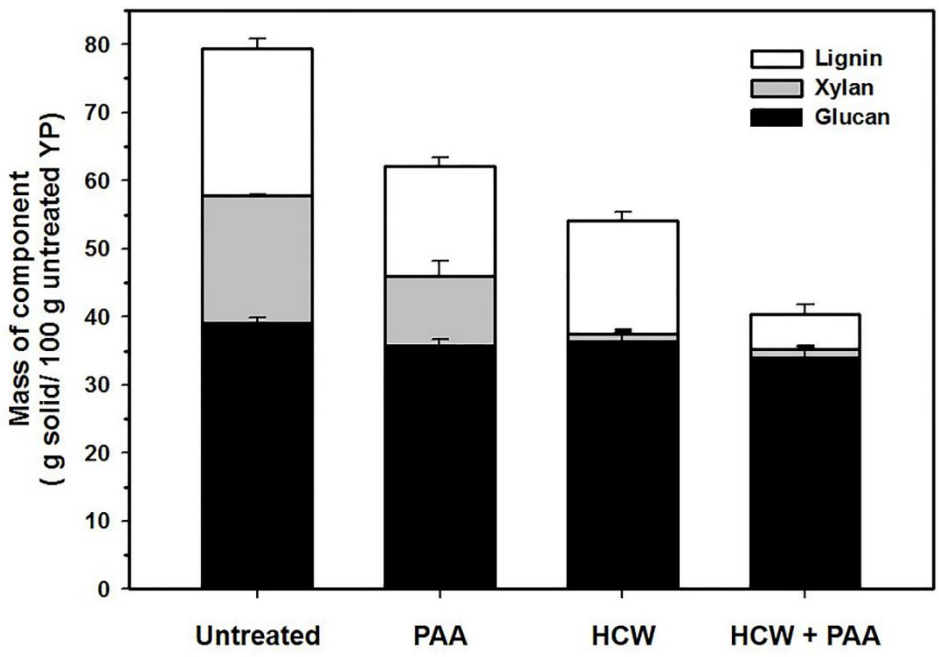

Figure 5.2 Composition of untreated yellow poplar biomass and solid recovered after various pretreatments. Hot compressed water (HCW) pretreatment removed almost all of the xylan. Peracetic acid (PAA) pretreatment removed some lignin, but removed more lignin when carried out after HCW pretreatment. The combined pretreatment removes more xylan and lignin than the sum of the single pretreatments Error bars correspond to the standard deviation for three measurements.

a single step of PAA at low concentration to effectively remove lignin.

The reverse order of pretreatment steps (PAA followed by HCW) would be inefficient. Pretreatment of biomass with PAA removed not just lignin, but also 40% of the xylan in this study. The lignin in this mixture inhibits fermentation, so this sequence of steps wastes 40% of the xylan. In contrast, pretreatment with HCW first yields a lignin-free xylose solution which could be used for fermentation.

The combined pretreatment did not release substantial amounts of compounds that could inhibit enzymatic hydrolysis or fermentation. Harsh pretreatment of biomass forms fermentation inhibitors like acetic acid, 5-hydroxymethylfurfural (HMF) and furfural. Previous work showed that HCW at 200 ^{o}C, 1.5 MPa does not generate significant amounts of these inhibitors [79]. The mild conditions of the PAA pretreatment also do not generate HMF or furfural, but do contain acetic acid. Filtration and washing removes this acetic acid from the insoluble glucan before enzymatic hydrolysis.

The light brown yellow poplar wood particles darkened during HCW pretreatment, but lightened during PAA pretreatment, Figure 5.3. The darkening during HCW pretreatment in consistent with caramelization of the sugar due to the high temperature [112], while the lightening is consistent with bleaching effects of oxidants. Oxidative cleavage of chromophores removes color.

Figure 5.3 Photographs showing color of untreated yellow poplar biomass (YP) and solids after single- and combined-pretreatments. PAA = peracetic acid; HCW = hot compressed water. Particle sizes range from 0.25 to 0.42 mm.

5.3 Enzymatic hydrolysis of single and combined-pretreated solids

Hydrolysis using a high loading of cellulase (60 FPU/ g glcuan) released only 9.3% of the glucose from untreated YP, but this increased to 23.2.% after PAA pretreatment, 78.3% after HCW pretreatment and 89.7% after the combined pretreatment, Figure 5.4. Each pretreatment deconstructed the YP to different extents and in that way improved the accessibility of the enzyme to cellulose. The effectiveness of a much lower loading of cellulase (4 FPU/ g glucan) depended on the amount of lignin in the sample, Figure 5.4. The yield of glucose released was 5.7% from untreated YP, 19.2% with PAA pretreatment, 47.7% with HCW pretreatment and 91.9% with the combined pretreatment. The yields for HCW-pretreated decreased significantly as compared to the higher loading of cellulase, likely due to presence of lignin, which hinders cellulase access and non-specifically binds cellulases [45]. The combined pretreated sample contained less lignin and released similar amounts of glucose even at lower cellulase loading.

The effectiveness of enzymatic digestion of the combined-pretreated solid with the low cellulase loading was higher than for the single-pretreated solids even with the high cellulase loading. While HCW alone can break up the structure and remove the xylan, it does not remove the cellulase-hindering

lignin. PAA alone removes only 20% of the lignin likely because it does not break up the biomass structure. Only the combination removes most of the lignin and breaks up the structure leading to effective enzymatic digestion at low cellulase loadings. Other combined pretreatments are also more effective than a single step. For example, successive dilute acid and alkali treatment on rice straw yield 61.8 – 92.7% glucose release [113].

The cost effectiveness of HCW+PAA pretreatment depends on its cost as compared to the traditional dilute acid pretreatment and balanced by the increased yield of glucose. HCW requires some special equipment and energy input, but does not require addition of sulfuric acid. While commercial PAA is expensive, on-site generation of PAA lowers material costs. The perhydrolase has not been optimized for industrial production, but one can expect the cost of this enzyme would be similar to that of other industrial enzymes such as cellulases and xylanases which are an important and cost-effective part of biomass processing.

5.4 Structural characterization of solids after single and combined pretreatment

The crystallinity index (CrI) correlates with the fraction of crystalline cellulose in the sample. The CrI of YP, measured by x-ray powder diffraction, increased after the HCW single pretreatments and even more after combined

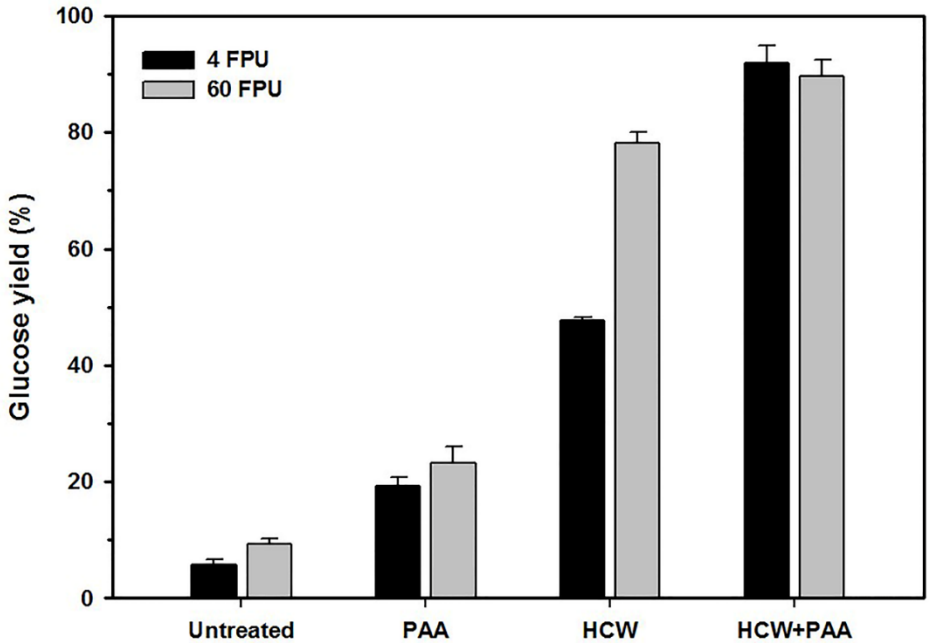

Figure 5.4 Yield of glucose from enzymatic digestion of untreated YP, single- and combined-pretreated substrates. The enzymatic digestion of the combined-pretreated biomass yielded the highest amount of glucose. In most cases, the amount of glucose released using low enzyme loading (4 filter paper units/g of cellulose) was similar to the amount released with high enzyme loading (60 FPU/g cellulose). The exception is the HCW-only pretreatment, which released more glucose with higher enzyme loading. The remaining lignin in this sample likely binds and inhibits the enzymes at low loading, but higher loading of enzyme overwhelms this binding and inhibition. Error bars correspond to the standard deviation for three measurements.

pretreatment, Figure 5.5. The CrI values of the untreated YP, PAA single-, HCW single- and combined-pretreated solid were 60.8, 60.5, 75.6 and 82.3%, respectively. The CrI is strongly affected by composition of materials. When amorphous components such as hemicellulose, lignin and amorphous cellulose are removed after pretreatment, the CrI would increase. This could be proved by the fact that HCW-pretreated solid had higher CrI than raw YP. But, the CrI of PAA-pretreated solid was similar to that of raw YP due to 20% lignin removal. The CrI of combined pretreated solid increased due to removal of residual lignin and increase in relative cellulose content. But, crystallinity of combined pretreated solid increased slightly compared to that of HCW-pretreated solid. The removal of xylan may be more significant on the CrI than that of lignin [70]. Therefore, the increase in CrI is consistent with the removal amorphous components xylan, lignin and amorphous cellulose leaving an increased proportion of crystalline cellulose. Crystalline cellulose is the main component of the solid residue after combined-pretreatment.

The relative infrared absorption spectra of the solid residues showed the expected decrease in functional groups corresponding to removal of xylan and lignin, Figure 5.6. Infrared spectra were measured using attenuated total reflectance. Comparison of spectral of the untreated and HCW-pretreated solid are consistent with an increase in relative amount of cellulose in the

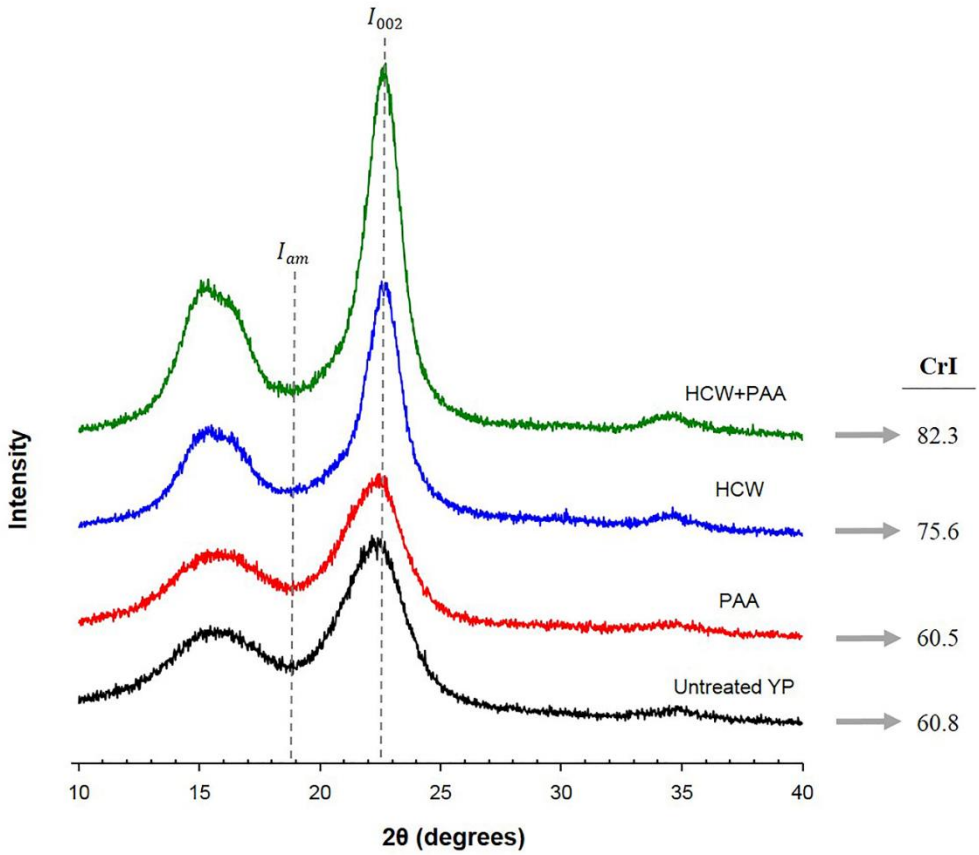

Figure 5.5 Cellulose crystallinity index (CrI) of untreated YP, single- and combined-pretreated solids as measured by X-ray powder diffraction. Removal of amorphous components - xylan and lignin - causes the CrI of the remaining solid to increase. CrI (%) = (I_{002} – I_{am})/I_{002} * 100 %. I_{002}: the maximum intensity of the crystalline portion of biomass (crystalline cellulose) at 2θ = 22.0-22.4°, I_{am}: the minimum intensity of the amorphous portion of biomass (amorphous cellulose, hemicellulose and lignin) at 2θ = 18°.

HCW-pretreated solid due to the removal of xylan. The bands at 1156 and 1054 cm^{-1} vibration (associated with C-O-C vibration in cellulose and hemicellulose, and C-O stretch in cellulose) were stronger in the HCW-pretreated solid than in untreated YP. The bands at 1720 cm^{-1} (attributed to C=O ester linkage between hemicellulose and lignin) [114] and 1245 cm^{-1} (associated with alkyl ester of acetyl group in hemicellulose) were absent in the HCW-pretreated solid consistent with the removal of xylan. The spectra of the combined-pretreated solid showed further changes consistent with the removal of lignin. The bands between 1610 and 1330 cm^{-1}, related to the lignin structure [115], were weakened in the spectrum of combined-pretreated solid residue.

The surface morphology of untreated YP showed a dense and well-ordered structure, while the pretreated solid show a collapsing of the cell wall, Figure 5.7. The scanning electron micrographs of untreated YP shows compact pores with a few cracks and a few flakes on the surface. After HCW pretreatment, the pores were partially open and secondary cell wall was collapsed, consistent with xylan removal. The entire surface showed visible debris and spherical droplets with diverse diameters (< 2 μm). These droplets were ascribed to deposited lignin; pseudo-lignin, which can hinder the accessibility of cellulase to cellulose [116]. This change in lignin shape, also observed during dilute acid pretreatment [117], increases the surface area of lignin,

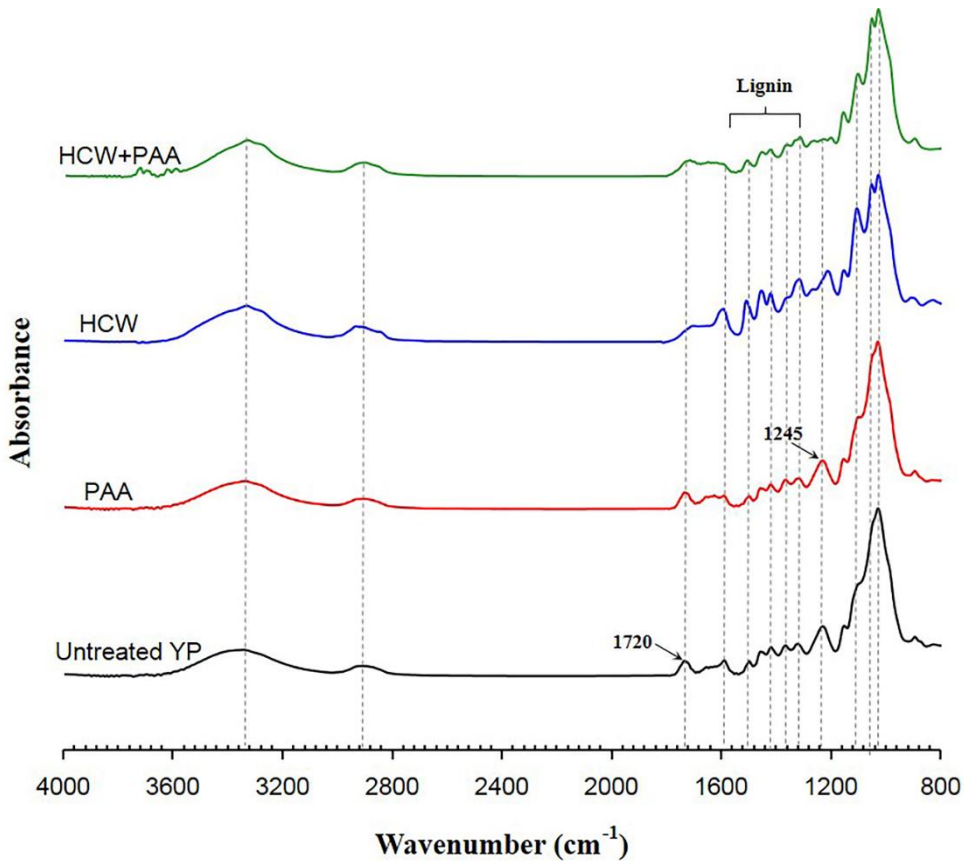

Figure 5.6 FTIR spectra of untreated YP, single- and combined-pretreated solids show changes in vibrations associated with various functional groups. After the combined pretreatment, the bands associated with hemicellulose at 1720 and 1245 cm^{-1} disappeared, and the bands associated with lignin between 1610 and 1330 cm^{-1} were diminished as compared to untreated YP.

which inhibits enzymatic hydrolysis of glucan. After combined-pretreatment, the opening of pores and secondary cell wall collapse increased consistent with further disruption of the structure by lignin removal. Spheres of deposited pseudo-lignin and cell wall flakes are gone, which indicates that PAA removed the residual lignin and pseudo-lignin [97, 118].

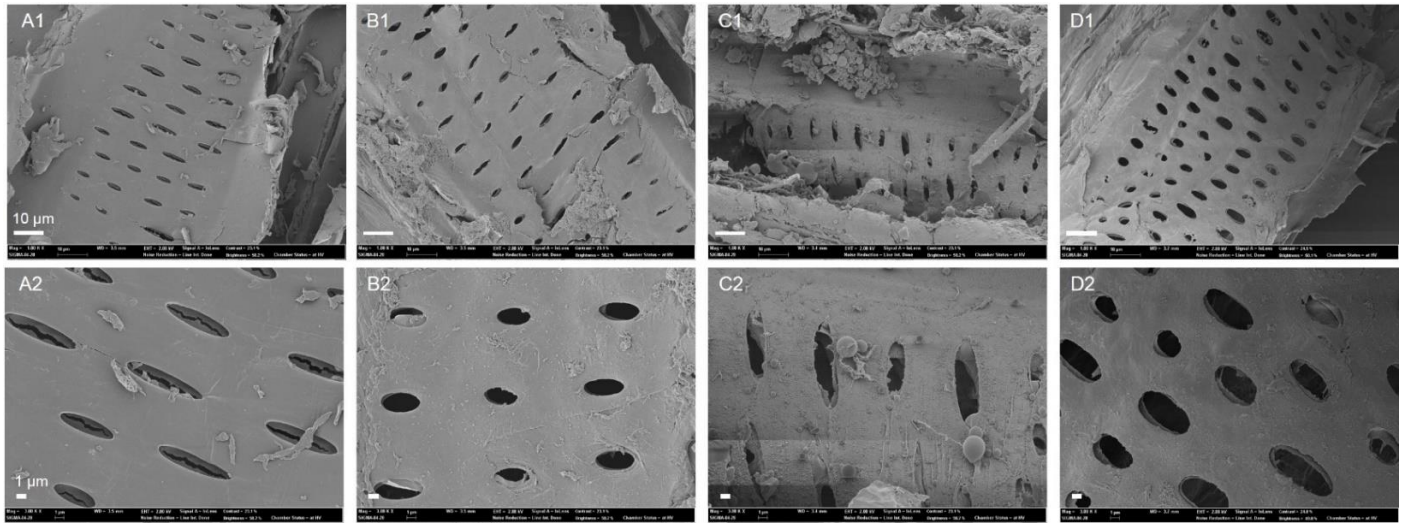

Figure 5.7 Scanning electron microscope images of (A) untreated YP biomass, and solids recovered after (B) PAA-pretreatment, (C) HCW-pretreatment, and (D) combined-pretreatment. The images on the left and marked 1 correspond to 1000 x magnification, while the images on the right and marked 2 correspond to 3000 x magnification. The white bar corresponds to 10 microns for the images on the left and 1 micron for the images on the right.

5.5 Conclusion

The combined-pretreatment fractionated YP particles into a xylan-rich solution with the HCW pretreatment and a lignin-fragment-rich solution with the PAA pretreatment, leaving a solid enriched in glucan (~75%). A low cellulase loading (4 FPU/g glucan) released only 5.7% of the glucose from untreated YP, but the yield increased to 47.7% after HCW-pretreatment and further to 91.9% upon adding the PAA pretreatment step. Both lignin removal and disruption of the structure contribute to making the cellulose accessible to cellulases.

Chapter 6.

Mild pretreatment of yellow poplar using sequential dilute acid and enzymatically-generated peracetic acid to enhance cellulase accessibility

Chapter 6. Mild pretreatment of yellow poplar biomass using sequential dilute acid and enzymatically-generated peracetic acid to enhance cellulase accessibility

6.1 Introduction

Lignocellulosic biomass is a renewable and sustainable alternative to fossil fuels for the production of energy, materials and chemicals [2, 3]. Lignocellulosic biomass is a complex matrix of three macromolecules: cellulose, hemicellulose and lignin [119]. Producing biofuels from lignocellulosic biomass requires first hydrolysis of the lignocellulose to soluble sugars, followed by microbial fermentation of the sugars to biofuels. The structural complexity of lignocellulose make it recalcitrant to hydrolysis needed to release soluble sugars [8]. Pretreatment steps reduce the biomass recalcitrance by making the cellulose component more accessible to enzymatic hydrolysis [9, 120]. Effective and economical pretreatment is essential for the conversion of lignocellulosic biomass to fuels.

Many pretreatment methods including physical, chemical, physicochemical and biological techniques can remove hemicellulose and/or lignin and improve yield of soluble sugar from lignocellulosic biomass. Dilute acid pretreatment is a leading and potentially commercial process [121]. Dilute

acid selectively hydrolyzes the hemicellulose to xylose [35, 122, 123], increases the porosity of remaining biomass and increases the yield of glucose in the subsequent enzymatic hydrolysis [9]. The disadvantages of dilute acid pretreatment are 1) the formation of byproducts, such as acetic acid, furfural and 5-hydroxymethylfurfural (HMF), which inhibit both enzymatic hydrolysis and fermentation [48] and 2) inhibition of cellulases by the lignin remaining in the solid residue. Milder conditions for the pretreatment and a lignin-removal step would solve these problems.

Peracetic acid is a strong oxidant, which selectively dissolves lignin leaving a cellulose-rich residue [12]. Enzymatic hydrolysis of these cellulose-rich residues yields glucose for bioethanol production. Peracetic acid pretreatment requires only mild conditions, thereby minimizing the formation of byproducts. However, previous single step peracetic acid pretreatment required 50 wt% peracetic acid based on initial dry weight [111]. In addition, concentrated peracetic acid is unstable and explosive [15]. Safety concerns increase the cost and complicate the production, transportation and storage of peracetic acid. Enzymatic on-site generation of peracetic acid avoids the hazards of concentrated form and may reduce costs [16, 17].

The present study focuses on yellow poplar (*Liriodendron tulipifera*) as the lignocellulosic biomass. Yellow poplar grows faster, sequesters more CO_2 and accumulates a higher fraction of polysaccharides than other wood species as

pine and larch [74]. The Korean Forestry Service recommends planting yellow poplar as a biomass crop. While previous work tested sequential subcritical water and formosolv pretreatment of yellow poplar [104], here sequential dilute acid followed by peracetic acid is tested. This novel approach more efficiently fractionates the biomass into xylan, lignin and cellulose fractions and reduces the amount of cellulase needed, Figure 6.1.

6.2 Dilute acid pretreatment of yellow poplar

The conditions for the dilute acid (DA) pretreatment of YP biomass (acid concentration, temperature and treatment time) were optimized using the combined severity factor (CSF) [124].

$$\mathrm{CSF} = \log\left(t \times exp\left(\frac{T_H - T_R}{14.75}\right)\right) - pH$$

where t is the reaction time in minutes, T_H is the pretreatment temperature in ^oC, T_R is the reference temperature in 100 ^oC and pH is the initial pH value calculated from H_2SO_4 concentration. CSF value efficiently compares operating conditions because it combines the treatment temperature, residence time and pH in one value to approximate harshness. In the study, the reaction temperature ranged from 120 to 160 ^oC, the reaction time from 1 to 30 min and the H_2SO_4 concentration was either 0.05 or 0.1 M. The combined severity factor ranged from 0.6 to 1.8.

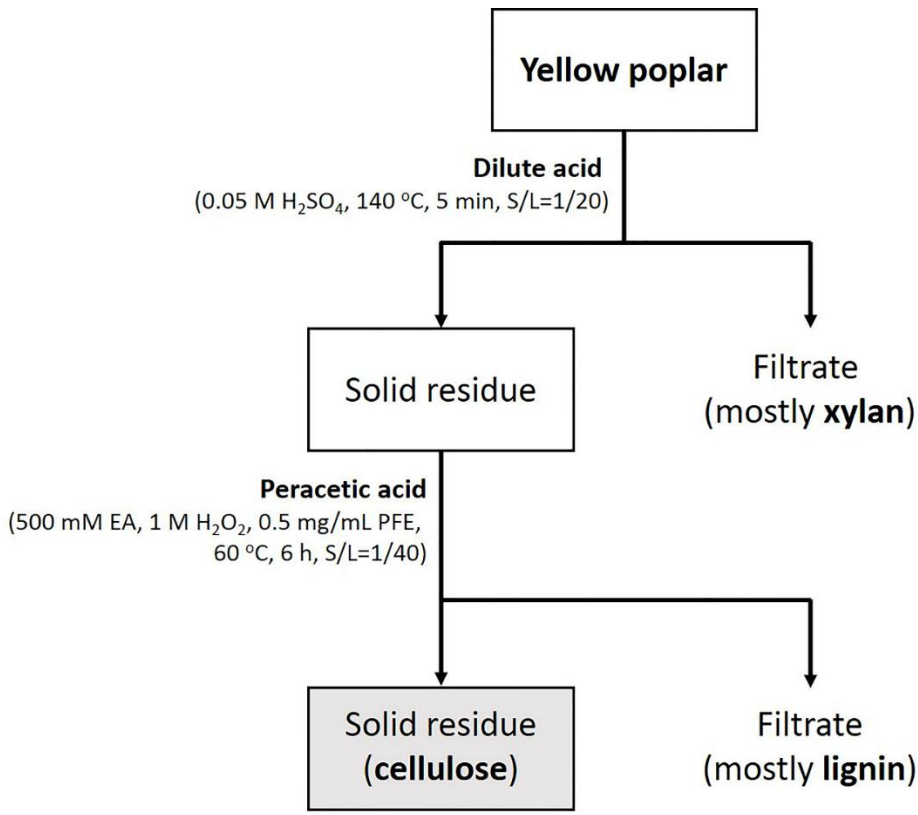

Figure 6.1 Schematic of sequential pretreatment of yellow poplar biomass. Dilute acid pretreatment mainly solubilizes the xylan fraction by hydrolysis of glycosidic links, and the subsequent peracetic acid pretreatment solubilizes the lignin fraction by oxidation, which breaks lignin into oligomers and adds polar oxygen functional groups. S/L: solid to liquid ratio, EA: ethyl acetate, PFE: *Pseudomonas fluorescens* esterase.

Raw YP biomass contains 39.3 g glucan, 18.4 g xylan, 21.4 g lignin and 20.9 g other components per 100 g. DA pretreatment hydrolyzes and solubilizes the xylan and glucan in the biomass. A decrease in the amount of solid recovered corresponds to more hydrolysis. The amount of solid recovered ranged from 72.7 to 40.8 g per 100 g of YP and decreased with increasing temperature and acid dose, Table 6.1.

The main chemical effect of the DA pretreatment was hydrolysis of the xylan component to xylose. Depending on the CSF, the amount of xylan in the solid decreased from 18.8 g per in raw YP to 7.6 to 0.8 g per 100 g. The solution contained xylose corresponding to hydrolysis of 58.9–95.7% of the original xylan. The xylan hydrolyzes more readily than the cellulose because it is amorphous, has low molecular weight and a branched structure with short side chains [29]. The maximum xylose yields were 76.1–83.1% at temperatures of 120 or 140 ^{o}C corresponding to CSF = 0.9-1.5. At 160 °C, the xylose yield decreased to 36.6-55.7% corresponding to CSF = 1.1-1.5, due to further degradation of xylose to furfural (> 2 g/L).

While mild DA pretreatment had little effect on the glucan, harsher DA pretreatment hydrolyzed this fraction. The glucan in the solid residues decreased from 39.3 g in raw YP to 38.2 to 21.9 g per 100 g of YP which corresponds to 2.8–44.4% removal of glucan. The glucose yield in the filtrate increased from 1.5 to 33.5% as the temperature and acid dose increased.

Although the concentration of HMF, a degradation product of glucose, also increased with increasing CSF [125], it remained low enough (0.01–0.71 g/L) to not be a concern as a fermentation inhibitor.

DA pretreatment removed only small amounts of lignin, but did change the location of the lignin. The lignin in the solid residues decreased from 21.4 g in raw YP to 19.1 to 14.3 g per 100 g, which corresponds to 10.5-33.1% removal of lignin. Above ~130 °C, lignin melts and then re-solidifies upon cooling [117]. Lignin can also rearrange by replacing carbon oxygen links with more stable carbon-carbon links under acidic conditions [126]. Electron micrographs of pretreated solid (see below) show changes in the physical structure of the lignin. The initially distributed lignin formed spherical droplets of lignin on the surface after pretreatment.

DA pretreatment selectively hydrolyzed the xylan thereby increasing in the relative amount of glucan and lignin in solid residue. The best conditions are those that release the highest amount of xylose while forming minimum amounts of degradation products. The best condition of DA pretreatment was 140 ^{o}C with 0.05 M sulfuric acid for 5 min. Solid material from these pretreatment conditions was further treated with peracetic acid.

6.3 Dilute acid-peracetic acid pretreatment of yellow poplar

Although DA pretreatment improves the enzymatic digestibility of the

Table 6.1 Optimization of conditions for dilute acid pretreatment

Temp (°C)	H_2SO_4 (M)	Time [a] (min)	CSF	Solid recovery (%)	Solid phase			Liquid phase (prehydrolysate)			
					Dry material (%) [b]			Glucose yield (%)	Xylose yield (%)	Inhibitory compound (g/L)	
					Glucan	Xylan	Lignin			HMF	Furfural
120	0.05	10	0.6	72.7	52.2±3.0	10.4±2.1	25.4±0.7	1.5	54.4	n.d.	0.07±0.02
	0.1	10	0.9	70.9	53.9±2.7	9.3±1.6	27.0±2.0	2.0	67.8	0.01±0.01	0.13±0.04
	0.1	20	1.2	65.6	55.7±4.5	6.6±1.5	27.0±0.5	2.9	76.1	0.02±0.01	0.28±0.08
	0.1	30	1.4	65.8	58.0±1.7	6.7±1.3	27.3±0.7	2.9	80.2	0.02±0.01	0.29±0.06
140	0.05	5	0.9	59.2	63.4±3.5	2.7±0.3	27.8±0.8	6.7	83.1	0.08±0.04	0.63±0.25
	0.05	10	1.2	59.7	60.6±2.2	4.1±1.2	28.5±1.1	8.3	80.7	0.10±0.04	0.74±0.29
	0.1	10	1.5	56.4	63.2±0.2	1.8±0.4	30.8±0.8	11.8	78.1	0.17±0.01	1.35±0.09
	0.1	20	1.8	53.7	60.3±2.8	1.5±0.2	32.5±0.7	14.6	65.8	0.21±0.02	1.77±0.13
160	0.05	1	0.8	49.4	62.6±3.0	2.0±1.0	29.0±0.4	19.3	66.1	0.28±0.08	1.35±0.36
	0.1	1	1.1	47.1	62.9±1.3	n.d. [c]	32.2±1.3	24.6	55.7	0.39±0.10	2.00±0.52
	0.05	5	1.5	45.6	57.6±4.4	n.d.	33.2±3.9	28.7	36.6	0.55±0.17	2.26±0.56
	0.1	5	1.8	40.8	53.6±2.1	n.d.	39.9±2.2	33.5	35.2	0.71±0.08	3.07±0.42

[a] Time does not include heating or cooling time. Heating time: within 2 min; cooling time: 1 min (120 °C), 2 min (140 °C), 2 min 30 s (160 °C), [b] Raw YP: Glucan (39.0%), Xylan (18.8%), Lignin (21.7%), [c] Not detected

remaining solid, the residual lignin still limits enzymatic hydrolysis by blocking access to the glucan and by non-specific adsorption of the enzyme to the lignin[45, 127]. Therefore, DA-pretreated solid was further treated with peracetic acid, which removed the residual lignin, Figure 6.2. The relative amounts of glucan and lignin in the solid changed markedly. The glucan, xylan and lignin content in sequentially pretreated solid was 34.1, 1.1, 3.4 g per 100 g of YP, respectively. As expected, the peracetic acid efficiently removed the lignin, up to ~84% of original lignin. The relative amount of glucan increased from 39.3 to 80.8%. The sequential pretreatment yielded a cellulose fraction with low amount of hemicellulose and lignin. Only 13.2% of the glucan was lost during the sequential pretreatment.

The DA pretreatment reduced the amount of PAA needed by approximately half. Single-step PAA pretreatment required ~50 wt% PAA based on initial dry weight [111]. The DA step removed the xylan allowing the PAA better access to the lignin so that only ~25 wt% PAA based on initial dry weight was needed.

The mild conditions of sequential pretreatment minimized formation of fermentation inhibitors such as HMF or furfural. Acetic acid, a byproduct of peracetic acid, was removed in the washing step. Therefore, the sequential pretreatment does not require detoxification steps before fermentation.

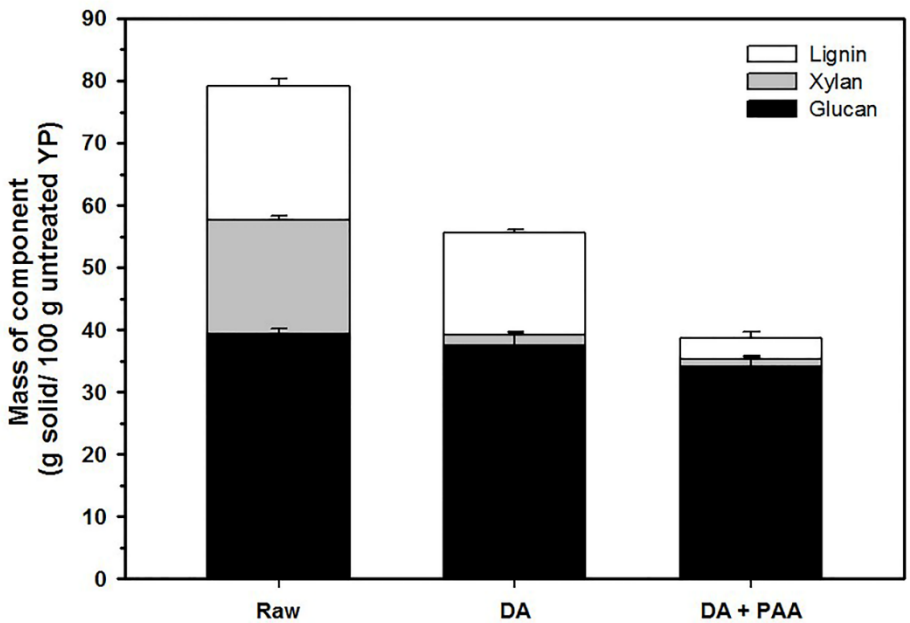

Figure 6.2 Composition of raw YP, dilute acid (DA)-pretreated solid and sequentially (DA + PAA) pretreated solid. Dilute acid pretreatment mainly solubilizes the xylan fraction, and the subsequent peracetic acid pretreatment selectively solubilizes the lignin fraction. After sequential pretreatment, relative amount of glucan was increased.

6.4 Enzymatic hydrolysis of pretreated solids

The yield of glucose from enzymatic hydrolysis of raw YP, DA-pretreated and sequentially pretreated solid measures the effectiveness of various pretreatments. The DA- and sequentially pretreated solids yielded more glucose than raw YP, showing that the pretreatment changed the structure of biomass and improved the enzymatic accessibility to cellulose. The enzymatic hydrolysis of raw biomass yielded 5.4% of the glucose within the biomass using 10 FPU/ g of cellulose. On the other hand, DA-pretreated and sequentially pretreated solid yielded 62.2 and 88.4% of the glucose within the solid, respectively. Sequential pretreatment enhanced the enzymatic hydrolysis efficiency approximately 1.4-fold over DA pretreatment alone. The improvement in the enzymatic hydrolysis of the cellulose-rich residues is likely because the removal of lignin increased the accessibility of cellulase to cellulose and reduced the inhibitory effect of residual lignin. The enzymatic digestibility of the sequentially pretreated solid was also greater than that of raw YP and DA-pretreated solid with lower cellulase loading (5 FPU/ g of cellulose), Figure 6.3. Although the enzyme loading reduced from 10 to 5 FPU/g of cellulose, enzymatic hydrolysis yield of sequentially pretreated solid was similar (90.5%) and was ~28% higher than DA pretreatment with the higher 10 FPU/g of cellulose loading. In addition, enzymatic saccharification of sequentially pretreated solid was complete at 48 h with 5

FPU cellulase loading, while the DA pretreated sample showed further increases at 72 h. Therefore, the advantages of sequential pretreatment over single DA pretreatment is the removal of the lignin fraction, resulting in reduced cellulase loading and faster saccharification.

Several different factors contribute to the overall cost of a pretreatment. While the added step and PAA increase costs, the lack of fermentation inhibitors [128], lower cellulase requirements and higher glucose yield reduce costs. In addition, the *in situ* generation of PAA could lower material costs since ethyl acetate and hydrogen peroxide are much less expensive than PAA. The cost of perhydrolase for industrial production expected to be similar to that of other industrial enzymes such as cellulases and xylanases. As an alternative, PAA can also be formed from acetic acid and hydrogen peroxide using sulfuric acid as catalyst, but this process is slow at room temperatures.

6.5 Structural characterization of sequential pretreatment

The structural characterization identified how the pretreatment changed the structure of the biomass and that these changes can enhance enzymatic hydrolysis.

The crystallinity of the solid residue following sequential pretreatment increased as compared to raw YP and DA-pretreated solid, Figure 6.4. The crystallinity index (CrI) of raw YP, containing a large fraction of amorphous

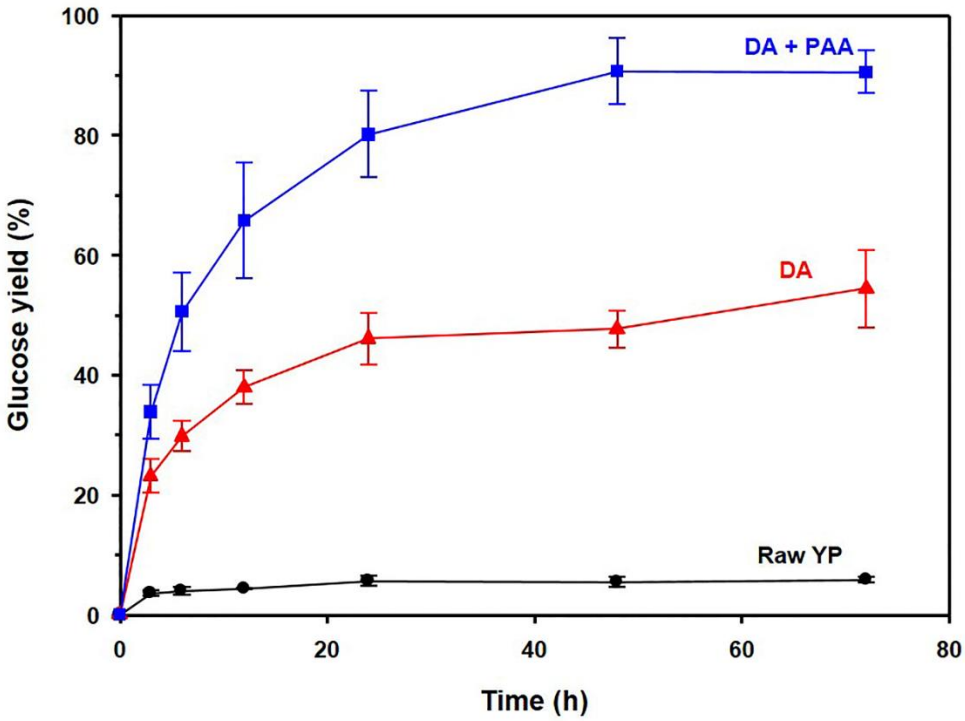

Figure 6.3 Yield of glucose from raw YP, dilute acid (DA)-pretreated solid and sequentially pretreated (DA + PAA) solid at an enzyme loading of 5 FPU/g of glucan. Glucose yield was only 5.1% from raw YP, but increased to 54.3% with DA pretreatment, 90.5% with sequential pretreatment at 72 h. The sequentially pretreated solid contained less lignin and released increased amount of glucose compared to DA-pretreated solid. In 48 h, sequential pretreatment reached its maximum glucose yield. Error bars correspond to the standard deviation for three measurements.

component (mainly xylan and lignin), was 49.9%. The CrI increased to 67.1 and 74.0% after DA and sequential pretreatment, respectively. The increase in the relative amount of cellulose in the pretreated solid residue due to removal of amorphous components, xylan and lignin, accounts for the increase in crystallinity index.

The absorption bands related to cellulose in the FTIR spectrum of DA-pretreated solid increased as compared to that of raw YP, Figure 6.5, consistent with the increase in the relative amount of cellulose. The hydrogen bonds of cellulose with O-H stretching at 3330 cm^{-1} and methylene of cellulose within C-H stretching at 2900 cm^{-1} were enhanced after DA pretreatment as compared to raw YP. The C-O-C glycosidic bond stretching at 1160 cm^{-1}, C-O-C ring skeletal vibration at 1100 cm^{-1} and C-O-H stretching of primary and secondary alcohols at 1030 cm^{-1} were also stronger in spectra of DA-pretreated solid than that of raw YP [122]. The absorption bands associated hemicellulose weaken after DA pretreatment, consistent with solubilization of xylan [129]. The ester linkage C=O between lignin and hemicellulose at 1720 cm^{-1} and the alkyl ester of the acetyl group in hemicellulose at 1245 cm^{-1} disappeared from the DA-pretreated solid. The absorption bands at 1300-1600 cm^{-1} related lignin structure increased after DA pretreatment, consistent with an increase of relative amount of lignin. In contrast, after sequential pretreatment, these band decreased, consistent with

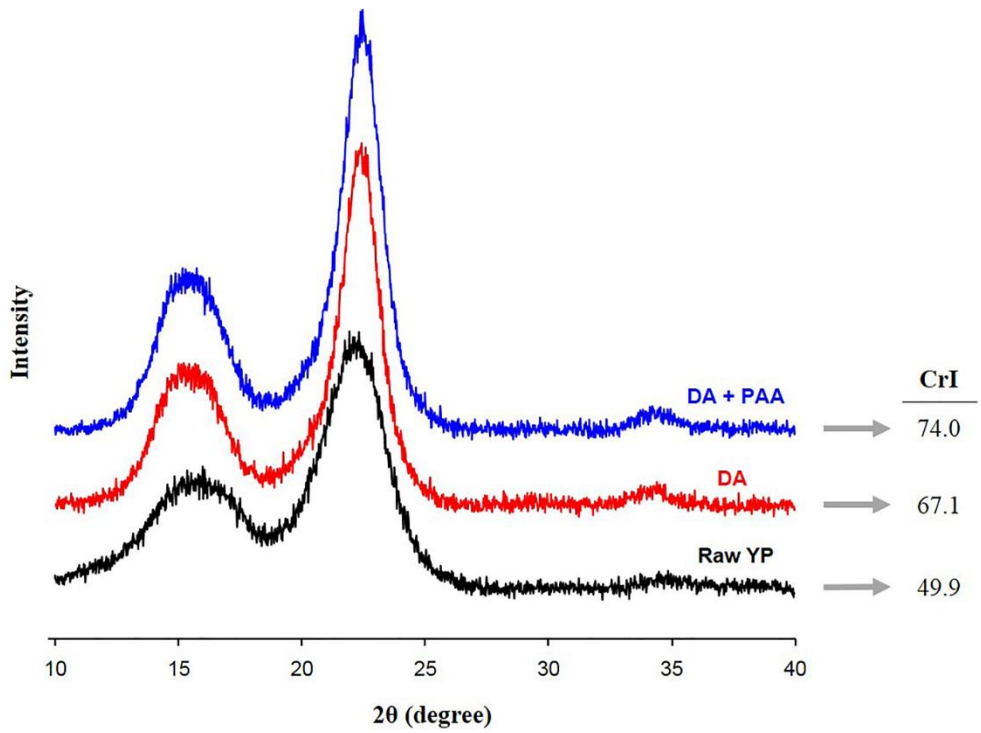

Figure 6.4 Crystallinity change after pretreatment with dilute acid (DA) and with sequential pretreatment (DA + PAA). Powder diffraction shows an increase in the crystalline index of the biomass upon pretreatment. CrI (%) = $(I_{002}-I_{am})/I_{002} \times 100\%$. I_{002}: maximum intensity of crystalline region at 2θ = 22.0 ~ 22.4 º, I_{am}: minimum intensity of amorphous region at 2θ = 18.7 º.

the removal of lignin by the PAA treatment.

The sequential pretreatment significantly changed the surface of the biomass, Figure 6.6. The raw YP has well-ordered and compact surface structure. The DA-pretreated solid showed irregular cracks and enlarged pores on the surface. Dissolution of xylan in the secondary cell wall during DA pretreatment created these cracks and enlarged these pores, which would enhance access of cellulase to its substrate. Diverse spherical droplets, called pseudo-lignin, covered almost all of the surface. This lignin can bind cellulase, thereby preventing it from binding to the cellulose [117]. The sequentially pretreated solid showed additional changes in the surface morphology. The pseudo-lignin droplets disappeared and the surface was now smooth. The fragmentation of fibril bundles may be separated from the larger tissue cluster, which is due to the removal of lignin from middle lamella. Delignification can collapse structure of lignocellulose with greater exposure of the fibers [130]. The remaining solid is mainly cellulose which is easily accessible to cellulases.

Overall, the structural changes are consistent with mainly xylan removal in the DA pretreatment and mainly lignin removal in the subsequent PAA treatment.

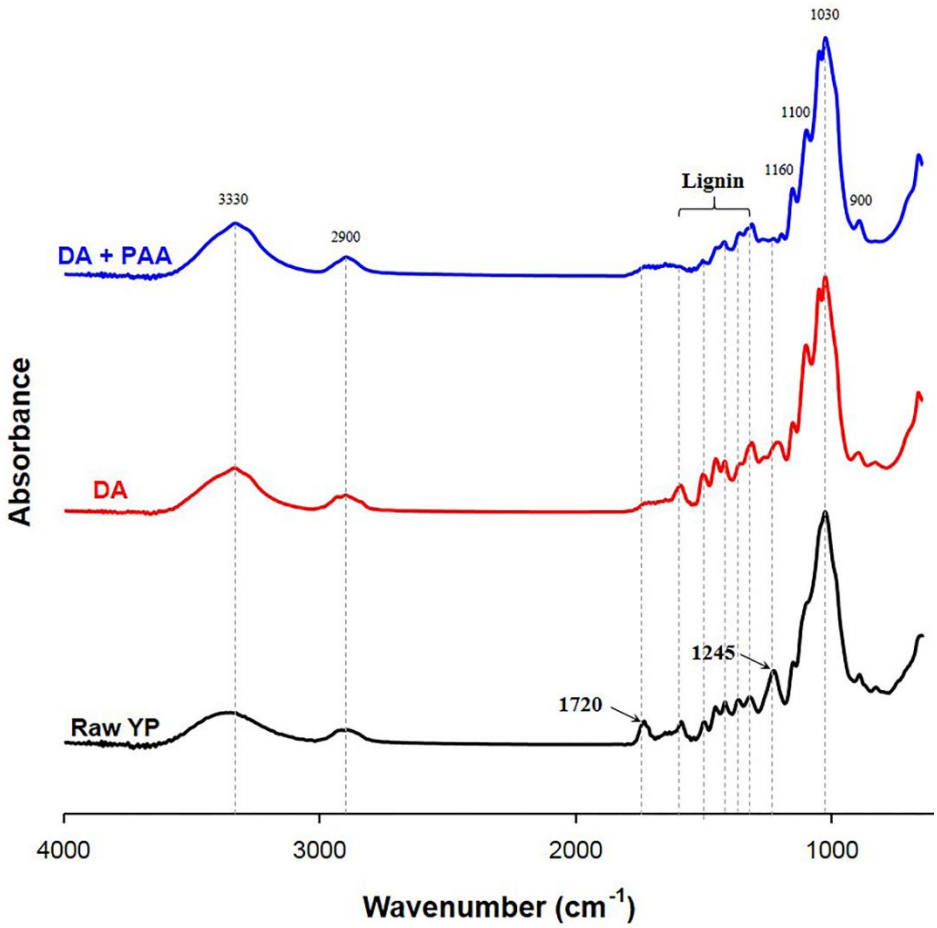

Figure 6.5 Infrared spectra of changes in functional groups in the biomass upon pretreatment with dilute acid (DA) and with sequential pretreatment (DA + PAA). After DA pretreatment, the bands at 1720 and 1245 cm^{-1} related to hemicellulose decreased. After subsequent PAA treatment, the bands at 1300 ~ 1600 cm^{-1} related to lignin structure decreased.

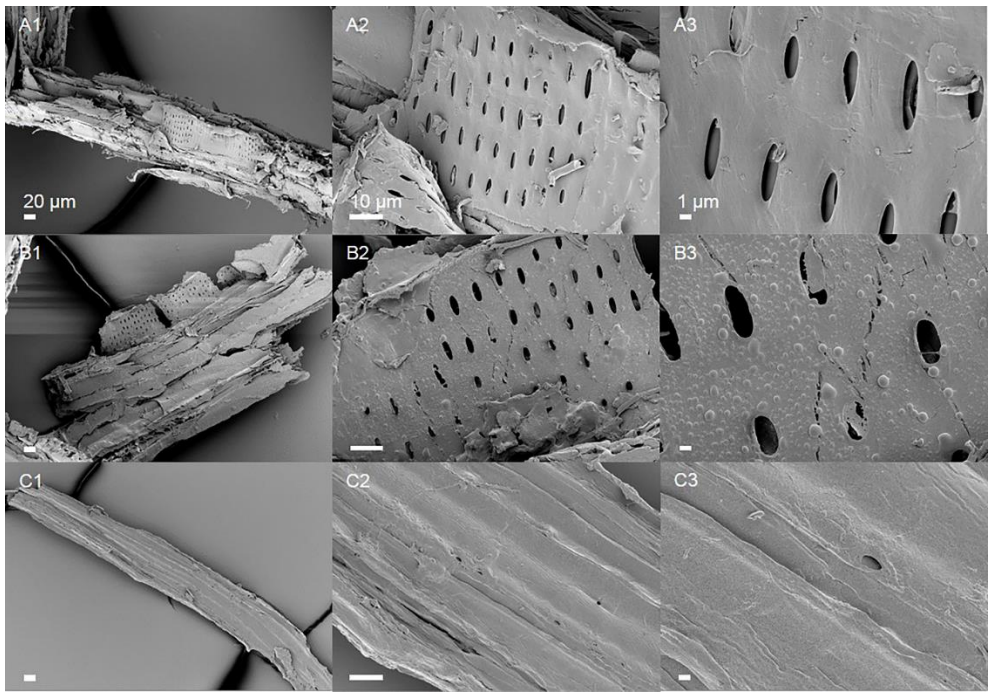

Figure 6.6 SEM images of raw YP (A), dilute acid pretreated solid (B) and sequential pretreated solid (C) at three different magnifications (× 500 labeled as 1, × 3,000 as 2 and × 10,000 as 3). The white bars indicate 20, 10, 1 microns. The raw YP has a well-ordered structure, while dilute acid pretreated solid has several irregular cracks and pores. Spherical droplets of pseudo-lignin covered almost all of the surface. After sequential pretreatment, the surface shows smooth cellulose fiber due to the removal of xylan and lignin.

6.6 Conclusion

The sequential pretreatment efficiently deconstructed yellow poplar biomass into its three major components. The initial DA pretreatment solubilized the xylan (~90% xylan), subsequent PAA treatment solubilized the lignin (~84% delignification) leaving a solid consisting of mainly cellulose (~81%). Although two treatment steps increase complexity, they increase efficiency. The DA step is well suited to remove xylan under mild conditions, achieving sufficient xylose recovery (83.1%). Increasing the harshness of this step to partially remove lignin is undesirable because the incomplete removal of lignin requires more cellulase and the harsher conditions create fermentation inhibitors. Similarly, PAA alone requires twice as much PAA to remove the same amount of lignin and PAA alone does not completely remove the xylan. Single step pretreatment also do not separate the major components of biomass for separate application. After the sequential pretreatment, enzymatic digestion of the cellulose fraction efficiently released glucose (90.5% yield) even using low enzyme loadings. The sequential pretreatment (DA+PAA) is a promising method to efficiently remove xylan and lignin with less carbohydrate loss under mild conditions, thereby increasing the enzymatic digestibility for biofuel production.

Chapter 7.

One-step pretreatment of yellow poplar using peracetic acid to enhance enzymatic digestibility

Chapter 7. One-step pretreatment of yellow poplar biomass using peracetic acid to enhance enzymatic digestibility

7.1 Introduction

Biofuels produced from lignocellulosic biomass can reduce dependence on petroleum-based fuels and greenhouse gas emissions [2, 131]. Pretreatment of lignocellulosic biomass is key step in the conversion of biomass to biofuels. Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin in an interwoven matrix. Conversion of biomass into biofuels requires enzymatic hydrolysis of cellulose to glucose followed by the microbial fermentation of this glucose to biofuels [5]. The complex structure of biomass makes it recalcitrant to enzymes making the hydrolysis of cellulose slow and inefficient. This recalcitrance is the major economic obstacle to conversion of biomass to sugar [7]. Pretreatment of biomass enhances the accessibility of cellulose to enzymes, thereby overcoming biomass recalcitrance [9]. Effective pretreatments should be energy efficient, not degrade the cellulose and yield a cellulose fraction that is easily hydrolyzed with low enzyme loadings [5, 132].

The most effective pretreatments – dilute acid and organosolv – still require high temperatures of >150 °C. Dilute acid pretreatment at high temperatures

(>160 ^{o}C) removes most of the hemicellulose with minimal degradation of cellulose and lignin [122, 133]. The remaining lignin hinders access of the enzymes to cellulose both by blocking access and by adsorbing the enzymes [134, 135]. Organosolv pretreatments using ethanol or THF remove both the hemicellulose and lignin [14, 136, 137], but still require high temperature (>150 ^{o}C) and additional steps to recover the organic solvents. Lower temperature pretreatments are desirable because they reduce energy requirements and capital costs of equipment because the pressures generated during pretreatment are lower. In particular, autoclaves conveniently and inexpensively generate temperatures of 121 °C using steam, so pretreatments that work at or below this temperature are desirable.

Peracetic acid is strong oxidant that effectively removes lignin from biomass [12]. Pretreatment with peracetic acid at 80 ^{o}C improved the cellulose digestibility of sugarcane bagasse [111] and pretreatment with hydrogen peroxide-acetic acid mixtures, which generate peracetic acid in situ, at 80 ^{o}C was also increased the enzymatic digestibility of rice straw, pine wood and oak wood as compared to dilute acid pretreatment [18]. Two-step pretreatment with alkali followed by peracetic acid increased the enzymatic digestibility and reduced the amount of peracetic acid needed [63]. Enhanced enzymatic digestibility of the solid after peracetic acid pretreatment is due to delignification and increased surface area of cellulose [14]. However,

pretreatment with only peracetic acid removed mainly lignin leaving most of the hemicellulose. This remaining hemicellulose hindered cellulase access to the cellulose. Removal of both lignin and xylan allows efficient enzymatic hydrolysis of the cellulose [8].

Yellow poplar (*Liriodendron tulipifera*) grows quickly even in poor soil and sequesters higher amounts of carbon dioxide than other biomass crops due to its extensive root structure. The Korea Forest Service recommended yellow poplar as a biomass crop and it is a major planting species in Korea [74].

In this study, the effectiveness of dilute acid pretreatment by adding peracetic acid was increased. This single step procedure simultaneously removes both hemicellulose and lignin from yellow poplar biomass under mild conditions. As compared to dilute acid under same conditions, this process reduces pretreatment time, pretreatment temperature and cellulase enzyme loading while providing high yields of glucose.

7.2 Optimization of one-step pretreatment

The four pretreatment variables are the concentrations of peracetic acid (PAA) and H_2SO_4, the temperature and the time of pretreatment. These variables were optimized stepwise for xylan and lignin removal by measuring the effect of one variable, while the other variables were fixed at harsh condition.

A PAA concentration of 300 mM gave a high xylan and lignin removal with minimal loss of glucan. The ratio of PAA solution to water was varied from 1:9, 2:8 and 3:7 (v/v), which corresponded to 200, 300 and 400 mM PAA, respectively. Caution: PAA decomposes at high temperatures. Solutions with >600 mM PAA rapidly evolved gas over 100 ^{o}C so were not used. Other pretreatment conditions were kept at 120 ^{o}C, 100 mM H_2SO_4 and 2 h pretreatment time. As the concentration of PAA increased, the amount of lignin removed increased from 63.1% in 200 mM, 85.4% in 300 mM, to 96.2% in 400 mM, but the amount of xylan removed remained similar (84.1, 79.4, 85.2%, respectively), Figure 7.1a. These results indicate that PAA reacted with the lignin [12], while the acidic conditions were responsible for xylan hydrolysis. With increasing PAA concentration, the losses of glucan also increased to 17.0, 20.8 and 33.6%, respectively. The optimal PAA concentration was 300 mM because it gave a high xylan and lignin removal and minimal loss of glucan.

The optimal temperature for removal of xylan and lignin was 120 °C, Figure 7.1b. Pretreatment at 100 or 110 ^{o}C with 300 mM PAA for 2 h only partially removed xylan and lignin, but pretreatment at 120 ^{o}C effectively removed both.

The higher acid concentration of 100 mM removed xylan more effectively, but had no effect on lignin removal, Figure 7.1c. High acid concentration (100

mM H_2SO_4), removed more xylan (79.4%) than at the low acid concentration (25 mM H_2SO_4: 60.7%, 50 mM H_2SO_4: 71.0%), indicating that H_2SO_4 catalyzes xylan hydrolysis. When combined with PAA, more effective xylan removal leads to more effective lignin removal. PAA with added H_2SO_4 removed lignin more effectively than PAA only [138], but the amount of lignin removal did not correlate with increasing concentration of H_2SO_4 at 120 ^oC. Therefore, optimal concentration of H_2SO_4 was selected to 100 mM based on xylan hydrolysis.

Surprisingly, pretreating for only 5 min was as effective as pretreating for 2 h, Figure 7.1d. Heating the sample to 120 °C and cooling required approximately 3 min, so it was not practical to reduce the pretreatment time below 5 min. Xylan and lignin removal were complete within 5 min and extending the pretreatment time had little further effect on xylan hydrolysis or delignification. Separate experiments revealed that the concentration of PAA decreased from 300 mM to ~30 mM after 5 min. Longer pretreatment times also had the disadvantage of removing some of the glucan due to acid hydrolysis. For example, ~15% of glucan was lost after a 30-min pretreatment time, Figure 7.1d.

The enzymatic digestibilities of the solids from different pretreatment times were similar, Figure 7.2. At an enzyme loading of 5 FPU/g glucan and 72 h digestion, the glucose yield increased slightly with increasing pretreatment

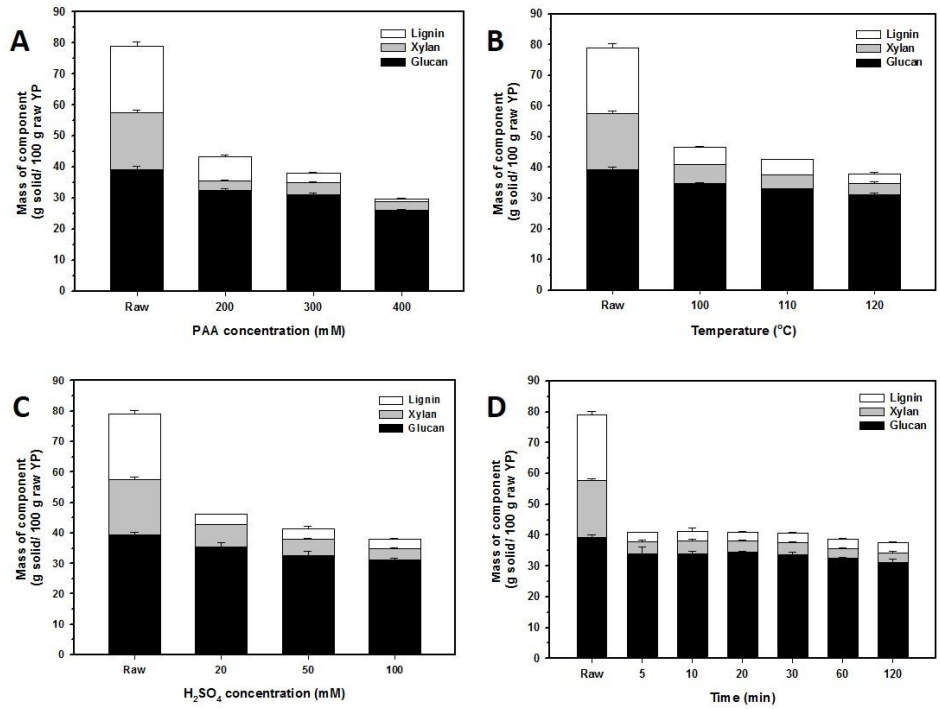

Figure 7.1 Optimization of one-step pretreatment. Increasing PAA concentration, pretreatment temperature and H_2SO_4 concentration increased xylan and lignin removal. The pretreatment time of 5 min was sufficient for the removal of xylan and lignin. Composition analysis of pretreated solid with (A) different PAA concentrations at 120 ^oC with 100 mM H_2SO_4 for 2 h, (B) different pretreatment temperatures with 300 mM PAA and 100 mM H_2SO_4 for 2 h, (C) different H_2SO_4 concentrations with 300 mM PAA at 120 ^oC for 2 h, (D) different pretreatment times with 300 mM PAA and 100 mM H_2SO_4 at 120 ^oC. Error bars correspond to the standard deviation for three measurements.

time (75.5, 76.2, 78.1 and 84.3%). Samples pretreated for 30 min yielded ~10% more glucose than those pretreated for only 5 min. At the higher enzyme loading of 30 FPU/g glucan, the glucose yield increased and the differences between the different pretreatment times decreased: 87.5, 86.1, 88.2 and 93.9%. These similar glucose yields from 5, 10 and 20 min pretreated solids suggests that the pretreatment removed similar amounts of xylan and lignin. The slightly higher glucose yield after 30 min pretreatment comes at the cost of longer pretreatment. 5 min as the optimal pretreatment time was selected.

Negligible amounts of sugar degradation products, furfural or HMF, formed under the selected pretreatment conditions. These degradation products, which can also inhibit subsequent fermentation, often form under hasher conditions [135].

Finally, optimal conditions were selected to minimize loss of glucan and formation of byproducts, and enhance xylan and lignin removal. Therefore, the optimal conditions for one-step pretreatment were 300 mM PAA (~2.3 wt%), 100 mM H_2SO_4 (~1 wt%), 120 ^oC and 5 min. These optimal conditions are milder than those for the standard acid hydrolysis of biomass (4 wt% sulfuric acid at 120 ^oC for 1 h) [81].

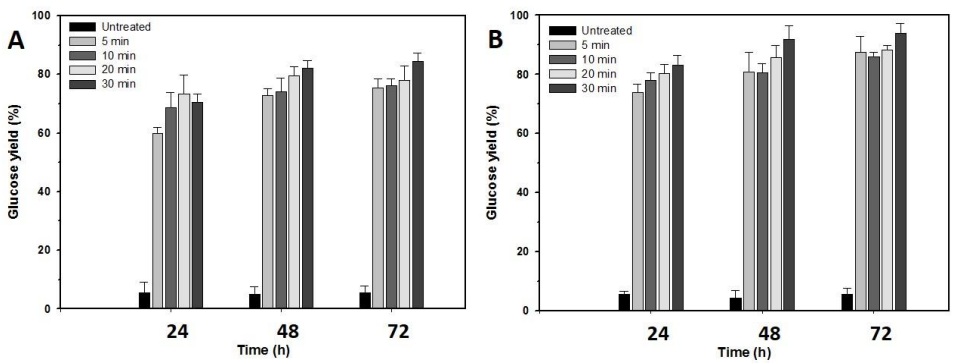

Figure 7.2 Effect of pretreatment time on enzymatic digestibilities of pretreated solid. The solid pretreated for 5 min released slightly lower amounts of glucose as did the solids pretreated for 10, 20 and 30 min. Yellow poplar was pretreated with 300 mM PAA and 100 mM H_2SO_4 at 120 ^oC. Enzymatic hydrolysis was conducted using enzyme loadings of (A) 5 FPU/g glucan (B) 30 FPU/g glucan and 30 *p*NPGU/g glucan. Error bars correspond to the standard deviation for three measurements.

7.3 Comparison with dilute acid pretreatment under same conditions

The effectiveness of the one-step pretreatment was compared to the standard dilute acid pretreatment under the same conditions of 120 ^{o}C for 5 min.

7.3.1 Chemical composition

Raw yellow poplar biomass contains 39.3 g glucan, 18.4 g xylan, 21.4 g lignin and 20.9 g other components per 100 g. Dilute acid (DA) pretreatment selectively solubilized and hydrolyzed xylan, thereby increasing the relative amounts of glucan and lignin in the remaining solid. The DA-pretreated solid contained 38.2 g glucan, 4.4 g xylan and 17.0 g lignin per 100 g of raw YP, which corresponds to removal of 75.8% of the original xylan and 20.8% of the lignin. DA pretreatment at higher temperatures removes 90 to 100% of the xylan [5, 14], but treatment removed only 75.8% of the xylan at the moderate temperature of 120 ^{o}C.

The DA/PAA pretreatment removed both xylan and lignin leaving a glucan-rich solid, Figure 7.3. The DA/PAA-pretreated solid contained 33.2 g glucan, 2.6 g xylan and 2.1 g lignin per 100 g of raw YP, which corresponds to removal of 85% of the original xylan and 90% of the lignin under moderate temperature. The relative amount of glucan in the solid fraction increased

from 39.3% in the raw biomass to 75.6% in the pretreated biomass. Only 15.4% of the glucan was lost during the one-step pretreatment. One-step pretreatment dramatically improved the lignin removal and also increased the xylan removal as compared to DA pretreatment under same conditions.

7.3.2 Enzymatic hydrolysis

Cellulase mixtures digested the one-step pretreated solid more effectively than the raw YP or the DA-pretreated solid, Figure 7.4. The enzymatic hydrolysis released only 5.9 and 30.0% of the glucose in raw YP and DA-pretreated solids, respectively, but released 81.7% of the glucose from the one-step pretreated solid after 72 h at low enzyme loadings. The one-step pretreated solid had 13.8 and 2.5 times higher enzymatic digestibility than raw YP and DA-pretreated solid, respectively. In the DA-pretreated solid, the residual lignin or pseudo-lignin likely blocked access of the enzymes to cellulose and non-productively adsorbed the cellulases thereby preventing them from acting on the cellulose [45, 127]. The higher removal of xylan and especially lignin in the one-step pretreated biomass enhanced its enzymatic digestibility by increasing the accessibility of cellulase to cellulose and reducing inhibition by residual lignin, thereby reducing the enzyme requirements.

The optimal conditions for one-step pretreatment (120 °C, 5 min) are milder

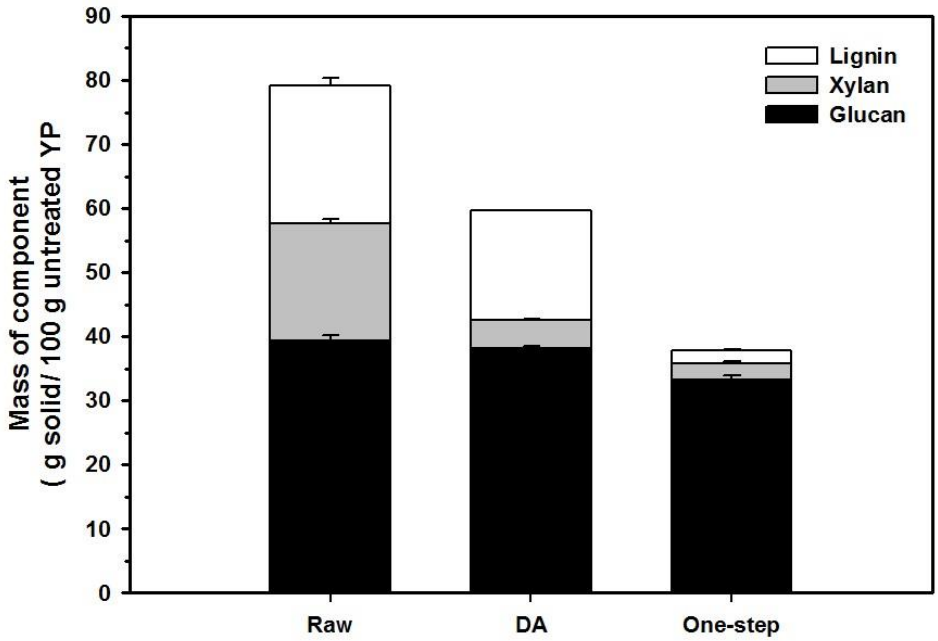

Figure 7.3 Composition of pretreated solid by DA and one-step pretreatment under same conditions. While DA pretreatment mainly hydrolyzed the xylan, one-step pretreatment solubilized xylan and lignin simultaneously. The values are based on the content of each component in 100 g of yellow poplar before pretreatment. Pretreatment conditions: DA: 100 mM H_2SO_4, 120 ^oC, 5 min; One-step: 300 mM PAA, 100 mM H_2SO_4, 120 ^oC, 5 min. Error bars correspond to the standard deviation for three measurements.

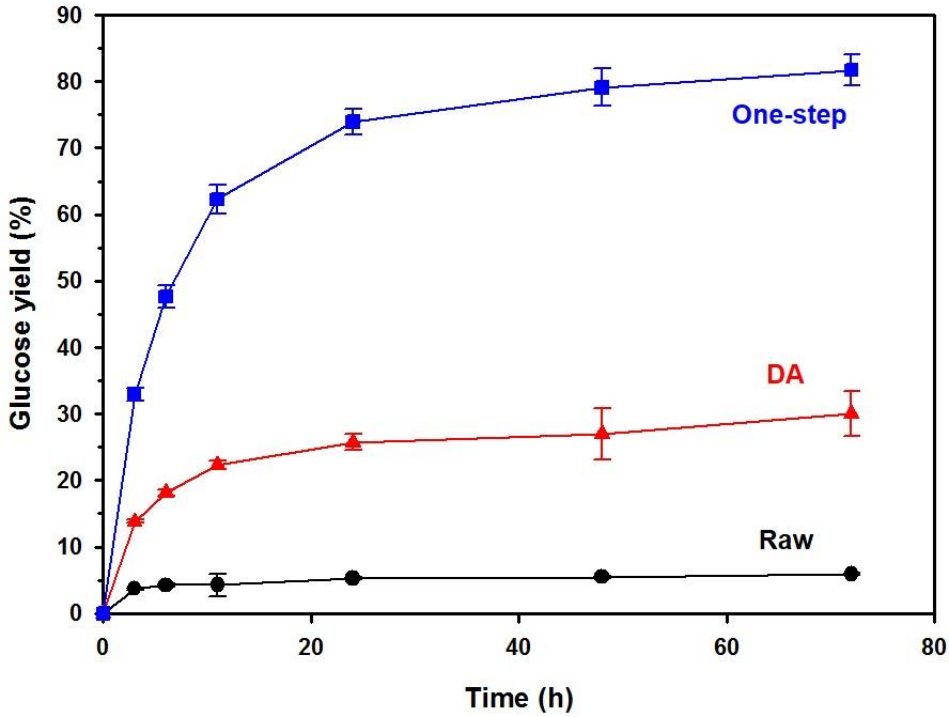

Figure 7.4 Comparison of glucose yield from enzymatic hydrolysis of solid from DA and one-step pretreatment of yellow poplar using 5 FPU/g glucan and 10 *p*NPGU/g glcuan. Glucose yield of raw YP and DA-pretreated solid was 5.9 and 30.0%, but increased up to 81.7% with one-step pretreatment at 72 h. Delignification in biomass increased the accessibility of cellulase to cellulose and reduced the inhibitory effect of lignin to cellulase compared as DA pretreatment. Pretreatment conditions: DA: 100 mM H_2SO_4, 120 ^oC, 5 min; One-step: 300 mM PAA, 100 mM H_2SO_4, 120 ^oC, 5 min. Error bars correspond to the standard deviation for three measurements.

than those for various organoslv pretreatment, Table 7.1. To remove comparable amounts of xylan and lignin, organosolv pretreatments required higher temperatures or longer times than the one-step pretreatment.

The one-step pretreatment yields a lower quality xylose fraction. First, the liquid, xylose-rich filtrate from the one-step pretreatment contained not just xylose, but also lignin and residual acetic acid from the PAA. In addition, HPLC analysis revealed three sugar oxidation products, likely due to reaction with the PAA. These products were not further characterized. The lower quality of this xylose fraction must be balanced by the short pretreatment time, higher quality of the glucose fraction, and high yield of glucose at low enzyme loadings.

7.3.3 Structural characterization

The structural changes in crystallinity, functional group distribution and surface morphology are consistent with removal of xylan and lignin with the one-step pretreatment.

The crystallinity index (CrI) of one-step pretreated solid increased from 49.9% for raw YP to 66.9% for DA-pretreated solid and to 75.1% for the one-step pretreated solid, Figure 7.5. The CrI indicates the relative amount of crystalline cellulose in the solid. This increase is consistent with the removal of the amorphous xylan and lignin fractions during pretreatment.

Table 7.1 Organosolv biomass pretreatment for saccharification

Biomass	Solvent	Catalyst	Temp. (°C)	Time (min)	Cellulose yield (%)	Hemicellulose removal (%)	Delignification (%)	Cellulose digestibility (%)	Ref.
Wheat straw	Ethanol 60% (w/w)	0.29% H_2SO_4	190	60	91.1	95.3	75.8	89.4	[139]
Sugarcane bagasse	Glycerol 80% (w/w)	0.94% H_2SO_4	190	60	89.3	96.6	53.5	>90	[140]
Sugarcane bagasse	FA [a] 88% (w/w)	-	107	60	87.5	90.7	74.5	53.2 (95.7 [b])	[141]
Sugarcane bagasse	PAA [c] 50% (on biomass)	-	80	120	-	-	82.0	82.1	[142]
Parairie cordgrass	MIBK [d] 9% (w/w)	0.69% H_2SO_4	154	39	-	-	87.0	84.0	[143]
Switchgrass	EA [e] 37%, ethanol 25% (w/w)	0.46% H_2SO_4	140	20	-	-	-	84.9	[109]
Corn stover	THF [f] 50% (v/v)	0.5% H_2SO_4	150	25	75.0	94.8	76.6	95.0	[136]
Beech wood	GVL [g] 80% (w/w)	0.75% H_2SO_4	120	60	95.0	78.5	77.0	55.0 (99.0 [h])	[75]
Yellow poplar	PAA 2.3% (w/v)	1% H_2SO_4	120	5	75.6	85.0	90.0	81.7	This study

[a] Formic acid, [b] Deformylation with NaOH incubation at 120 °C for 1 h, [c] Peracetic acid, [d] Methyl isobutyl ketone, [e] Ethyl acetate, [f] Tetrahydrofuran, [g] γ-Valerolactone, [h] NaOH incubation at 50 °C for 1 h and neutralization with acetic acid.

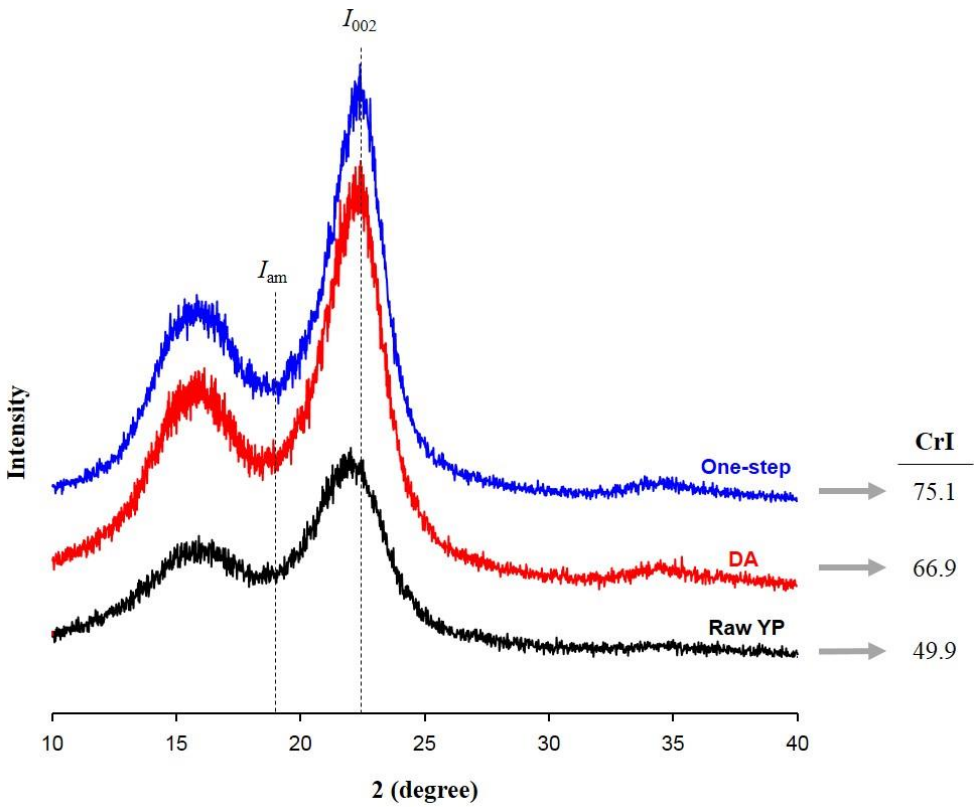

Figure 7.5 Crystalline change after DA pretreatment and one-step pretreatment. The crystallinity of one-step pretreated solid increased due to high removal of xylan and lignin. I_{002}: maximum intensity of crystalline portion at ~22° and I_{am}: minimum intensity of amorphous portion at ~18°. Pretreatment conditions: DA: 100 mM H_2SO_4, 120 °C, 5 min; One-step: 300 mM PAA, 100 mM H_2SO_4, 120 °C, 5 min.

The absorption bands in the FTIR spectrum related to functional groups in hemicellulose and lignin decreased in the one-step pretreated solid as compared to that of raw YP, Figure 7.6. The ester linkage C=O between lignin and hemicellulose at 1720 cm^{-1} and the alkyl ester of the acetyl group in hemicellulose at 1245 cm^{-1} disappeared. The absorption bands at 1300-1600 cm^{-1} related to lignin also decreased. These decreases indicate that the one-step pretreatment removed xylan and lignin. In addition, several absorption bands associated with cellulose increased in the one-step pretreated solid as compared to that of raw YP. The O-H stretch at 3330 cm^{-1} and C-H stretch at 2900 cm^{-1} associated with cellulose were stronger after one-step pretreatment than raw YP. Similarly, the C-O-C glycosidic bond stretching at 1160 cm^{-1}, C-O-C ring skeletal vibration at 1100 cm^{-1} and C-O-H stretching of primary and secondary alcohols at 1030 cm^{-1} were also more intense than in raw YP. These increases are consistent with an increase in the relative amount of glucan. In contrast, after DA pretreatment, only the absorption bands related to hemicellulose decreased, while the bands related to lignin increased. This difference is consistent with selective removal of xylan by the DA pretreatment, leaving a glucan- and lignin-enriched solid.

The surface morphology of the solid after one-step pretreatment showed an extensively disrupted structure with exposed cellulose fibers, Figure 7.7. The surface of raw YP appeared compact with rigid and highly ordered fibrils,

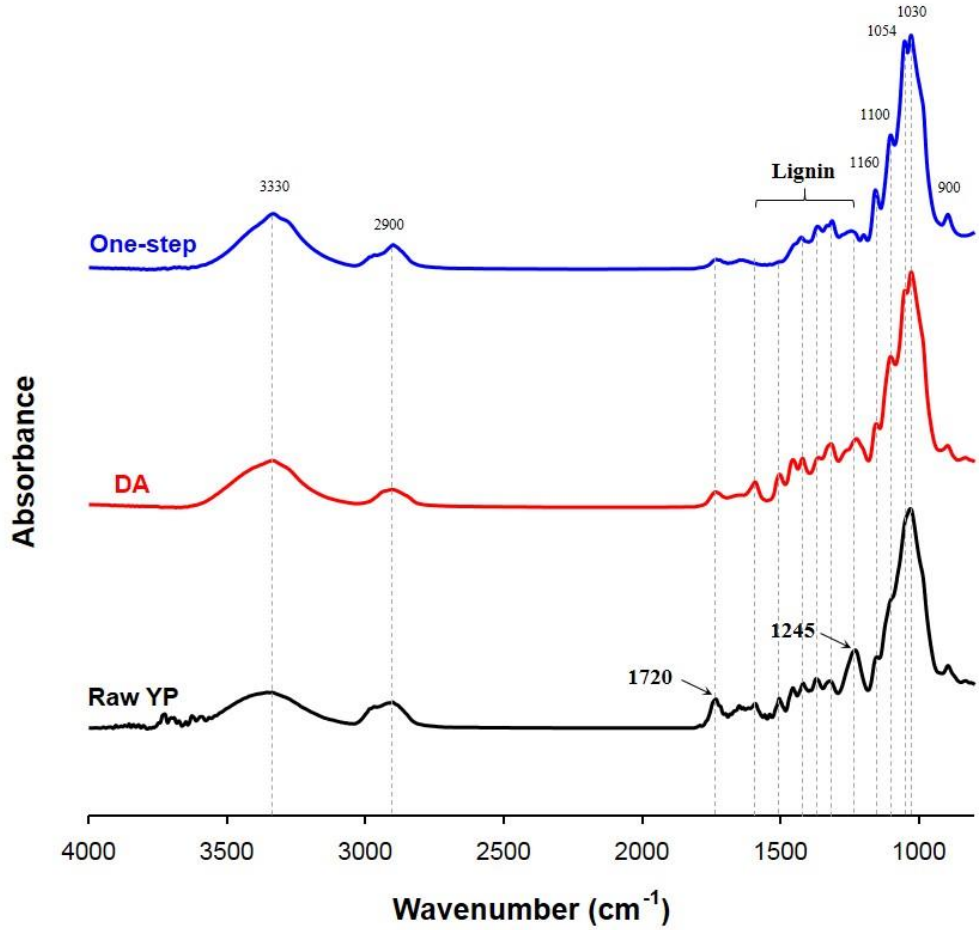

Figure 7.6 FT-IR spectra of raw yellow poplar, DA-pretreated solid and one-step pretreated solid. After one-step pretreatment, the absorption band related hemicellulose and lignin decreased, and the band associated with cellulose increased. Pretreatment conditions: DA: 100 mM H_2SO_4, 120 ^oC, 5 min; One-step: 300 mM PAA, 100 mM H_2SO_4, 120 ^oC, 5 min.

and some flakes on the surface, Figure 7.7A. The surface of the solid pretreated with DA showed enlarged pores and irregular cracks, but preserved the major features of raw YP. A thin layer covered the surface, likely consisting of redeposited lignin, which can inhibit binding of the cellulase to cellulose [117]. The surface of the one-step pretreated solid showed completely different surface morphology, Figure 7.7C. Defibrillation and fiber size reduction occurred upon one-step pretreatment. The structure separated into defibrillated fibers with the width of typical fragment decreasing from >250 μm in raw YP to ~10 μm. These SEM images indicate that the role of one-step pretreatment is to break down the lignin and carbohydrate linkages as well as xylan removal. Similar decreases in the width of fibers in sugar cane bagasse occurred upon pretreatment with concentrated acetic acid and hydrogen peroxide [144]. In addition, the visible 'wrinkling' on the surface indicate the solid may be structurally weaker by extensive removal of xylan and lignin. Hence, one-step pretreatment enhanced fiber defibrillation and delignification, exposed the cellulose fibers, thereby increasing accessibility of cellulase to cellulose.

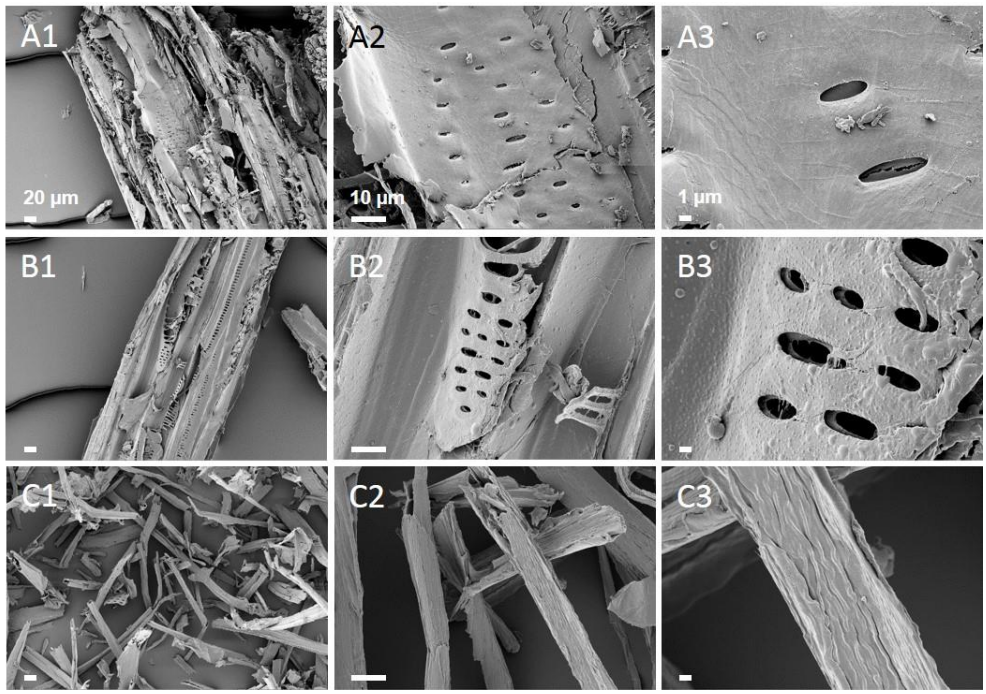

Figure 7.7 SEM images of raw yellow poplar (A), DA-pretreated solid (B), and one-step pretreated solid (C) at magnification 500 × (1), 3,000 × (2), and 10,000 × (3). Scale bars are shown. The raw YP had a rigid and compact surface. DA-pretreated solid showed enlarged pores and surface covered by a thin layer of deposited lignin. One-step pretreated solid showed completely defibrated structure and smooth cellulose fiber. Pretreatment conditions: DA: 100 mM H_2SO_4, 120 ^oC, 5 min; One-step: 300 mM PAA, 100 mM H_2SO_4, 120 ^oC, 5 min.

7.4 Conclusion

A short (5 min) one-step pretreatment at 120 ^{o}C with 300 mM PAA and 100 mM H_2SO_4 removed both xylan and lignin from yellow poplar biomass in contrast to dilute acid pretreatment, which removed mainly xylan under same conditions. Enzymatic hydrolysis of solid from this one-step pretreatment released ~80% of the glucose, which was 2.5 and 13.8 times higher than from solids from DA-pretreatment and raw YP, respectively. The difference between one-step and DA pretreatment was high lignin removal, which dramatically enhanced enzymatic hydrolysis at low enzyme loadings. In addition, one-step pretreatment decreased the formation of fermentation inhibitors. This novel one-step pretreatment under mild conditions will be promising methods that could be more effective in biofuel production.

Chapter 8.

Overall discussion and further suggestions

Chapter 8. Overall discussion and further suggestions

Depletion of fossil fuels and serious environmental pollution are a need for the replacement of petroleum and development of renewable energy. Ethanol does not generate pollution and is considered as a promising clean energy. However, production of ethanol from grains is not sustainable because of competition with food production. Production of ethanol from non-grain feedstock, especially lignocellulosic biomass, has received the interest in many countries.

Lignocellulosic biomass is a type of renewable and abundant resources. However, the complex structure of lignocellulosic biomass make it recalcitrant to enzymes making the hydrolysis of cellulose slow and inefficient. Effective pretreatment disrupts cell wall, increase enzymatic digestibility and affects the cost of downstream. Many processes have been developed for lignocellulosic feedstocks, but some processes seem to be promising.

Currently, biorefinery has focused on all sugars from lignocellulosic biomass for producing cellulosic ethanol. Other residues such as lignin are used as burning fuels for generating steam and electricity. One-product plant will be difficult to establish profitable biomass biorefinery. In the petrochemical industry, petroleum refineries produce multiple products

(liquefied petroleum gas, gasoline, diesel, etc) for high economic efficiency. Therefore, multi-product biorefineries are necessary for economically feasibility. In biorefinery, lignin will be used for phenolic precursors, xylose for high-value products as well as glucose for ethanol. Complete utilization of lignocellulosic biomass will enhance the economy of biomass refineries.

In this thesis, integrated process based on peracetic acid was developed for utilization of lignocellulosic biomass. The optimization of supercritical or hydrothermal process supports the performance of peracetic acid-based process, and thus enhances degradation of lignin and increases hydrolysis of cellulose in lignocellulosic biomass through combination with peracetic acid process.

In chapter 4, combining peracetic acid with supercritical water was developed to improve degradation of Kraft lignin. Peracetic acid (90 mM, 24 h) and supercritical water (420 ^{o}C, 50 MPa, 20 min) degraded to ~40 and ~25% of original Kraft lignin, respectively. The combinatorial method with peracetic acid and supercritical water was more effective than either single treatment by ~20% degradation of Kraft lignin. In addition, the combinatorial method produced catechols from Kraft lignin, which was not existed in either single treatment. The synergistic effect of combinatorial method led to reduce the reaction time of supercritical water to 3 min.

In chapter 5, combination of hot compressed water with peracetic acid was

established for enhancement of lignin degradation and cellulase accessibility to cellulose in lignocellulosic biomass. Hot compressed water (200 ^{o}C, 1.5 MPa, 15 min) selectively hydrolyzed up to 90% of the xylan. Peracetic acid (90 mM, 60 ^{o}C, 6 h) solubilized ~20% of the lignin, but increased the solubilization of the lignin to ~70% by using hot compressed water prior to peracetic acid. The combined pretreatment with hot compressed water and peracetic acid was more effective than the sum of the single pretreatment to remove xylan and lignin. The remaining solid consisted of mainly glucan (~75%). Enzymatic hydrolysis released only 5.7% of the glucose from untreated yellow poplar, but the yield increased to 47.7% after hot compressed water pretreatment and further to 91.9% upon adding the peracetic acid pretreatment step. Enhancement of lignin removal contribute to making the cellulose accessible to cellulases.

In chapter 6, sequential process with dilute sulfuric acid and peracetic acid was developed for mild operating conditions. Sulfuric acid was added for reducing the pretreatment time and temperature. In the first step, dilute acid with microwave heating (50 mM, 140 ^{o}C, 5 min) hydrolyzed ~90% of xylan. The xylose yield in hydrolysate after dilute acid pretreatment was 83.1%. In the second step, peracetic acid (90 mM, 60 ^{o}C, 6 h) removed ~80% of lignin. This sequential pretreatment fractionated biomass into xylan and lignin, leaving a solid residue enriched in cellulose (~80%). The sequential

pretreatment enhanced enzymatic digestibility of the cellulose by removal of the other components in biomass. The glucose yield after enzymatic hydrolysis was 90.5%, which is 1.6 and 18 times higher than for dilute acid-pretreated solid and raw biomass, respectively. This novel sequential pretreatment efficiently separates the three major components of yellow poplar, and reduces enzyme requirement and energy consumption.

In chapter 7, we have reported for the first time the one-step pretreatment, in which a peracetic acid pretreatment is coupled to a dilute acid pretreatment, in order to remove the xylan and lignin at moderate temperature, simultaneously. Pretreatment of biomass with dilute acid requires high temperatures of >160 ^{o}C for several hours to remove xylan and does not remove lignin. Addition of peracetic acid to mild dilute acid pretreatment reduces the temperature to 120 ^{o}C. Pretreatment of yellow poplar with peracetic acid (300 mM) and dilute acid (100 mM) at 120 ^{o}C for 5 min removed 85.7% of the xylan and 90.4% of the lignin leaving a solid consisting of 75.6% glucan. This solid was converted to glucose with an 84.0% yield, which was 2.5 and 13.8 times higher than with dilute acid-pretreated solid and raw yellow poplar, respectively. Therefore, the addition of peracetic acid led to high lignin removal and dramatically increase the effectiveness of dilute acid pretreatment of biomass. This novel one-step pretreatment at moderate temperatures will be promising methods for biofuel production.

In this thesis, an integrated process based on peracetic acid was established for the application to biorefinery. The performance of the peracetic acid-based process through enhancement of lignin degradation and cellulose hydrolysis was evaluated. It is believed that this research will improve the availability of the integrated process in the lignocellulosic biorefinery for the application of cellulose, hemicellulose and lignin.

Single-step peracetic acid pretreatment required high concentration of peracetic acid or several cycles of dilute peracetic acid to remove lignin effectively. An alternative two-step pretreatment with alkali and low concentrations of peracetic acid yielded a xylose fraction containing lignin and lignin derivatives that inhibit the fermentation of xylose, Table 8.1.

Sequential pretreatment has the advantage of efficient recovery of the xylose fraction and requiring only single treatment with dilute peracetic acid to remove the lignin. Although the dilute acid pretreatment does not remove lignin, it enhance the subsequent removal of lignin with low concentration of peracetic acid. In other words, when preceded by hydrothermal/dilute acid pretreatment that modified the biomass structure, requirement of peracetic acid can be decreased to 25 wt% based on initial dry materials and accessibility of peracetic acid to lignin can be more simply done. In addition, the most common pretreatment is acid at high temperatures for several hours. The development of process under mild operating conditions at 120 ^{o}C for 5

min will be useful for biorefinery system. Addition of peracetic acid to acid pretreatment dramatically improves its efficiency, thereby lowering the required temperature and the time. Thus, performance of the integrated process under mild operating conditions has a significant potential in biorefinery alternative to conventional process.

In this research, it is not the crystallinity of cellulose but degree of delignification to have a significant influence on the enzymatic digestibility of lignocellulosic biomass. By confirming the enhancement of enzymatic hydrolysis after integrated process compared to single process, we showed that almost complete lignin removal was the most noticeable difference.

Although the integrated process could receive a competitive evaluation, addition of peracetic acid process may increase cost of overall process. An economic evaluation considering overall process from biomass to sugars is essential for further comparison of the integrated process with conventional process. It is crucial for the improvement of integrated process to commercial scale. It can be better if the cost of perhydrolase for industrial production are similar to that of other industrial enzymes.

Optimization of pretreatment using various variables will be helpful to expect the results from pretreatment. But, more generalized data should be established for application to future process. Therefore, it is necessary for planning further characterization of the pretreated solid on various

lignocellulosic feedstocks (herbaceous plan, softwood and hardwood) to confirm the potential of integrated process. The optimal conditions of post treatment for pre-treated materials are also needed as well as those of raw materials.

The pilot-scale of pretreatment and high solids enzymatic hydrolysis is essential to show the possibility of integrated process in industrial scale. In addition, purification process is needed to increase the purity of fractionated components by separating impurities.

When these additional evaluation and processes are established, we expect this research to contribute to constructing a biomass-based society which is economically feasible.

Table 8.1 Summary of peracetic acid-based pretreatment

Biomass	Pretreatment conditions						Hemicellulose removal (%)	Delignification (%)	Cellulose digestibility (%)	Ref.
	Previous treatment			Peracetic acid treatment						
	Solvent	Temp. (°C)	Time (min)	Solvent [a]	Temp. (°C)	Time (min)				
Sugarcane bagasse	-	-	-	PAA 50%	80	120	59.6	82.0	82.1 [b]	[111]
Sugarcane bagasse	NaOH 10%	90	90	PAA 10%	75	150	-	95.6	56.2 [c]	[63]
Aspen	-	-	-	PAA 28.2%	60	360	6.0%	85.0	95.1 [d]	[17]
Pine	-	-	-	PAA 100% (v/v) [e]	80	120	-	98.1	-	[18]
Yellow poplar	Water	200	15	PAA 25.2%	60	360	93.6	76.0	91.9 [f]	This study
	H_2SO_4 0.5%	140	5	PAA 25.2%	60	360	94.0	84.1	90.5 [g]	
	-	-	-	PAA 45.6%	120	5	85.0	90.0	81.7 [h]	

[a] Based on initial dry materials, [b] 20 FPU/g glucan, [c] 15 FPU/g solid, [d] 60 FPU/g glucan and 64 *p*NPGU/g glucan, [e] liquid/solid ratio (w/w) = 10:1, [f] 4FPU/g glucan and 30 *p*NPGU/g glucan, [g] 5FPU/g glucan and 30 *p*NPGU/g glucan, [h] 5 FPU/g glucan and 10 *p*NPGU/g glucan.

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국 문 초 록

과산화아세트산 처리를 통한 리그닌 분해 및 셀룰로오스 가수분해의 향상

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본 연구에서는 리그닌 분해를 향상시키고, 목질계 바이오매스의 구성 성분들을 분리하여 셀룰로오스 가수분해를 향상시키는 과산화아세트산 기반의 통합적인 공정의 개발을 시도하였다.

효소로 생산된 과산화아세트산을 이용한 단독 공정은 과산화아세트산의 낮은 농도로 인해, 크래프트 리그닌과 목질계바이오매스의 리그닌 산화에 비효율적이다. 리그닌을 효과적으로 산화시키기 위해서는 고농도 과산화아세트산 혹은 저농도 과산화아세트산의 반복 처리가 필요하다. 이에, 크래프트 리그닌의 분해를 위해 과산화아세트산과 초임계수 처리의 최적화 과정을 통하여 조합 공정을 개발하였다. 최적화된 조합 공정은 크래프트 리그닌의 분해를 향상시켰고, 카테콜 생산의 가능성을 제시하였다. 또한, 조합 공정의 시너지 효과는 단독 초임계수 공정의 반응 시간을 20분에서 3분으로 감소시켰다.

목질계 바이오매스의 리그닌 분해만 가능한 과산화아세트산 처리의 한계를 극복하기 위해 열수처리를 도입함으로써 백합나무의 셀룰로오스, 헤미셀룰로오스 및 리그닌을 분획할 수 있는 순차적인

공정을 개발하였다.

우선, 백합나무의 구성성분들을 분리하기 위해, 효소로 생산된 과산화아세트산과 열수의 조합 공정을 구축하였다. 열수 처리 (200 °C, 15 분) 공정을 통해, 대부분의 자일란을 용해시켜 자일로스를 얻을 수 있었다. 단독 과산화아세트산 처리 공정 시 리그닌 제거율은 20% 였지만, 조합 공정을 통해 리그닌 제거율을 80%까지 증가시켰다. 자일란과 리그닌의 분리를 통해, 셀룰로오스의 순도를 증가시킴으로써 보다 효율적인 셀룰로오스 가수분해를 유도하였다.

다음으로 열수공정에 황산을 추가하여 보다 온화한 전처리 공정을 개발하여, 구성성분들을 분리하였다. 산 처리 공정의 최적화 과정 (140 °C, 5 분)을 통해, 80% xylose recovery를 얻었고, 추가적인 과산화아세트산 처리로 리그닌도 성공적으로 분리하였다. 순차적인 전처리 공정은 다른 성분들의 분획을 통해 셀룰로오스의 순도를 증가시켰고, 효소 당화력을 향상시켰다.

기존의 과산화아세트산을 이용한 공정과 비교하여, 순차적인 공정은 각 성분들을 분획할 수 있다는 장점이 있다. 또한, 순차적인 공정에서 열수처리는 리그닌을 제거하지 못하지만, 저농도의 과산화아세트산만으로 리그닌 분해를 향상시켰다. 이는 열수처리로 인해, 바이오매스의 구조가 변하여 과산화아세트산의 요구량을 감소시킬 수 있었다. 또한, 자일란이 제거됨으로써, 리그닌에 대한 과산화아세트산의 접근성을 증가시킨 것으로 판단된다.

마지막으로, 묽은 황산을 이용한 바이오매스 전처리 공정은 헤미셀룰로오스를 제거하기 위해 고온이 필요하지만 리그닌을 제거할 수 없다. 과산화아세트산을 첨가하여 리그닌도 함께 제거함으로써 순도 높은 셀룰로오스를 얻을 수 있는 단일 (one-step) 공정을 개발하였다. 단일 공정 (120 °C, 5분)의 최적화를 통해 자일란, 리그닌을 동시에 제거하였고, 셀룰로오스의 순도를 증가시킴으로써 보다 효율적인 셀룰로오스 가수분해를 유도하였다. 과산화아세트산의 첨가는 산 처리 공정의 효율을 극적으로 증가시켰고, 온도 및

반응시간 요구조건을 감소시켰다. 과산화아세트산을 이용한 단일 공정은 기존의 ethanol, glycerol, ethyl acetate 및 THF 등을 이용한 유기용매 전처리 공정보다 낮은 온도 및 짧은 반응시간 조건에서, 유사한 헤미셀룰로오스, 리그닌 제거율 및 효소 당화력을 보일 수 있다는 장점이 있다.

본 연구는 기존의 열수공정과 과산화아세트산의 조합을 통하여 리그닌 분해를 향상시켜 목질계 바이오매스에 적용하였고, 헤미셀룰로오스와 리그닌을 대부분 제거하여 고순도의 셀룰로오스를 생산하였다. 개발된 공정은 에너지 소비량 및 당화효소 요구량을 감소시킬 수 있다. 이러한 과산화아세트산을 기반으로 한 통합적인 공정의 개발은 석유 기반의 연료 및 화학물질에 대한 의존도를 줄이고, 목질계 바이오매스의 모든 성분들을 활용하기 위한 바이오리파이너리 시스템 개발을 위해 앞으로 나아가야 할 하나의 방향을 제시할 것으로 기대된다.

주요어: 목질계 바이오매스, 크래프트 리그닌, 백합나무, 과산화아세트산, 초임계수, 열수, 묽은 황산, 전처리

학번: 2010-21008