CATALYZED OXIDATIVE DELIGNIFICATION TO OVERCOME PLANT CELL WALL RECALCITRANCE TO BIOLOGICAL CONVERSION

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ABSTRACT

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Biomass from agricultural/forestry waste and energy crop plantations is available in large quantities for the production of renewable fuels and chemicals. Utilization of biomass delivers many ecological and agronomical benefits, and supports the growth of a sustainable economy. The cell wall polysaccharides in biomass can be enzymatically hydrolyzed to monomeric sugars, which in turn can be used as an intermediate platform chemical for the production of biofuels and biochemicals via catalytic transformation and microbial fermentation processes. A major challenge faced by many biomass conversion strategies is the low enzymatic digestibility of cell wall polysaccharides, which is caused by the plants' natural defense against enzymatic attack and deconstruction. To impair this defense and to prepare biomass for efficient enzymatic conversion, many pretreatment technologies have been designed and employed.

We have developed a novel catalytic oxidative pretreatment technology, a.k.a. the Cu(bpy)-AHP pretreatment. The enzymatic hydrolysis yields of sugars from woody biomass (e.g. hybrid poplar) can be improved by two to three folds as the result of the Cu-catalyzed hydrogen peroxide oxidation during Cu(bpy)-AHP pretreatment. Under particular reaction conditions, we achieved high efficacy of pretreatment in about 1 hour of pretreatment with modest consumption of chemicals. Through tuning of operation variables and improvements in process integration, a scheme for bio-ethanol production from Cu(bpy)-AHP pretreated hybrid poplar has been established for techno-economic evaluation and further development.

Detailed characterization of Cu(bpy)-AHP pretreated biomass with heteronuclei NMR spectroscopy and TEM microscopy reveals oxidative modifications of lignin as the result of the pretreatment, as well as

disruption of lignified cell wall structure. As the result of modest Cα oxidation and depolymerization reactions, a significant proportion of lignin in the plant cell wall is solubilized during Cu(bpy)-AHP pretreatment. Microscopic and spectroscopic analyses highlight the role of metal-catalyzed oxidation reactions in close vicinity of the biomass surface. Analysis of biomass degradation products released during pretreatment suggests that Cu-catalyzed oxidation is a viable technology as both a biomass pretreatment and a process for sustainable production of aromatic chemicals such as vanillin.

Man follows Earth.
Earth follows heaven.
Heaven follows the Tao.
Tao follows what is natural.
— *Lao-Tsu*

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KEY TO ABBREVIATIONS

AA	auxiliary activity (enzyme family)
ABTS	2,2’-azinobis(3-ethylbenzthiazoline-6-sulfonate)
AEAHPSG	AHP-pretreated AESG
AESG	alkali-extracted switchgrass
AHP	alkaline hydrogen peroxide
BPPA	N,N-bis(6-pivaloylamido-2-pyridylmethyl)-N-(2-pyridylmethyl)amine
CBM	carbohydrate binding module (enzyme family)
CBM33	carbohydrate binding module family 33
CC	cell corner
Cel7A	cellubiohydrolase I
CML	compound middle lamella
Cu(bpy)	copper 2,2'-bipyridine complexes
Cu(bpy)-AHP	alkaline hydrogen peroxide pretreatment catalyzed by copper 2,2'-bipyridine complexes
Cu-APL	lignin solibilized during Cu(bpy)-AHP pretreatment that is recovered via acidification
DMSO	dimethyl sulfoxide
DTNE	1,2-bis-(4,7-dimethyl-1,4,7-triazacyclonon-1-yl)ethane
EDS	energy dispersion spectroscopy
EELS	electron energy loss spectroscopy
EIA	U.S. Energy Information Administration
ESEM	environmental scanning electron microscopy
FPU	filter paper unit
FXU	Farvet xylan unit
G	guaiacyl (lignin subunit)
GalA	galacturonic acid

GH	glycosyl hydrolase (enzyme family)
GH61	glycosyl hydrolase family 61
GXU	Genencor xylanase units
H	p-hydroxyphenyl (lignin subunit)
HBT	1-hydroxybenzotriazole
HSQC	heteronuclear single quantum coherence spectroscopy
ICM-MS	inductively coupled plasma mass spectrometry
L/M	ligand-to-metal molar ratio
LiP	lignin peroxidase
LPMO	lytic polysaccharide monooxygenase
MnP	manganese peroxidase
MTO	methyltrioxorhenium
NMR	nuclear magnetic resonance
POM	polyoxometalate
PTL	recovered liquid from liquid-solid separation after pretreatment
S	syringyl (lignin subunit)
SEC	size exclusion chromatography
SEM	scanning electron microscopy
SPORL	sulfite pretreatment to overcome recalcitrance of lignocellulose
SSF	simultaneous saccharification and fermentation
TACN	1,4,7-trimethyl-1,4,7-triaza-cyclononane
TEM	transmission electron microscopy
UV	ultraviolet
UV-Vis	ultraviolet-visible (spectroscopy)

CHAPTER 1

INTRODUCTION

The term "biomass" to be discussed refers to the ensemble of many types of organic material with origin in plantae. Plants harvest solar energy and mitigate carbon dioxide to provide energy and organic carbon for the entire biosphere. Until the Industrial Revolution in the 18th century, the majority of the energy need of humanity was fulfilled by utilization of biomass. In the Unites States, a predominant proportion of the energy consumption came from wood until 1850s.[1] Biomass materials such as cotton and flax were (and still are) important feedstocks for the textile industry. Since the discovery of fossil resources (e.g. coal, petroleum and natural gas), the availability of energy has soared in coincidence with the global energy consumption. During the same time period, the role of biomass as an energy source significantly diminished and the industrialized countries became heavily dependent on fossil energy. The olefin chemicals in petroleum are used to produce various types of plastics synthetic fibers, which substituted metal material and biomass fibers in many industries and applications. Fossil resources have been at the center of the chemical industry in the past century and largely defined our current way of living.

As far as the sustainability human development is concerned, the dependence on fossil resources becomes problematic. Coal, petroleum and natural gas are products from the decay of buried flora and fauna under ground over epochs. The rate at which fossil resources regenerate is typically slow (the regeneration process takes 10^3-10^9 years) and cannot catch up with the consumption rate, and therefore the depletion of these resources is inevitable. Should such depletion occur in the future, new renewable resources would need to be discovered and exploited in a sustainable fashion to fulfill the everlasting global need for energy and organic materials. One of such resources is cellulosic biomass (referred to as "biomass" in this context), which stores solar energy as well as organic carbon as the result of biological carbon sequestration. Production of biomass can be performed on various types of landscapes (e.g. biomass plantations, agricultural farmland, and marginal land), and therefore the feedstock for biomass-based energy and materials will be abundant. Moreover, growth of cellulosic biomass improves land vegetation and creates ecological benefits such as CO_2 mitigation and increased biodiversity.

Biomass can be obtained from many sources, and the availability of biomass varies geographically due to the variability in local ecology and agricultural landscape. Forestry land produces woody biomass such as logging residues and industrial by-products (bark, slabs, sawdust etc.), while agricultural land produces other types of biomass such as straw, stover and husks. The morphological, mechanical, and chemical properties significantly differ between different types of biomass, and the biomass material itself is also heterogeneous in nature. Although biomass materials vary by source and nature, they are all constituted by the plant cell wall, which provides plant with structural support and mechanical rigidity. Plant cell wall is a complex matrix of structural molecules including polysaccharides (cellulose, hemicellulose, pectin, etc.), lignin, and glycosylated proteins. The chemical identity and properties of these components and their distribution in the plant cell wall will be discussed in detail in Section 1.3. Some technologies target all components of biomass as a whole and produce a less refined product such as "pyrolysis bio-oil" and "biogas from biomass gastification", while some other technologies fractionate biomass into relatively pure streams (e.g. sugars from enzymatic hydrolysis of polysaccharides, lignin from solvent extraction) before refining these streams into fuels and chemicals.

1.1 Current Technologies

Heating biomass to an elevated temperature in the absence of oxygen releases steam, volatile organic compounds, and oxocarbons (CO and CO_2) from the biomass. During pre-industrial ages, woody biomass was torrefied to generate heat and to produce char for smelting minerals. Relatively more recent research on deconstructive dry distillation of wood started early in the 20th century and led to the development of more advanced technologies including biomass gastification and pyrolysis. Exposing biomass to heat induces thermochemical changes in biomass structure and partially volatilizes the biomass. The vapor released from biomass includes biomass syngas (CO_2, CO, H_2) as well as some other volatilized compounds that can be condensed into a liquid a.k.a. "pyrolysis bio-oil". The pyrolysis bio-oil is not stable and has high content of water, acids, and oxygenated carbon.[2] Therefore, it cannot be used directly as a drop-in fuel. This challenge can be overcome by an advanced catalytic pyrolysis process which hydrogenates the pyrolysis vapor *in situ*.[3] Alternatively, pyrolysis bio-oil can be chemically upgraded via

hydrotreating and hydrocracking to meet the standards for diesel and gasoline fuels.[2] The syngas released from biomass during thermochemical treatment can be combusted directly to generate heat and power, while it can also be converted to organic fuels via Fischer-Tropsch synthesis[4] or microbial syngas fermentation.[5]

The solid carbonaceous residual after biomass pyrolysis is commonly referred to as "biochar." As a potential soil amendment, biochar retains the carbon that is fixed by plants, and contributes to the mitigation of CO_2 from the atmosphere.[6] Biochar can also be used directly as the carbon fuel for direct carbon fuel cells.[7] The porous structure and adsorption properties of biochar have attracted increasing interest in the development of biochar-based sorbents for water contaminants[8,9] and soil pollutants.[10,11] With high surface area and conductivity, biochar can also be used to manufacture electrodes in fuel cells[12] and supercapacitors.[13-15]

Utilization of agricultural biomass via anaerobic digestion has been performed for centuries.[16] Under anaerobic conditions, organic matter in biomass can be fermented by heterotrophic microorganisms to produce "biogas" which is rich in methane and carbon dioxide. The microorganisms involved in methanogenic anaerobic digestion is a microbial consortium with high diversity and great complexity, but they can be roughly categorized into four families: cellulose lytic microbes, non-cellulose sacharride lytic microbes, hydrogen-producing bacteria, and Archaea methanogen.[17] During anaerobic fermentation of biomass, the carbon source of the microbial community is the polysacharrides. As the result, the hydrolysis of polysaccharide is a rate-limiting step in the conversion of biomass to biogas,[17] and the yield of biogas during anaerobic digestion is affected by the digestibility of biomass by microbes.

Plant cell wall materials are the carbon source for various types of saprotrophic bacteria and fungi. Most of these organisms extract the carbohydrates from plant cell wall by deconstructive reactions facilitated by extracellular enzyme systems. These enzyme systems are vastly diverse among species and consist of families of proteins with miscellaneous enzymatic activities. Some of the enzyme systems are ensembles of secreted carbohydrate-active enzymes that cleave covalent bonds in carbohydrates, and many of these free enzymes have been identified, isolated, and characterized. Based on their functions,

carbohydrate-active enzymes are categorized into families including the glycosyl hydrolase family (GH), the carbohydrate bonding module family (CBM), and the auxiliary activity family (AA). These enzymes have different specificities and function synergistically during biomass hydrolysis. For example, *Hypocrea jecorina* secretes endoglucanases that are capable of cleaving cellulose chains and creating new chain ends. *Hypocrea jecorina* also secretes both cellobiohydrolase Cel7A, which progressively cleaves cellulose chain and produces cellubiose molecules, and β-glucosidase, which catalyzes the hydrolytic reaction of cellubiose yielding monomeric glucose. Another known carbohydrate-acting enzyme system is the cellulosome which is a protein complex attached to the outer surface of certain types of bacteria and fungi. These protein complexes are biomass-degrading enzymes linked together by a protein scaffold (a.k.a. scaffoldin). Although cellulosomes are still under intensive research and have only been rudimentarily understood, microscopic studies of cellulose degradation suggested that cellulosomes employ very different mechanisms compared to free enzymes during cellulose hydrolysis.[18]

Enzymatic hydrolysis of biomass to monomeric sugars followed by fermentation to biofuels and biochemicals is a viable choice as a biomass conversion technology. Homologous and heterologous expression of carbohydrate-acting enzymes enabled commercial scale production of enzyme cocktails dedicated for biomass saccharification. Renewable sugars from biomass saccharification can again be used to produce enzymes sustainably. Enzymatic hydrolysis of biomass features high reaction selectivity and low loss of sugars, and the hydrolysis process has low requirement in energy input. Despite all these advantages, the production of sugars from biomass via enzymatic hydrolysis still faces many challenges, particularly when the sugars are dedicated for a low-value product such as biofuel. Due to the contemporary market trends and the limitations in technology, the cost of enzymes for biomass conversion is a significant factor in causing the high minimum selling price of biofuels. As part of the natural defense of plants against biological blight, plant cell walls possess chemical and structural properties that protect plants from microbial and enzymatic deconstruction. Such defensive properties (commonly referred to as "recalcitrance" of the cell wall[19]) significantly compromise the efficiency of enzymatic degradation of the cell wall by limiting enzyme access, enzyme inhibition, and enzymes

deactivation. Mechanistic details of the defensive cell wall properties against enzymatic deconstruction will be discussed in more detail in Section 1.3. To facilitate the enzyme reactions and to prevent the waste of enzymes, a physical or chemical treatment of biomass prior to enzymatic hydrolysis (i.e. pretreatment) is often necessary to overcome the call wall recalcitrance to enzymatic conversion. Many pretreatment processes have been proposed and developed, and all pretreatment processes affect plant cell wall components and alter plant cell wall structure in such a way that the yield of products from enzymatic conversion (a.k.a enzymatic digestibility) of the pretreated plant cell wall is higher compared to the plant cell wall before pretreatment.

1.2 Constituents of Plant Cell Wall

About 40-60% of the total dry mass of plant cell wall is cellulose,[20] a 1,4-linked β-glucan polymer commonly found in plant cell wall. Glucose units are linked by glycosidic bonds forming a linear polymer with an aldehyde/hemiacetal chain end a.k.a. the "reducing end." The glucan chains interact via intramolecular hydrogen bonds and Van der Waals forces to form crystalline cellulose microfibrils, which are packed glucan fibrils typically with 2-5 nm in diameter.[21-23] Native crystalline cellulose in plant cell walls is mostly in the cellulose I allomorph in the form of I_α (with a triclinic unit cell) and I_β (with a monoclinic unit cell).[23] Glucan chains in crystalline cellulose I are arranged in parallel with the reducing ends on the same side of the chains, and this crystalline structure is thought to hinder the performance of reducing end-specific cellulases (e.g. *T. reesei* Cel7A) and presumably impedes enzymatic hydrolysis of cellulose.[24] After chemical treatment and irreversible regeneration/mercerization, cellulose I can be transformed to the thermodynamically more stable cellulose II allomorph with antiparallel arrangement of glucan chains. By treating cellulose with liquid ammonia, ethylene diamine and glycerol, other crystalline cellulose allomorphs (III_I, III_{II}, IV_I, IV_{II}) with different unit cell dimensions and structural conformations have also been synthesized.[25] The difference in molecular structure among cellulose allomorphs results in distinct hydrogen bond configurations and different enzymatic digestibilities in aqueous solutions.[26] In addition to crystalline cellulose, non-crystalline cellulose is also present in the plant cell wall. Cellulose microfibrils have periodic weak regions that are much more labile to acidolysis compared to crystalline

cellulose, and such regions are often identified as "amorphous cellulose." The structure of amorphous cellulose in native cellulose is less characterized compared to crystalline cellulose, partly because amorphous cellulose only represents a small proportion of the microfibrils.[27] It has been hypothesized that crystalline cellulose is connected by intermittent disordered regions of amorphous cellulose, resulting in longer cellulose "nunchuks" and "sansetsukons." Amorphous cellulose hydrolyzes more easily than crystalline cellulose, and the hydrolyzability of crystalline cellulose can be improved by amorphogenesis with phorsphoric acid[28] or non-hydrolytic proteins.[29]

As the scaffolding material in plant cell walls,[30,31] cellulose microfibrils have high crystallinity, low water solubility, and strong structural rigidity. These properties of cellulose are important factors in the recalcitrance of plant cell walls to microbial and enzymatic conversion. As a result of intramolecular hydrogen bonds and hydrophobic interactions, cellulose microfibrils coalesce into bundles[32,33] and sheets[22] that impede water penetration. The interaction between cellulose and other cell wall components is also a natural defense of cellulose against degradation. Cellulose is sheathed by amorphous polymeric hemicellulose which forms hydrogen bonds with cellulose and protects cellulose from degradation. Lignin also adsorbs to the hydrophobic surface of cellulose via ring-stacking interactions,[34] thus possibly limiting the accessible area for cellulases with specificity to hydrophobic surfaces.[35]

Hemicellulose is an ambiguous concept used to identify some of the non-cellulosic polysaccharides in biomass. As a complex polymer, hemicellulose consists of various types of structural units including hexoses (glucose, galactose, fucose), pentoses (xylose, arabinose) and organic acids. In most contexts, hemicellulose is the ensemble of the following types of plant cell wall polysaccharides: xylan, xyloglucan, arabinoxylan, mannan, glucomannan, and mixed-linkage glucan (a.k.a β-glucan).[36,37] A common feature of these hemicellulosic polysaccharides is that they all contain a backbone of β-1,4-linked sugar units, which is not common in other non-cellulose polysaccharides such as starch and pectin. The backbone of hemicellulose is usually covalently associated with branching residues (e.g. acetyl groups, ferulate ester) and side chains (e.g. feruloylarabinofuranoside). The chemical properties of these branches play an important role in the extractability of hemicellulose and the crosslinking patterns of hemicellulose with

other cell wall components. Hemicellulose is capable of forming multiple hydrogen bonds with cellulose microfibrils, as well as feruloyl and *p*-coumaryl ester linkages with lignin. These bonds and linkages are critical in maintaining the rigidity of the cell wall and limiting the biodegradability of the cell wall polysaccharides.[38] During enzymatic hydrolysis of biomass, hemicellulose depolymerization releases xylooligomers, which are strong inhibitors to cellulolytic enzymes.[39]

Pectin is another type of heterosaccharide which distributes in plant cell wall of plants. The backbone of pectin is a 1,4-linked polymer of α-D-galacturonic acid (GalA) units, and some of these units are methyl-esterified or acetylated.[40] Other forms of pectin that have been discovered include rhamnogalacturonan (e.g. RG-I, RG-II)[41] and branched xylogalacturonan.[40] Calcium ions coordinate with non-esterified unbranched galacturonan pectin and form junction zones called "calcium bridges."[42] Calcium galacturonate and water molecules crosslink in the polymer network to form a hydrophilic pectin gel in plant cell wall, a structure commonly found in compound middle lamella (the junction area between the walls of adjacent cells).

Lignin is commonly found in the cell wall of terrestrial plants and represents ca. 30% of the organic carbon in the ecosystem.[43] The structure of lignin is a polymeric complex of phenylpropanoid structural units known as "monolignols." The monomeric units of native lignin include *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The lignin structures formed by these units are also referred as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits, respectively. Some other phenylpropanoid units such as ferulates and hydroxycinnamyl aldehydes are also incorporated in the lignin of some plants as chain ends or crosslinks with other cell wall components.[44,45] Unlike cellulose and xylan, which have recurring polymeric structure with single type of glycosidic linkages, lignin is an amorphous polymer with many types of linkages. Research on lignin structure has suggested the involvement of a radical coupling process during lignin biosynthesis and the role of nucleation sites in lignin deposition,[43,45] while protein-regulated polymerization and the existence of primary sequences in lignin polymers have also been hypothesized.[46-50] The lignin in plant cell walls varies among species, and it can be roughly categorized as "softwood lignin", which contains mainly guaiacyl subunits and some *p*-hydroxyphenyl

subunits (particularly in compression wood),[51-53] "hardwood lignin", which has a higher content of syringyl subunits and much less guaiacyl subunits,[54] and "grass lignin", which has characteristic lignin-ferulate crosslinks.[55,56] In addition to the quantitative proportion of lignin subunits, the type of linkages between lignin units also varies with plant species and the spatial location of lignin in the cell wall.[52] The most frequent type of lignin interunit linkage is arylglycerol-β-aryl ether (a.k.a β-O-4 linkage),[53,57] and some other types of linkages such as β-β' resinol and phenylcoumaran structures are also found in lignin.[58]

Lignin is generally hydrophobic in nature and is responsible for the low water permeability of the cell wall. The hydrophobicity of lignin is critical to the function of xylem tissues as water transport vessels.[59] Suppression of lignin biosynthesis has been reported to negatively affect the growth of plants.[60] Research has suggested that lignin possibly plays an important role in the defense of plants to many types of abiotic and biotic stresses including excessive light,[61] ultraviolet radiation,[62] mechanical injury,[63-65] and pathogen attack.[66-69] Lignin fortifies the mechanical strength of the cell wall, absorbs UV radiation, and scavenges reactive oxidative species in coordination with phytoalexin and flavanoid antioxidants. As part of *in planta* natural defense to heterotrophic pathogens, lignin hinders the degradation of cell wall polysaccharides by cellulytic enzymes.[70,71] Enzyme inhibition is possibly the result of non-productive enzyme adsorption to lignin,[72-74] as well as lignin-induced protein unfolding and deactivation.[75] Cellulose is also less accessible to enzymes due to the adsorption of lignin on cellulose surface.

1.3 Oxidative Strategies for Overcoming Cell Wall Recalcitrance

Chemical oxidation of biomass has been shown to be an effective pretreatment which facilitates subsequent bioconversion processes.[76,77] Oxidative pretreatment produces lower amount of fermentation inhibitors (e.g. aromatic aldehydes and furan aldehydes) than some themochemical pretreatment methods,[78-80] and the fractionation of cell wall components during oxidation enhances the processability of the complex biomass feedstock.[76,81] Common oxidants include air,[82] pressurized oxygen,[79,83-85] hydrogen peroxide,[77,86-91] and ozone.[92-94] Oxidation reactions modify lignin and solubilize cell wall components (mostly hemicellulose and lignin), yielding a solid residue with high cellulose content and high enzymatic digestibility. Oxidation with hydrogen peroxide or oxygen under alkaline pH also results

in cleavage of ester linkages and facilitates fractionation of the cell wall in grasses.[95] Some major challenges to the implementation of oxidative pretreatment include the requirement of pressurized reactors (>10 bar) and the high consumption of costly chemicals (e.g. hydrogen peroxide). When alkaline hydrogen peroxide oxidation is used as a pretreatment under ambient pressure and temperature, a relatively high loading of hydrogen peroxide (10-50% w/w of biomass) is required for a significant improvement in the enzymatic digestibility of the pretreated biomass.[77,88] Considering the cost of hydrogen peroxide as a chemical, production of cheap sugars via such a pretreatment process is challenging. In addition, although the oxidation of lignin might compromise the value of lignin as a combustion fuel,[96] such effect can be partially countered by the value of vanillin and syringaldehyde as lignin oxidation by-products.

Metal-catalyzed oxidation has long been used as a strategy for biomass fractionation in the pulp and paper industry.[97,98] As processes for pulp delignification, metal-catalyzed and metal-activated lignin oxidation using molecular oxygen[99,100] and hydrogen peroxide[101,102] have been well studied. Catalytic oxidation is effective in solubilizing and removing lignin in woody biomass, but the application of catalytic oxidation as a pretreatment for woody biomass has received little attention. This is possibly due to the cost involved in the process, and the loss of carbohydrates to carboxylic acids during catalytic oxidation.[99] Recently, Hakola et al. revisited copper-phenanthroline catalyzed oxidation using pressurized oxygen as a pretreatment strategy, and the pretreated biomass exhibited high enzymatic digestibility.[103] The catalytic oxidation of biomass was performed under elevated pressure (10 bar) and temperature (120 °C) with a relatively high concentration of base (26 g/L sodium carbonate), and these conditions induce high cost in energy, process equipment, and chemicals. In another recently published work, Lucas et al. demonstrated the improvement in the enzymatic digestibility of poplar after soaking the biomass in 8.1% w/w H_2O_2 solution together with a manganese catalyst.[104] After prolonged pretreatment, the improvement in enzymatic glucose yield from poplar was limited to 20-40% relative to the yield from water-rinsed non-oxidized poplar.

The warfare between heterotrophic organisms and cell wall recalcitrance has lasted for eras, with both sides developing sophisticated strategies against each other.[18,19,105,106] Compared with other types of plants, woody plants are particularly resistant to microbial degradation due to its structural rigidity and high content of lignin. For those organisms that feed on woody biomass for carbon and energy, the deconstruction and assimilation of biomass is facilitated by enzymes that effectively overcome lignin recalcitrance. Only a few types of microorganisms have been identified which excrete lignin-degrading enzymes, and most of these organisms belong to the wood-decaying Basidomycota taxa of fungi. As an example, the white-rot fungus *Phanerochaete chrysosporium* produces lignin peroxidase (LiP) and manganese peroxidase (MnP), both of which are metalloenzymes with lignolytic activity in presence of extracellular H_2O_2.[107-109] LiP catalyzes electron transfer from a reducing substrate (e.g. lignin, veratryl alcohol) to H_2O_2,[110] and the oxidation results in the formation of lignin radical cations as well as the bond cleavage in lignin (e.g. C_α-C_β cleavage).[109,111,112] MnP catalyzes the oxidation of Mn(II) ions to Mn(III) ions by H_2O_2, and Mn(III) ions in turn oxidizes lignin substrates.[113] In addition to lignin oxidation enzymes such as LiP and MnP, *Phanerochaete chrysosporium* also secretes H_2O_2-generating enzymes such as glyoxal oxidase and glucose oxidase,[114,115] as well as a wide range of polysaccaride lytic enzymes (e.g. cellulase,[116] xylanase[117] and mannanase[118]). The synergistic effects of these enzymes enable *Phanerochaete chrysosporium* to degrade both lignin and cell wall polysaccharides effectively during saprotrophic growth.

Laccase is a type of oxidoreductase secreted by many species of white-rot fungi (which probably[119,120] does not[121,122] include *Phanerochaete chrysosporium*) during oxidative lignin degradation. Although lignin can be effectively degraded by enzymes other than laccase (as seen in the case of *Phanerochaete chrysosporium*, *Trametes versicolor*,[123] and *Fomes annosus*[124]), laccase plays an important role in the lignin degradation process of some microorganisms. Loss of lignolytic activity has been observed in laccase-negative mutants of lignin-degrading fungi such as *Sporotrichum pulverulentum* and *Pycnoporus cinnabarinus* (which does not secrete LiP or MnP).[125-127] As a multicopper oxidase with four copper ions, laccase is a metallozyme which catalyzes the oxidation of phenolic substrates by molecular oxygen.[128]

During catalytic oxidation, electrons are transferred from the reducing substrate to an electron-accepting copper center in laccase, and are then passed on to the trinuclear copper center, which binds, activates and reduces dioxygen.[128] In the catalysis cycle, laccase also catalyzes the oxidation of redox mediators (e.g. 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 1-hydroxybenzotriazole (HBT)), non-phenolic lignin model compounds (mediated by ABTS),[129] substituted phenol, and Mn^{2+}.[130] During in vitro lignin oxidation, direct electron transfer from lignin to laccase might be limited by the size of the enzyme (which hampers catalysis at inaccessible sites) and the low redox potential of laccase (E_0 < 800mV). It has been proposed that low molecular weight compounds (e.g. lignin degradation products such as syringaldehyde and acetosyringone) possibly serve as redox mediators that shuttle electrons from lignin to laccase during oxidative catalysis.[130]

In contrast to white-rot fungi, brown-rot fungi is a family of wood-decaying Basidomycota that employs a different set of strategies to degrade biomass. Unlike white-rot fungi which degrade lignin and bleach the decayed wood (leaving the cellulose-rich white rot on wood), brown-rot fungi do not incur extensive lignin removal but are still able to degrade cellulose efficiently.[131] More interestingly, some important lignolytic and cellulolytic enzyme activities that have been found in white-rot fungi are missing from the secretome of brown-rot fungi.[132] Brown-rot fungi generally lack cellobiohydrolase[133] (with few exceptions[134]) and laccase,[130] which implies that brown-rot fungi employ strategies different from those of white-rot fungi. Evidence suggests that the ability of brown-rot fungi to circumvent recalcitrant lignin is associated with biologically produced reactive oxidation species which attack cell wall polysaccharides and lignin.[135] Although a thorough understanding of extracellular reactive oxidative species of brown rot has not yet been fully established, the occurrence of hydroxyl radicals (•OH) has been repeatedly reported, and the involvement of Fenton chemistry has been suggested (Fenton chemistry will be discussed in more detail in Section 2.3).[136-138] Radical-induced lignin oxidation is possibly related to the cleavage of the dominant arylglycerol-β-aryl linkages in lignin,[139] and the modification of lignin possibly increases the accessibility of cell wall polysaccharides to hydrolytic enzymes. Recent characterization of enzymes from glycosyl hydrolase family 61 (GH61) and carbohydrate-binding modules family 33 (CBM33) suggested

the involvement of lytic polysaccharide monooxygenases (LPMO) in the biological degradation of cellulose.[140,141] Analysis of the GH61 enzyme isolated from cellulolytic fungi revealed the enzyme's identity as a copper-dependent LPMO and its synergistic effects with other cellulases during cellulose hydrolysis.[140,142]

CHAPTER 2

DISCOVERY OF A NOVEL CATALYTIC OXIDATIVE PRETREATMENT

2.1 Alkaline Hydrogen Peroxide Pretreatment: History and Recent Developments

Alkaline hydrogen peroxide (AHP) delignification of pulp and pretreatment of biomass has been studied since the 1980s. As an environmentally benign oxidant, H_2O_2 produces water as the only output, and the oxidation reaction can be performed under ambient conditions. Under alkaline pH, H_2O_2 can deprotonate and produce oxidative radicals (e.g. •OH, •OOH) which attack lignin and carbohydrates, resulting in delignification and cellulose oxidation.[143,144] Gould et al. and Williams et al. reported the increase in the swelling volume and the improvement of water penetration in AHP treated grasses,[145,146] possibly due to the removal of lignin from the cell wall. These chemical and structural changes may be responsible for the increased sugar conversion of AHP-pretreated biomass including corn stover,[77] switchgrass,[147] bamboo[148] and sugarcane bagasse.[149]

The cost of H_2O_2 is largely defined by the predominant[150] process for commercial production of H_2O_2, i.e. hydrogenation of oxygen mediated by anthraquinone. The use of metal catalysts (e.g. Ni, Pd) and the high consumption of energy in this process result in the high selling price of H_2O_2. As an alternative, alkaline H_2O_2 can be produced from a cost-efficient electrochemical process,[151] but the high alkalinity of the product (1.7-1.8 NaOH:H_2O_2 weight ratio) limits its applicability in AHP pretreatment (0.4-1.3 NaOH:H_2O_2 weight ratio). Given these considerations, the need for an advanced H_2O_2-efficient pretreatment process is compelling.

Although AHP pretreatment is effective on many types of grasses, the efficacy of AHP pretreatment on some recalcitrant feedstock is very limited even when high loading of H_2O_2 is used (250-500 mg H_2O_2/g biomass).[152] In order to prepare these types of feedstock for efficient enzymatic hydrolysis, strategies have been developed where AHP pretreatment is used as a post-treatment, i.e. a unit operation subsequent to another pretreatment. After the preceding pretreatment which partially removes cell wall recalcitrance, AHP treatment can target the more recalcitrant lignin in the biomass. The pretreatment prior to AHP disrupts the cell wall structure and facilitates better mass transfer during H_2O_2 oxidation. By

using the AHP process as a post-pretreatment following steam-explosion pretreatment of hardwood,[153] softwood,[154] and *Laspedeza* stalk,[155] the enzymatic digestibility of biomass can be significantly improved with modest consumption of H_2O_2 in the processes (e.g. < 5% H_2O_2 w/w of biomass).

One important cause of the waste in H_2O_2 is the high reactivity and low selectivity of the oxidant, and the oxidation reactions do not always contribute to the alleviation of cell wall recalcitrance. Biomass delignification can be achieved solely by alkaline treatment, and any further oxidation on the alkali-soluble lignin might be unnecessary. During AHP pretreatment, H_2O_2 is capable of reacting with both insoluble biomass material and the dissolved biomass material. Were a liquid-solid separation process in place after pretreatment to recover the pretreated solids, the oxidation of the dissolved biomass would have very limited effect on the enzymatic digestibility of the pretreated solid biomass. To improve the atomic efficiency of H_2O_2, peroxide oxidation can be applied to alkali pre-washed biomass instead of raw, untreated biomass. By removing alkali-soluble lignin in a mild alkaline extraction step, less H_2O_2 is needed to oxidize only the residual alkali-insoluble lignin.[156] Adoption of such a two-step strategy can significantly reduce the consumption of H_2O_2 and improve the economic feasibility of the pretreatment (Andrea Orjuela, personal correspondence).

2.2 AHP Pretreatment of Alkali Pre-Extracted Switchgrass

As previously stated, H_2O_2 can react with the more residual recalcitrant lignin which cannot be removed during a mild alkaline extraction. Thus, the oxidation efficiency of this "alkali-insoluble" lignin is the key to improving the economic feasibility of a two-step AHP pretreatment. We selected alkali-extracted switchgrass (AESG) as the model biomass substrate for the preliminary studies to further improve AHP pretreatment. To prepare AESG, untreated switchgrass (*Panicum virgatum*, cv. Cave-In-Rock) was soaked in an aqueous solution of 5 g/L NaOH at 80 °C for 2 hours. The weight-to-volume (w/v) solids loading of switchgrass during alkaline extraction was 10% (e.g. 10 g of biomass in 100 mL NaOH aqueous solution). After the alkaline extraction, the residual solid biomass was recovered via filtration and washed with deionized water until the washing effluent had a neutral pH. The washed solids was

recovered as AESG and air-dried. The composition of AESG was determined with an assay based on biomass acidolysis.[157]

To study the effect of AHP pretreatment on AESG, AHP pretreatment was performed in a 100 g/L aqueous suspension of AESG. The reaction mixture contained 0.04 M Na_2HPO_4-NaOH buffer to maintain the pH at 11.5 during pretreatment. After 24 h of incubation at 30°C without mixing, the pretreated biomass solid was washed using deionized water to remove the alkaline phosphate buffer.

The enzymatic digestibility of AHP-pretreated AESG (AEAHPSG) was estimated by the yield of glucose during the enzymatic hydrolysis of AEAHPSG. The percentage yield was calculated on the basis of the glucan content in AESG prior to AHP pretreatment. As a result, the theoretical maximum hydrolysis yield is strictly lower than 100% due to the loss of sugars in the washing step between AHP pretreatment and enzymatic hydrolysis. For enzymatic hydrolysis, 500 μL of 1 M Na-citrate buffer (pH 5.0) and 40 μL of 10 mM tetracycline (Sigma-Aldrich) were added to AEAHPSG that had never been dried after AHP pretreatment. Accellerase 1500 (42 FPU/mL; Danisco-Genencor, Palo Alto, CA) was added at 50 mg protein per gram of glucan in AESG. The total volume of the mixture (solid and liquid) was adjusted with deionized water to reach a 5% (w/v) solids concentration, and the samples were incubated at 50 °C during enzymatic hydrolysis.

As shown in Figure 1, the enzymatic digestibility of AEAHPSG is positively correlated with the amount of H_2O_2 used during the AHP pretreatment of AESG. By increasing the H_2O_2 loading from 0.05 to 0.10 g per g of AESG, the digestibility of AEAHPSG can be improved by 4-6%. This implies that the pretreatment effect is not yet saturated when the H_2O_2 loading is 0.5 g per g of AESG. In other words, by implementing new oxidation strategies that use H_2O_2 more efficiently, higher digestibility could possibly be achieved at the H_2O_2 loading of 0.5 g/g AESG.

2.3 Catalytic AHP Pretreatment

(This section has been published in *Biotechnology and Bioengineering*, 2013, **110**, 1078)

As a method for biomass pretreatment, very little research on catalytic AHP pretreatment has been reported in literature. Nevertheless, catalytic peroxide oxidation has long been studied and used in various

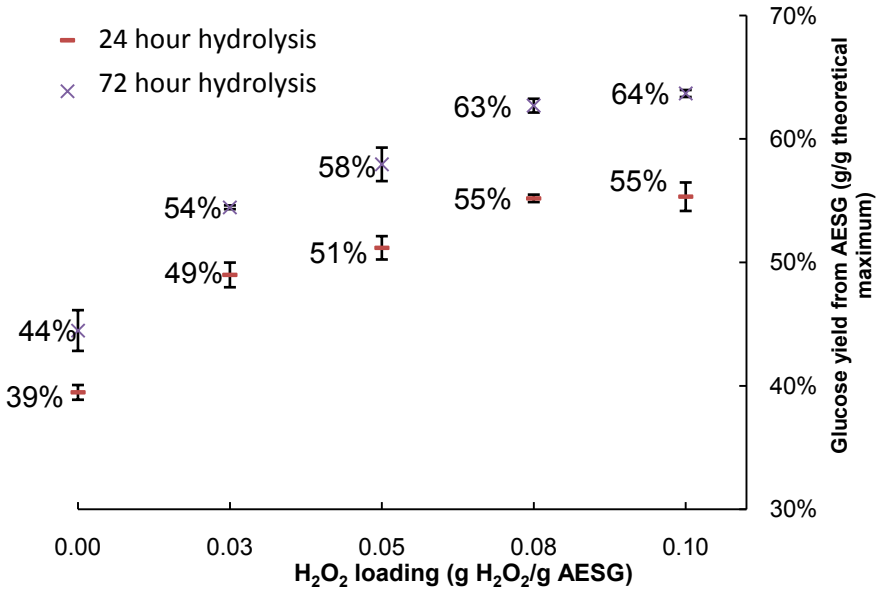

Figure 1. Effect of H_2O_2 loading on the enzymatic digestibility of AEAHPSG.

applications. In the late 1900s, Henry J. H. Fenton discovered the strong oxidation capability hydrogen peroxide in the presence of an iron catalyst, and this catalytic oxidant system was named after him to honor his discovery.[158] Decades later, Haber and Weiss discovered that the iron salt in the Fenton system catalyzes the decomposition of hydrogen peroxide and the formation of radicals:[159]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

$$Fe^{3+} + OOH^- \rightarrow Fe^{2+} + \bullet OOH$$

The hydroxyl radical (•OH) is a potent oxidant (E^0(•OH/H_2O) = 2.8 V) and is responsible for the strong oxidizing property of the Fenton system. Bentivenga et al. reported extensive degradation of lignin via oxidation by Fenton's reagent (ferrous ion and H_2O_2).[160] A recent publication by Kato et al. demonstrated that Fenton's reagent removes lignin from biomass and improves the microbial digestibility of cellulose.[161] In addition to ferrous ions, many other types of redox-active catalysts have been discovered to be capable of catalyzing the H_2O_2-meditated oxidation of lignin or lignin models. As biomimetic catalysts, Fe-porphyrin complexes catalyze the oxidation of veratryl alcohol (a lignin model compound which is also a metabolite of lignolytic fungi) and ferulic acid in the presence of H_2O_2 at high conversions.[162] Similar to metalloporphyrin, metal-phthalocyanine complexes have received attention as

suitable homogeneous catalyst systems, since they are more robust than porphyrin complexes during oxidaton with respect to temperature and pH range. Catalytic phthalocyanine complexes have been studied over a range of pH values, metals (Cu, Mn, Fe, Co), and oxidizing sources including H_2O_2[163] and dioxygen.[164] Fe-phthalocyanine complexes catalyze the H_2O_2 oxidation of lignin model compounds under acidic pH.[163] Many types of iron complexes with non-porphrin nitrogen-donating ligands (e.g. macrocyclic tetraamides) are documented in the patent literature as H_2O_2 bleaching catalysts/activators.[165] Chen et al. developed several types of catalytic Mn complexes with synthetic macrocyclic ligands that are highly effective in H_2O_2 bleaching of wood pulp (Figure 2).[166] Homogeneous Lewis acids have long been used as catalysts for H_2O_2 activation and epoxidation reactions.[167] Methyltrioxorhenium (MTO) and polyoxometalates (POM) are reported as activators for H_2O_2 oxidation of lignin.[100,168] Busch et al. described several Mn complexes with cross-bridged macrocyclic complexes that are effective catalysts for H_2O_2 epoxidation reactions using H_2O_2 as the oxidant.[169]

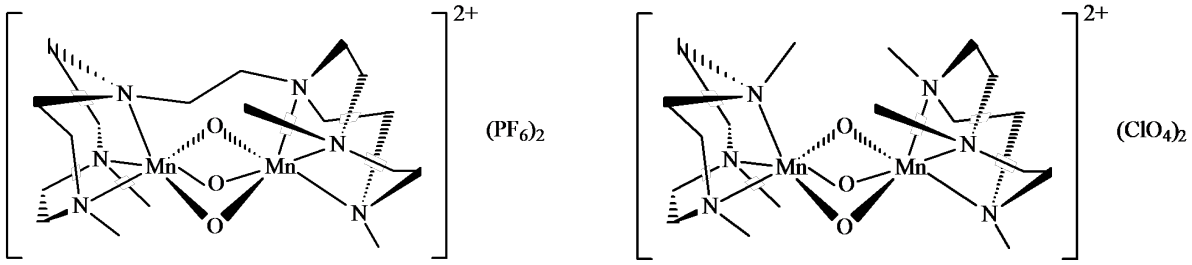

Figure 2. Structures of $[(Me_4DTNE)Mn(IV)_2(\mu\text{-}O)_3](PF_6)_2$ and $[(Me_3TACN)Mn(IV)_2(\mu\text{-}O)_3](ClO_4)_2$.

Due to the complex ultrastructure of the plant cell wall, the ability of water-soluble homogeneous catalysts to diffuse into the porous cell wall is highly desirable as far as spatial specificity of the catalyst is concerned. We screened a number of metal catalysts for AHP pretreatment of AESG in search of a catalyst that promotes the efficiency of hydrogen peroxide activation. The effect of the catalyst was evaluated by the enzymatic digestibility of the AEAHPSG after catalytic pretreatment. The loading of H_2O_2 during catalytic pretreatment was 0.05 g per g of AESG. Catalytic pretreatment was performed under various pH (in 0.04 M Na_2HPO_4-NaOH buffer solutions) for 24 hours at 30 °C prior to enzymatic hydrolysis using Accelerase 1500 cellulase cocktail at 50 °C. For conditions with xylanase

supplementation, 15 mg of Multifect Xylanase (8200 GXU/mL according to the manufacturer; Danisco-Genencor) was used at a 1:1 ratio with cellulase on a protein basis. The enzymatic hydrolysis lasted 24 or 72 hours, and the loading of cellulase during enzymatic hydrolysis was 15 mg protein per glucan in AESG before pretreatment. Yields of monosaccharides in the hydrolysate were quantified by HPLC following the method described by Banerjee et al.[77]

Catalyst	Catalyst concentration (mM)	pH	Glucose yield of enzymatic hydrolysis (% of theoretical maximum)	
			24 h	72 h
No catalyst	-	3.0	30.06±1.96	36.12±2.33
		9.0	32.37±1.20	37.97±0.57
		10.5	31.25±0.16	38.77±0.28
		11.5	37.74±0.33	42.70±0.59
		13.0	36.82±1.23	not determined
Fe(III)-phthalocyanine	0.05	3.0	30.91±0.24	36.80±2.84
		11.5	34.21±0.23	38.75±1.52
Fe(III)-tetraphenylporphyrin	0.05	3.0	31.55±1.60	42.13±1.89
		11.5	36.98±1.30	42.14±0.63
Fe(III)-tetrakis-pentafluorophenyl)-porphyrin	0.05	3.0	29.18±0.33	35.38±0.43
		11.5	35.37±0.45	40.25±0.19
$[NEt_4][Fe(III)(bpb)Cl_2]$	2	9.0	27.07±0.60	34.27±0.21
		10.5	25.54±0.21	31.97±0.13
		11.5	27.44±1.54	31.54±0.23
[Al(III)(3,5-^{t}Bu$_2$-salophen)Cl]	2	10.5	28.90±0.17	not determined
		11.5	29.80±0.25	not determined
		13.0	34.19±0.41	not determined
$[Zn^{II}$(BPPA)Cl]Cl	2	10.5	26.43±0.31	not determined
		11.5	27.33±0.62	not determined
		13.0	33.36±0.30	not determined
Cu (bpy), L/M=3:1	5	10.5	38.67±0.30	42.98±0.88
		11.5	41.65±0.14	44.97±0.42
		13.0	41.56±0.06	43.95±0.17

Table 1. Effect of metal catalyst addition to the 24hr AHP pretreatment of AESG.

Among the catalyst candidates chosen for screening, Fe(III)-phthalocyanine, Fe(III)-tetraphenylporphyrin and Fe(III)-tetrakis-pentafluorophenyl)-porphyrin are purchased from Sigma-Aldrich and used without further purification. The [Al(III)(3,5-^{t}Bu$_2$-salophen)Cl] (3,5-^{t}Bu$_2$-salophen = *N,N'*-bis(3,5-di-*tert*-butylsalicylidene)-1,2-phenylenediamine), [Zn(II)(BPPA)Cl]Cl (BPPA = *N,N*-bis(6-pivaloylamido-2-pyridylmethyl)-*N*-(2-pyridylmethyl)amine), and $[NEt_4][Fe(III)(bpb)Cl_2]$ (H_2(bpb) = *N,N*-

bis(2-pyridinecarboxamide)-1,2-benzene) complexes were synthesized according to the literature procedures and verified by NMR and/or UV-Vis spectral analysis.[170-174] Stock solutions of the [Al(III)(3,5-^{t}Bu$_2$-salophen)Cl, [Zn(II)(BPPA)Cl]Cl, and [NEt_4][Fe(III)(bpb)Cl_2] catalysts were prepared in methanol, 1:1 water:methanol, and DMSO respectively. Cu(bpy) catalyst was prepared in an aqueous solution by mixing cupric sulfate pentahydrate and 2,2'-bipyridine at a ligand:metal molar ratio (L/M) of 3:1.

As shown in Table 1, AHP pretreatment of AESG at pH 11.5 was not improved via addition of metal catalysts except for the case of the Cu(bpy) catalyst, where the pretreatment gave a very moderate improvement in the enzymatic digestibility of AEAHPSG. Based on this observation, AHP pretreatment using Cu(bpy) was identified as a promising approach warranting further study. We named this new pretreatment method which involves alkaline peroxide and Cu(bpy) catalyst as the **Cu(bpy)-AHP pretreatment**.

Subsequently, studies were performed on biomass from three taxonomically diverse plants including AESG, silver birch (*Betula pendula*), and a hybrid poplar (*Populus nigra* var. *charkoviensis* x *caudina* cv. NE-19) to compare the efficacy of Cu(bpy)-AHP pretreatment on different types of biomass. Both silver birch and hybrid poplar are hardwood biomass and are very recalcitrant to enzymatic hydrolysis prior to pretreatment. For hardwood AHP and Cu(bpy)-AHP pretreatment, 500 mg dry weight of biomass was pretreated in 5 mL aqueous solution containing 10.8 g/L NaOH and 10 g/L H_2O_2 (equivalent to 10% w/w loading on biomass, or 0.1 g H_2O_2 per g of untreated biomass). The concentration of the Cu(bpy) catalyst for hardwood pretreatment was the same as in the Cu(bpy)-AHP pretreatment of AESG. After 48 h of pretreatment, 20 μL of 72% w/w H_2SO_4 aqueous solution was added to the sample mixture to decrease the pH to 5.0 prior to enzymatic hydrolysis. For pretreated hardwood, an enzyme mixture of Novozyme Cellic CTec2 (227 FPU/mL) and HTec2 (1090 FXU/mL according to the manufacturer) of the same protein content was used for hydrolysis. The total protein content was 70 and 60 mg for silver birch and hybrid poplar, respectively. The total volume was adjusted to 10 mL with deionized water, and the

samples were incubated at 50° C during enzymatic hydrolysis. Enzymatic digestibility of the pretreated hardwood was quantified following the same procedure used for AEAHPSG.

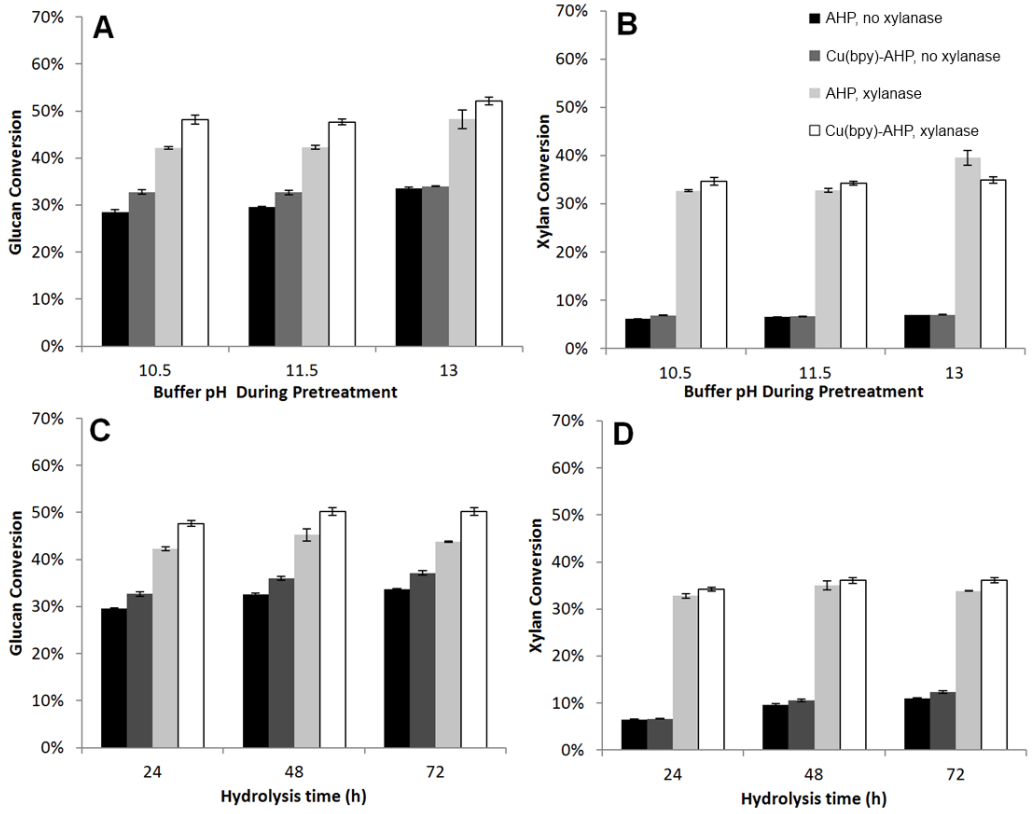

Figure 3. Pretreatment of AESG with 0.05 g H_2O_2/g AESG loading.
Effect of pretreatment buffered at various alkaline pH was assessed by (**A**) enzymatic glucose yield and (**B**) enzymatic xylose yield after 24 hours of hydrolysis. Effects of hydrolysis time on (**C**) enzymatic glucose yield and (**D**) enzymatic xylose yield were assessed after pretreatments performed at a pH of 11.5.

Figure 3 demonstrates improvements in 24 hour enzymatic digestibilities for AESG using this pretreatment approach. The polysaccharides conversions are calculated based on the amount of carbohydrates available in the AESG prior to pretreatment. Increased hydrolysis times (48 or 72 hours) only marginally improved sugar yields (Figure 3C and 3D) from AEAHPSG after pretreatment at pH 11.5. Xylanase supplementation improves both glucan and xylan conversion for all pretreatments, and this phenomenon is well-established in the literature.[175] Interestingly, the results reveal that xylanase supplementation results in greater improvements in enzymatic glucose yields for Cu(bpy)-AHP pretreatment relative to uncatalyzed AHP, implying that more polysaccharides (i.e. both cellulose and

xylan) became enzymatically accessible after Cu(bpy)-AHP pretreatment. There are minimal differences in the glucose and xylose release for pretreatment buffered at a pH of either 10.5 or 11.5. Increasing the buffer pH to 13.0, however, results in noticeable increases in glucose conversions by catalyzed pretreatment relative to uncatalzyed AHP (Figure 3A).

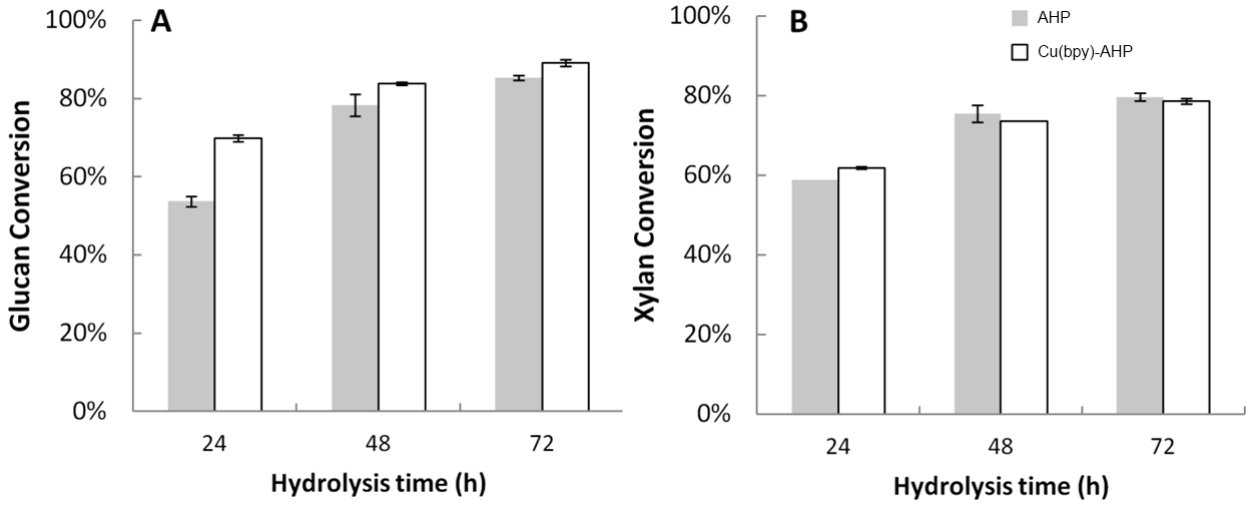

Figure 4. Effect of hydrolysis time and xylanase supplementation on silver birch pretreated with Cu(bpy)-AHP pretreatment at 10% w/w H_2O_2 loading and an initial pH of 11.5.
Data show (**A**) enzymatic glucose yield and (**B**) enzymatic xylose yield.

Woody plants typically have thicker cell walls, a denser vascular structure, a higher lignin content, and less alkali-soluble lignin than monocot grasses such as switchgrass and corn stover. As a consequence of their greater recalcitrance, woody plants typically require harsher chemical pretreatments to achieve enzymatic conversions comparable to herbaceous plants. With the use of Cu(bpy)-AHP pretreatment, significant improvement in the enzymatic digestibility of woody biomass can be achieved with a mild pretreatment. As shown in Figure 4, digestibility gains for silver birch are most apparent for the initial stage of hydrolysis with differences decreasing later as hydrolysis approaches 90% glucose conversion, suggesting that Cu-catalyzed oxidation reactions affect the kinetics of cellulolytic enzymes on pretreated biomass and shorten the time prior to saturation while still retaining the final hydrolysis yield.

The largest differences between catalyzed and uncatalyzed pretreatments were observed for the hybrid poplar (Figure 5). AHP pretreatment alone leads to only modest improvements in enzymatic

glucose yield from 21% for hydrolysis of untreated poplar to 27% for uncatalyzed AHP (72 h hydrolysis, no xylanase). This is further increased, however, to 50% with addition of Cu catalyst (72 h hydrolysis, no xylanase). In this instance, these substantial gains are not only gains in the initial rate, but they are also represented by final yields after the reaction has proceeded to its maximum achievable extent. Like the results in Figure 3, supplementation of xylanase results in more pronounced improvements in enzymatic glucose yield for the catalyzed pretreatment approach relative to uncatalyzed AHP (61% versus 30% glucan conversion for 72 hours of hydrolysis).

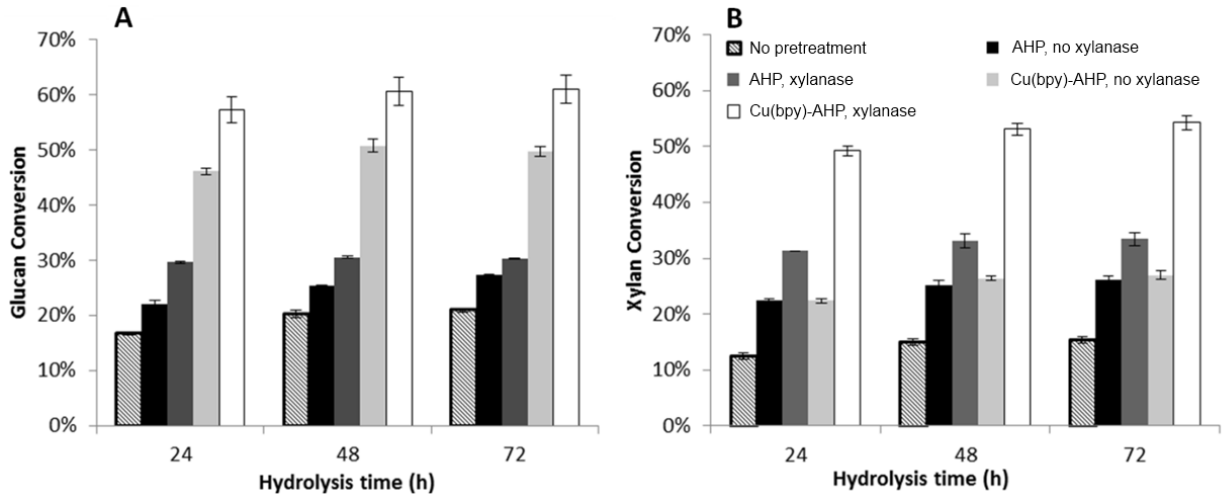

Figure 5. Effect of hydrolysis time on sugar yields for hybrid poplar pretreated with Cu(bpy)-AHP pretreatment at 10% w/w H_2O_2 loading and an initial pH of 11.5.
Data show (**A**) enzymatic glucose yield and (**B**) enzymatic xylose yield.

Although the mechanism of Cu(bpy)-AHP pretreatment and the details of the oxidation chemistry are not completely known, it is possible that Cu catalysis increases the reactivity of H_2O_2 towards plant cell wall components and thereby favors the targeted oxidation of cell wall versus non-productive H_2O_2 decomposition. Given this hypothesis, high pretreatment efficacy should be achievable using less H_2O_2 with the help of a Cu catalyst. Such potential will be exploited in Chapter 4. In terms of mechanism, Cu(bpy)-AHP pretreatment could conceivably act on the plant cell wall at a variety of different levels to improve glucan and xylan digestibility. These possibilities include: (1) modifications to lignin that improve its hydrophilicy, water solubility, depolymerization, and/or removal from the cell wall, (2) modifications to lignin that decrease cellulolytic enzyme adsorption to the lignin, (3) reactions that break

ester and ether cross-links between lignin and xylan, (4) reactions that improve xylan removal, possibly through any of the previously stated mechanisms, (5) chaotropic effects on cellulose microfibril crystalline regions,[176] and (6) oxidative modifications/decrystallization of cellulose in the manner of GH61[142] that would increase enzyme accessibility to sites for glycosidic bond cleavage. To investigate these hypothetical biomass pretreatment mechanisms, the impact of Cu(bpy)-AHP pretreatment on plant cell wall structure will be studied in Chapter 5.

CHAPTER 3

KEY VARIABLES AFFECTING THE CATALYTIC OXIDATIVE PRETREATMENT

(Sections 3.1, 3.2, and 3.3 have been published in *Biotechnology for Biofuels*, 2013, **6**, 119)

3.1 Hybrid Poplar as a Feedstock for Renewable Sugars

Production of renewable sugars from woody biomass is an attractive alternative to the utilization of corn grain as a sugar source. In particular, short-rotation woody crops such as willow (*Salix* spp.) and hybrid poplar (*Populus* spp.) that are currently grown in temperate regions for combined heat and power bioenergy applications represent as important feedstocks for liquid transportation fuels with agronomic and logistical advantages. Specifically, it has been shown that hybrid poplar can be grown on marginal agricultural lands with low energy and chemical input and produce biomass with high energy density at moderately high productivities,[177,178] thereby providing significant motivation for developing effective and economical conversion technologies that can be coupled with woody feedstocks.

Woody biomass such as hybrid poplar presents special challenges for the development of pretreatment technologies because of its thick cell walls, dense vascular structure, and high lignin content. As a result, the improvement in enzymatic digestibility of hybrid poplar after pretreatment is limited,[86,89] and this lack of efficacy on woody biomass is a ubiquitous challenge faced by many pretreatment methods.[179-181] Although a few methods including organosolv, dilute acid, and SPORL (a sulfite pretreatment combined with mechanical size reduction) have been reported to be effective pretreatments for hybrid poplar,[182,183] all of these methods suffer from drawbacks such as a high consumption of chemicals and the generation of fermentation inhibitors.[184] As a result, there is great interest in identifying effective pretreatment methods for hybrid poplar.

3.2 Consumption of Chemicals and Catalyst during the Cu(bpy)-AHP Pretreatment Process

As discussed in Chapter 2, Cu(bpy)-AHP pretreatment is an effective method to improve the enzymatic digestibility of hybrid poplar. The enzymatic digestibility of Cu(bpy)-AHP pretreated has 100% higher enzymatic digestibility compared to the hybrid poplar after uncatalyzed AHP pretreatment.[185] As Cu(bpy)-AHP pretreatment followed by enzymatic hydrolysis is being considered as a process for

producing sugars and inexpensive commodities such as biofuels, the economical feasibility of the process strongly depends on some of the key parameters in the pretreatment and hydrolysis process. Chemicals such as base (e.g. NaOH), H_2O_2, and sulfuric acid are consumed during the pretreatment process. Moreover, the enzymes for cellulose and hemicellulose hydrolysis also impose a significant portion of the cost in sugar and biofuel production. The consumption of water during pretreatment and hydrolysis affects the amount of energy required to concentrate biomass hydrolysate and to separate biofuel from fermentation broth via distillation, and a pretreatment process at higher solids loading followed by high-solids hydrolysis will be more cost-effective. In addition, as the reaction time during the pretreatment and hydrolysis positively affect the size of reactors needed at a given processing throughput, accelerated processes with a short retention time will result in lower capital investment in process equipment and enable higher throughput of production.

To exploit the potential of Cu(bpy)-AHP pretreatment as a cost-effective unit operation for preparing biomass for sugar production, we studied the effect of key operation parameters during pretreatment and enzymatic hydrolysis. The heartwood of hybrid poplar with high glucan content was chosen as the model biomass for this study. As preliminary proof-of-concept research, operation parameters are examined one at a time to acquire basic knowledge of their impacts.

As discussed in Section 2.1, the high price of H_2O_2 is a major obstacle in the application of AHP pretreatment as a commercially relevant process. Apart from one study employing AHP of wheat straw at low peroxide loadings (less than 26 mg/g biomass) to improve ruminant digestibility,[186] much of the prior work on AHP as a pretreatment for biofuels applications employed economically prohibitive high loadings of H_2O_2 on biomass to facilitate effective delignification and high enzymatic digestibilities. During Cu(bpy)-AHP pretreatment, however, oxidation chemistry of H_2O_2 is affected by the presence of the copper catalyst, and the oxidation stoichiometry as well as kinetics is potentially different from that in uncatalyzed AHP pretreatment.

To study the effect of H_2O_2 concentrations on Cu(bpy)-AHP pretreatment, hybrid poplar was pretreated in 10.8 g/L aqueous solution NaOH containing 2 mM of $Cu(SO)_4$, 10 mM 2,2'-bipyridine, and

various concentrations of H_2O_2. The concentration of solid biomass during pretreatment was 10% (w/v). After 24 hours of pretreatment, the pH of the reaction was adjusted to 5.0 via addition of 20 µL 72% w/w sulfuric acid and 500 µL of 1.0 M Na-citrate buffer. Next, 40 µL of 10 mM tetracycline (Sigma-Aldrich) stock solution was added to inhibit microbial growth, followed by addition of the enzyme cocktail consisting of Cellic CTec2 and Cellic HTec2 (Novozymes A/S, Bagsværd, DK) at a loading of 30 mg protein/g glucan each on the basis of hybrid poplar prior to pretreatment. The solids concentration during enzymatic hydrolysis was 5%. Biomass hydrolysate was sampled after 24 h and 72 h hydrolysis to calculate the yield of monomeric sugars.

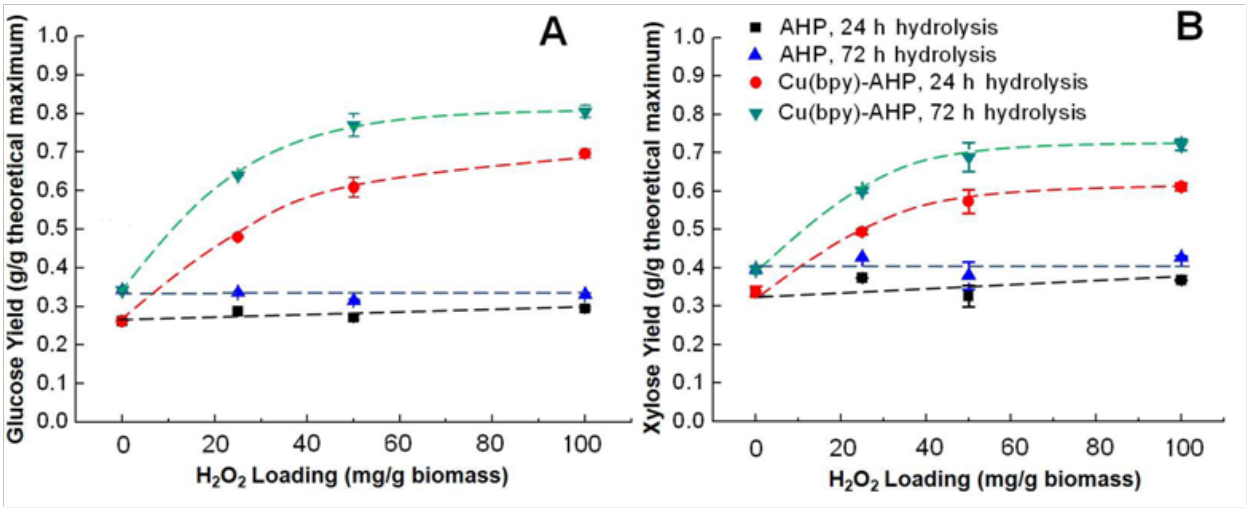

Figure 6. Effect of H_2O_2 loading during pretreatment on enzymatic hydrolysis yield.
Data show **(A)** glucose and **(B)** xylose yields after 24 h and 72 h of hydrolysis.

Results in Figure 6 demonstrate that, while there was only minimal improvement in glucose and xylose yields with increasing H_2O_2 loadings for uncatalyzed AHP, the presence of a small amount (< 5 mM) of Cu(bpy) resulted in monomeric glucose yields of more than 80% (of the theoretical maximum) and monomeric xylose yields of more than 70% at the highest H_2O_2 loading (100 mg/g biomass) after 72 h of hydrolysis. Importantly, these results demonstrate that the H_2O_2 loading can be halved (from 100 to 50 mg/g biomass) with less than a 4% decrease in the 72 h glucose and xylose yields. Additionally, the trend predicts that the H_2O_2 loading could be further decreased to as low as 35 mg/g biomass (comparable to loadings used in commercial pulp bleaching sequences[187]) and still result in more than 70% glucose

yields for 72 h of hydrolysis. Considering that the cost of H_2O_2 would likely be one of the primary contributions to the raw materials costs (along with biomass feedstock, enzyme, and catalyst cost), this 50-65% decrease in the H_2O_2 loading is substantial. By reducing the H_2O_2 demand of the process, the NaOH:H_2O_2 weight ratio during Cu(bpy)-AHP pretreatment can be increased to 2:1 to 3:1. Thus, using alkaline hydrogen peroxide electrochemically generated on-site becomes a viable and economically attractive approach.

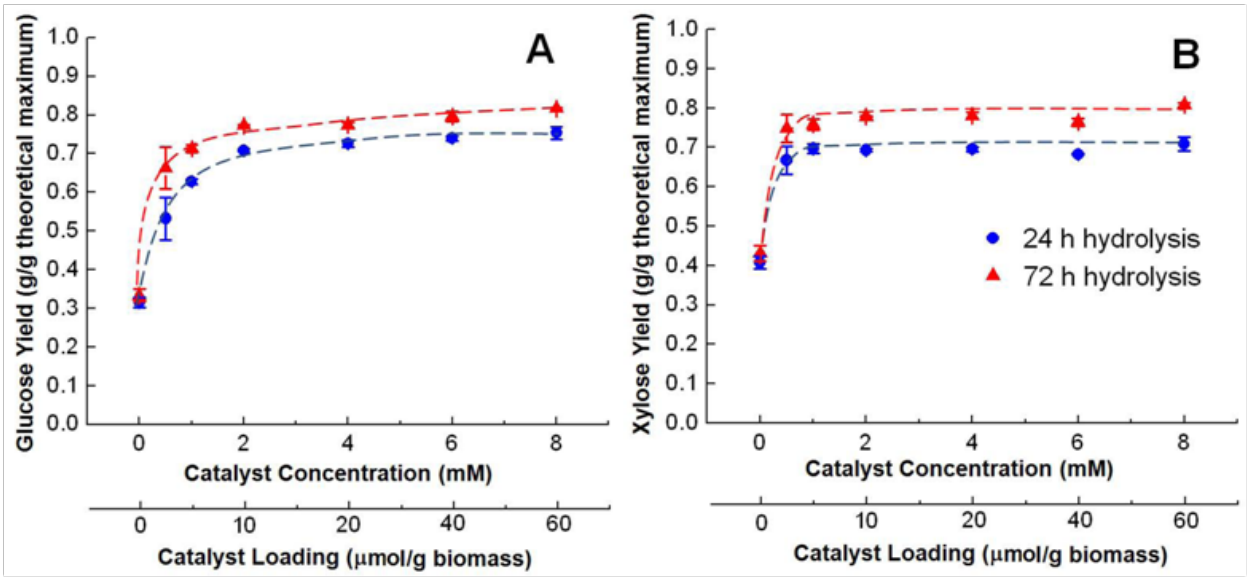

Figure 7. Effect of catalyst concentration during Cu(bpy)-AHP pretreatment.
Data show the enzymatic hydrolysis yield of **(A)** glucose and **(B)** xylose.

The concentration of the Cu(bpy) catalyst utilized during pretreatment is another variable that can be optimized. The water-soluble Cu(bpy) metal complexes have many advantages including their ease of synthesis[188] from $CuSO_4$ and 2,2'-bipyridine, and the fact that they are small enough to diffuse into nanoscale pores within plant cell walls to perform catalysis *in situ*. Reducing Cu(bpy) loadings would be advantageous because this would reduce input costs, alleviate potential inhibition to fermentation microorganism, and diminish environmental concerns about the fate of the catalyst in process water treatment streams. To this end, the effect of catalyst loading on the enzymatic digestibility of pretreated hybrid poplar was tested (Figure 7). The results demonstrate that after 24 h pretreatment with 10% H_2O_2 loading at 20% solids loading, the glucose and xylose yields both saturate at a Cu(bpy) concentration of

2.0 mM (corresponding to a catalyst loading of 10 μmol/g biomass) regardless of the hydrolysis time. In addition, the catalyst concentration can be further halved to 1.0 mM (5.0 μmol/g biomass) with only a 10% loss in the 72 h glucose conversion (Figure 7A) and essentially no loss in the xylose conversion (Figure 7B).

Bipyridine is an important component of the catalyst, as well as a major factor in the catalyst cost. Lowering the loading of 2,2'-bipyridine used during Cu(bpy)-AHP pretreatment will result in lower catalyst cost, although the efficacy of the pretreatment might also be affected. The types of 2,2'-bipyridine coordinated Cu complexes present in aqueous solution vary depending on the ligand-to-metal ratio (L/M), as well as on the pH of the aqueous system.[188,189] The reactivity of the Cu complexes is influenced by the electronic and steric effects introduced by the ligands. As the result, the catalytic oxidation chemistry of the Cu(bpy) system is affected by both L/M and pH. Korpi et al. studied the efficacy of Cu(bpy) complexes with different L/M as catalysts for oxygen delignification of wood pulp under various pH, and discovered the dependence of veratryl alcohol oxidation efficiency on L/M and pH.[190] As the dominant species for the condition under which maximum extent of veratryl alcohol oxidation was achieved, $[Cu(bpy)_2OH]^+$ was proposed as the active catalyst species during oxygen activation.

Using the enzymatic digestibility of pretreated biomass as a metric for pretreatment efficiency, the effect of L/M and pH during Cu(bpy)-AHP pretreatment was investigated (Figure 8). By pretreating the hybrid poplar heartwood in 22 g/L, 10.8 g/L, 0.8 g/L and 0.048 g/L of NaOH, the pH of the reaction mixture at the start of Cu(bpy)-AHP pretreatment was set at 13.0, 11.5, 10.0 and 8.5, respectively. The L/M ranged from 0 (2 mM $CuSO_4$, no 2,2'-bipyridine) to 5 (2 mM $CuSO_4$ and 10 mM 2,2'-bipyridine) among different reaction conditions. The loading of H_2O_2 on biomass is 10% (w/w) and the solids concentration during pretreatment was 10% (w/v). After 24 hour of pretreatment with different L/M at different pH, enzymatic hydrolysis was performed using the same procedure as previously described in the H_2O_2 loading experiment.

At pH 10.0 and 8.5, Cu(bpy)-AHP did not improve the enzymatic digestibility of hybrid poplar heartwood. This is possibly caused by the low reactivity of H_2O_2 and phenolic lignin at neutral pH. Under alkaline pH, L/M higher than 2 did not give improved hydrolysis yields compared to L/M = 2. It was also observed that for pretreatment with higher initial pH, higher L/M is needed for optimum pretreatment efficiency. This phenomenon is possibly caused by the competitive coordination of OH^- anion and 2,2'-bipyridine to copper.

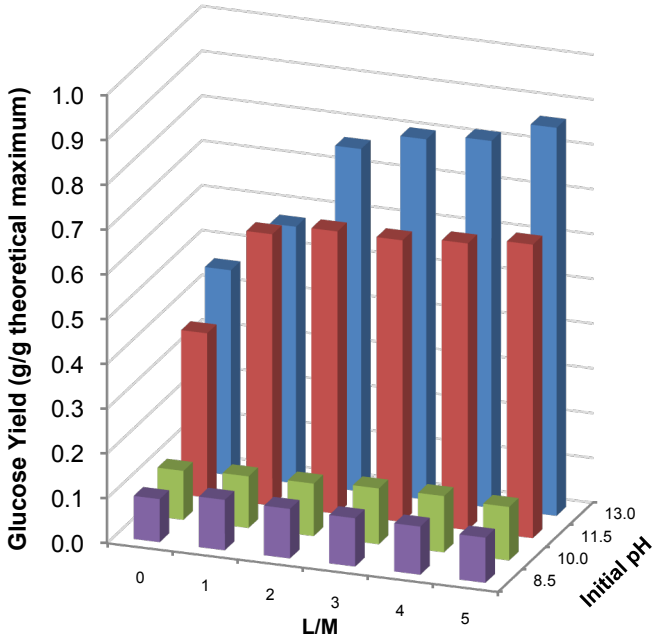

Figure 8. Effect of L/M and initial pH during 24 h Cu(bpy)-AHP pretreatment.

Addition of 2,2'-bipyridine facilitates effective pretreatment compared to AHP pretreatment catalyzed by $CuSO_4$ only (L/M = 0). Such evidence could possibly the result of the involvement of 2,2'-bipyridine in the oxidation catalysis, e.g. via formation of catalytically active coordinated Cu complexes. It is also possible that 2,2'-bipyridine helps targeting the copper into the cell wall and enhances the spatial selectivity of the oxidation. Sawyer et al. proposed a reaction mechanism in which bis-chelated Cu(bpy) complex forms mononuclear adducts with alcohol and dioxygen during alcohol oxidation,[191] and Czapski suggested the formation of H_2O_2 in catalytic dioxygen activation by Cu(bpy) complexes.[192] The ability of

Cu(bpy) complexes to activate dioxygen potentially improves the overall atomic efficiency during Cu(bpy)-AHP pretreatment.

3.3 Kinetics of Pretreatment and Enzymatic Hydrolysis

Performing pretreatment and hydrolysis at high solids concentrations with no subsequent washing imparts a number of process benefits, including a decrease of process water usage and catalyst consumption (on the basis of biomass being processed), a decrease in required reactor volumes, and an increase in sugar titers from hydrolysis and subsequently ethanol titers from fermentation. Intriguingly, uncatalyzed AHP pretreatment with 10% w/w H_2O_2 loading resulted in noticeable increase in glucan and xylan digestibilities as the hybrid poplar solids were increased from 10% to 20% (w/v), with further modest increases continuing even up to 50% (w/v) solids concentration (Figure 9). The catalyzed AHP pretreatment (with 2 mM Cu and 10 mM 2,2'-bipyridine in the aqueous phase) at the same H_2O_2 loading showed a different trend in that the maximum enzymatic digestibility of hybrid poplar was achieved for solids concentrations in the range of 10% to 20% (w/v) solids with pretreatment efficacy decreasing above 30% solids (w/v) concentration. It is likely that at higher solids concentrations (> 20% w/v), the efficacy of the catalyzed pretreatment may be affected by limited mass transfer due to the lack of free water,[193] low loading of catalyst on biomass, as well as decreased selectivity of H_2O_2 for the biomass oxidation versus non-productive disproportionation due to the change in reactant concentrations.

Pretreatment reaction kinetics is important for the economics of a process since the reactor volume and hence the capital equipment requirement is proportional to the residence time of the reactor (besides the effect of solids concentrations). An advantage of Cu(bpy)-AHP pretreatment is that the rate of pretreatment is very rapid. The enzymatic glucan digestibility of pretreated hybrid poplar heartwood rapidly increases to approach a near maximum value within only 10-30 min at 10% solids (w/v) concentrations, while increasing the solids to 20% (w/v) results in achieving the maximum value in less than 10 min (Figure 10A). Comparable increases in the xylan digestibilities can also be achieved within the same short period of time (Figure 10B). Conversely, uncatalyzed AHP pretreatment results in

considerably lower digestibility improvements and requires significantly longer pretreatment time for maximum efficacy.

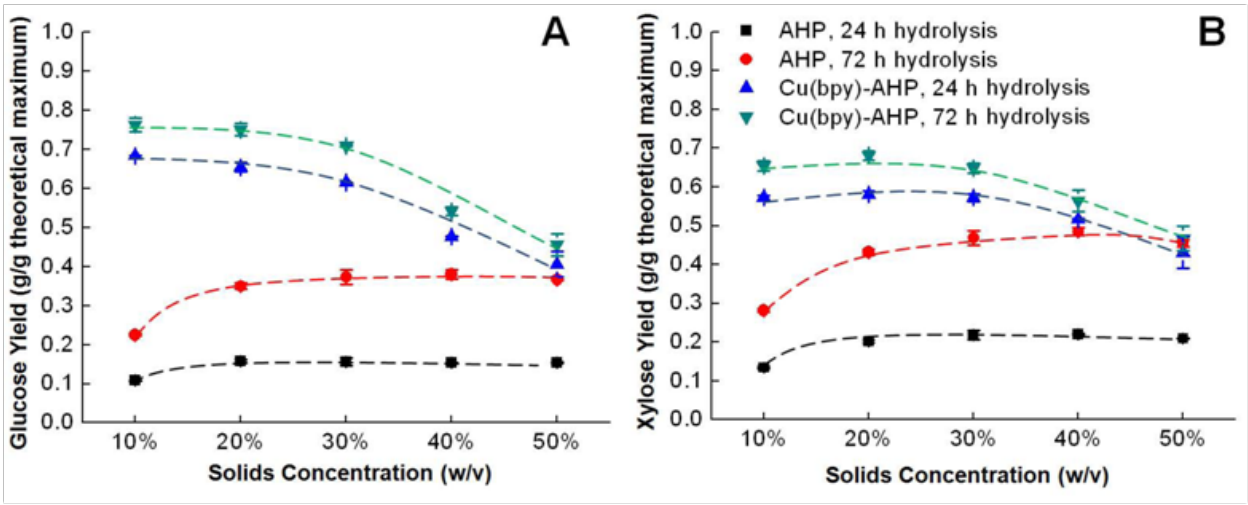

Figure 9. Effect of biomass solids concentration during 24 h AHP and Cu(bpy)-AHP pretreatment. Data show the enzymatic hydrolysis yield of **(A)** glucose and **(B)** xylose.

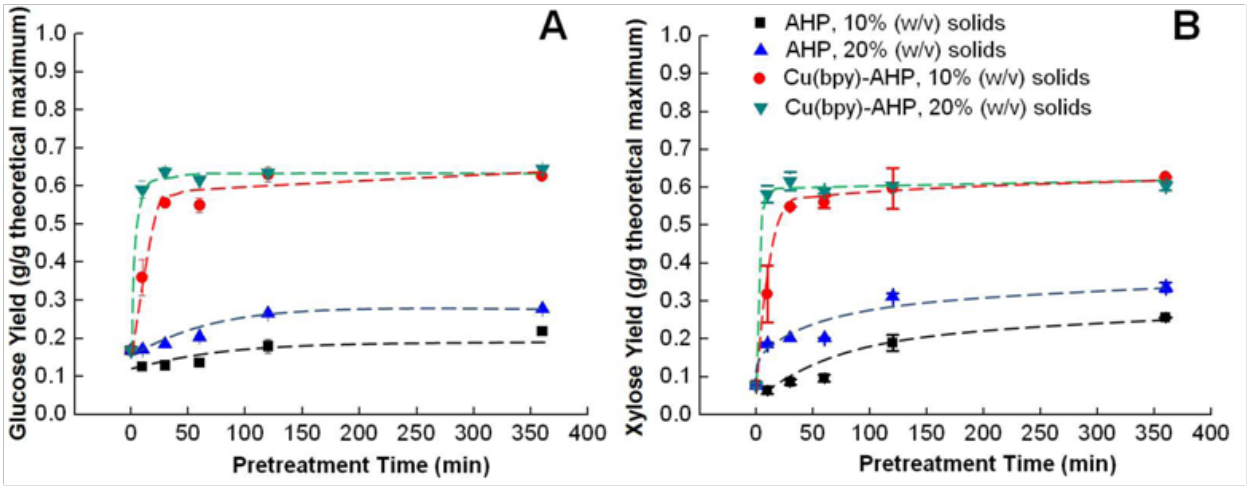

Figure 10. Effect of pretreatment time on the efficacy of AHP and Cu(bpy)-AHP pretreatment. Data show enzymatic hydrolysis yield of **(A)** glucose and **(B)** xylose. The H_2O_2 loading is 10% during pretreatment, and the catalyst concentration is 5 mM (L/M=5:1).

As Cu(bpy)-AHP pretreatment improves the digestibility of hybrid poplar, the amount of enzyme needed for effective hydrolysis is also decreased (Figure 11). Substantially less enzyme is needed to achieve higher digestibilities (i.e. less mass enzyme protein per mass sugar generated) using Cu(bpy)-catalyzed AHP treated poplar relative to the AHP pretreated material. Another observation is that the

xylanase supplementation provides improvement in both the glucose or xylose yields with the synergy between xylanases and cellulases increased at limiting enzyme loadings. This indicates that, like other xylan-retaining pretreatments, xylanase leveraging is possible.[175] The results also indicate that for the given pretreatment conditions, glucan and xylan conversions nearly saturate at their maximum achievable levels with respect to enzyme loading. Additionally, the enzyme dosage can be decreased by at least 50% to a total enzyme loading of 30 mg protein/g glucan with only minor losses in glucose and xylose yields. This decrease is important considering that enzyme costs are anticipated to be one of largest contributions to cellulosic biofuels costs.[194]

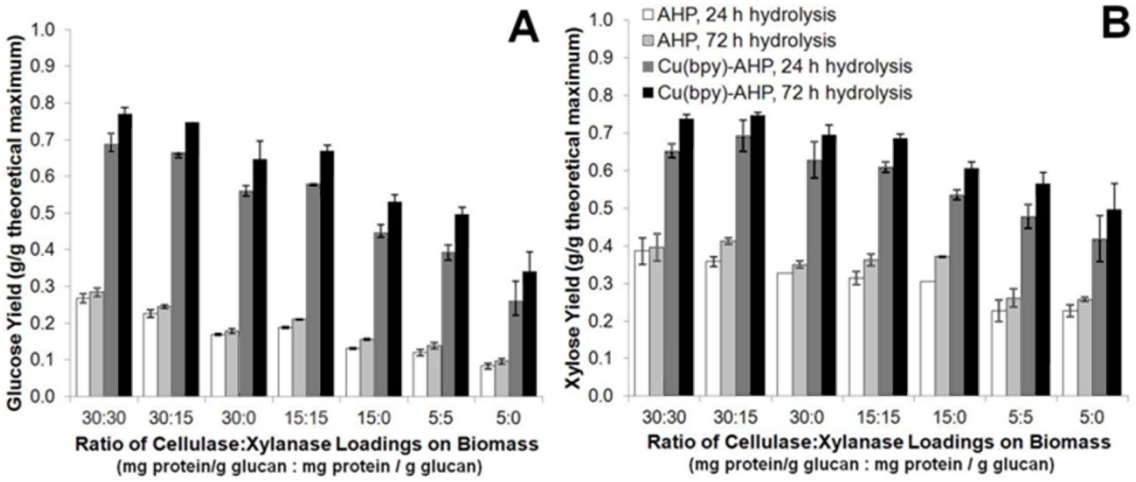

Figure 11. Effect of enzyme loading and xylanase supplementation on enzymatic hydrolysis. Data show hydrolysis yield of **(A)** glucose and **(B)** xylose from biomass after pretreatment performed for 24 h with 0.1 g H_2O_2 per g of biomass, 10% (w/v) solids concentration, and a Cu(bpy) concentration of 2.0 mM for the catalyzed reaction. The molar ration between ligand and copper ion is 5:1.

The kinetics of the enzymatic hydrolysis following catalyzed and uncatalyzed pretreatment during pretreatment was also investigated (Figure 12), highlighting a number of important outcomes of the pretreatments. As demonstrated in the results, both the rate and extent of enzymatic hydrolysis are significantly improved following Cu(bpy)-catalyzed AHP treatment relative to uncatalyzed treatment. After 3 hours of hydrolysis, the enzymatic conversion of glucan in Cu(bpy)-catalyzed AHP pretreated hybrid poplar heartwood is approximately two-fold higher than that in hybrid poplar heartwood after uncatalyzed AHP pretreatment, and this ratio increases even further with longer hydrolysis time. Another

key finding is that while longer pretreatment times result in higher monomeric glucose yields for both catalyzed and uncatalyzed AHP pretreatment, the majority of the glucan digestibility improvement by pretreatment takes place within the first 30 minutes. Additionally, the differences in sugar yield between 1 h and 24 h pretreatment times nearly disappear at 20% solids, which is in agreement with the results shown in Figure 10.

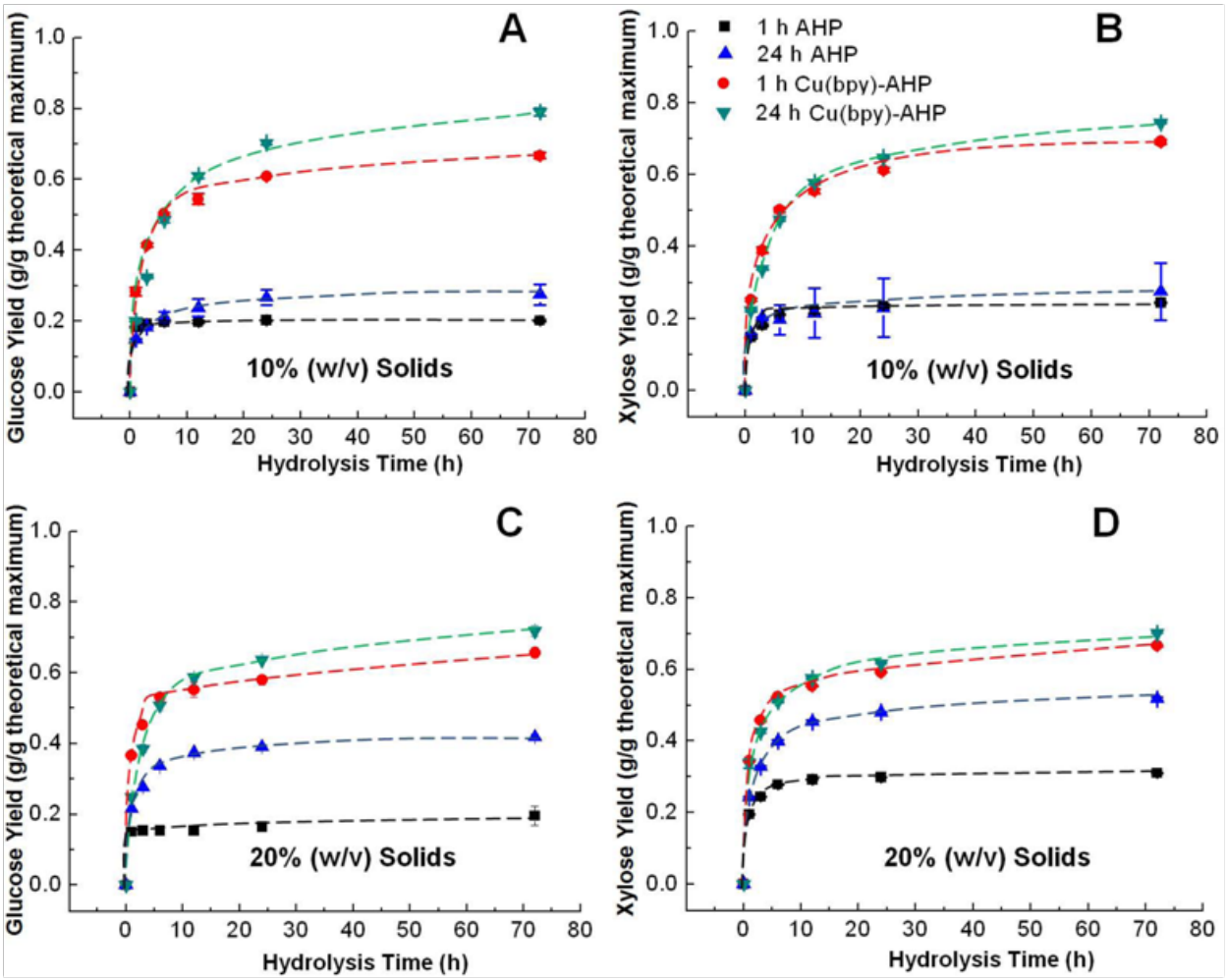

Figure 12. Effect of enzymatic hydrolysis time.
Data show the yield of glucose (**A, C**) and xylose (**B, D**) from biomass after 24 hpretreatment with 0.1 g H_2O_2 per g of biomass, at solids loading of 10% w/v (**A, B**) and 20% w/v (**C, D**), and a Cu(bpy) concentration of 5.0 mM for the catalyzed reaction. The molar ratio between ligand and copper ion is 5:1.

As presented above, the effect of Cu(bpy)-AHP pretreatment on increasing the enzymatic digestibility of hybrid poplar is very significant. The enzymatic digestibility of Cu(bpy)-AHP pretreated hybrid poplar is similar to (or even higher than) the enzymatic digestibility of hybrid poplar pretreated by some of the

most effective pretreatment technologies.[195,196] Cu(bpy)-AHP pretreatment is a rapid process which reaches optimum efficacy within 30 minutes when operated under batch setting. The catalytic pretreatment with low demand of H_2O_2 (35-50 mg per g of biomass), which is an important advantage compared to uncatalyzed AHP pretreatment with high H_2O_2 demand. The Cu(bpy) catalyst is highly active, and relatively low loading of catalyst is needed for effective retreatment (10 μmol per g of biomass). The dosage of Cu catalyst during Cu(bpy) pretreatment can potentially be further decreased via implementation of catalyst recovery strategies, as well as the use of more active catalysts at lower loadings. Lowering Cu consumption is important not only because of its impact on process economy, but also because of its effect on downstream sugar fermentation process (i.e. Cu toxicity to fermentation organisms). Hybrid poplar pretreated with Cu(bpy)-AHP has high enzymatic digestibility, and much less enzyme is required for the effective hydrolysis of pretreated biomass compared to untreated biomass. The rapid hydrolysis rate of pretreated biomass also suggests significant removal of cell wall recalcitrance during Cu(bpy)-AHP pretreatment. Some mechanistic studies on pretreatment-induced cell wall modification and recalcitrance removal will be discussed in more detail in Chapter 5.

3.4 Fermentation of Enzymatic Hydrolysate from Cu(bpy)-AHP Pretreated Hybrid Poplar

(This section was based on collaborative work with Dr. Yaoping Zhang and Dr. Trey Sato.)

After proper pretreatment, hybrid poplar can be used for producing fermentable sugars and bio-ethanol. Due to the recalcitrant nature of hybrid poplar, however, the yield of sugars and ethanol from poplar is limited by the low enzymatic digestibility of hybrid poplar. Ballesteros et al. produced ethanol from liquid hot water pretreated hybrid poplar via a simultaneous saccharification and fermentation (SSF) process, and the final ethanol titer was 17-20 g/L depending on the operation conditions.[197] Similar yield of ethanol was achieved after 5 days SSF of hybrid poplar after SPORL pretreatment.[182] Hybrid poplar after oxidative lime pretreatment is highly digestible by enzymes, and the hydrolysate can be fermented to ethanol with a final titer of 39.9 g/L.[195]

To produce ethanol at high titer, the hydrolysate should have a high concentration of fermentable sugars (predominantly glucose and xylose) prior to fermentation. Therefore, enzymatic hydrolysis needs

to be performed at relatively high solids loading. Moreover, the concentration of residual Cu catalyst in the hydrolysate needs to be minimized to reduce the toxicity of the hydrolysate to fermentation microorganisms. To prepare poplar hydrolysate for fermentation, Cu(bpy)-AHP pretreatment with reduced catalyst loading (2 mM $CuSO_4$, 4 mM 2,2'-bipyridine) was performed on hybrid poplar (mix of heartwood and sapwood) prior to enzymatic hydrolysis. The solids concentration during pretreatment was 20% (w/v) and the loading of H_2O_2 was 10% (w/w) on biomass. Following the 24 hour pretreatment, the pH of pretreated biomass slurry was adjusted to 5.5 using sulfuric acid. Next, 40% of the liquid volume in the pretreated biomass slurry was replaced with deionized water with pH adjusted to 5.5. This was done by a liquid-solid separation step (to remove 40% of the liquids) followed by addition of pH 5.5 water. Insoluble biomass was not removed from the slurry during liquid-solid separation. Novozymes Cellic Ctec2 and Htec2 enzymes were then added to the biomass slurry, both at a loading of 30 mg protein per g of glucan in the biomass prior to pretreatment. The enzymatic hydrolysis was conducted at 50 °C for 48 hours. After the enzymatic hydrolysis, the aqueous hydrolysate was recovered via centrifugation and filtered through 0.22 μM mixed cellulose esters membrane. $(NH_4)_2SO_4$ at the concentration of 30 mM was supplemented to the hydrolysate as a nitrogen source. Ethanol fermentation was performed in an Applicon MiniBio fermenter (Applikon Biotechnology Inc., Foster City, CA) under anaerobic condition. A genetically modified xylose-fermenting *Saccharomyces cerevisiae* strain (GLBRC-Y73)[198] was used for ethanol fermentation. After inoculation, the initial OD_{600} of the broth is ca. 2.0.

GLBRC-Y73 is a metabolically engineered *S. cerevisiae* strain in which xylose reductase and xylitol dehydrogenase are expressed.[156] As the result, both xylose and glucose can be metabolized during fermentation. After 23 hours of fermentation, the glucose in the hydrolysate had already been completely consumed, and about 20% of the xylose was also consumed. The slow uptake of xylose was possibly caused by the inhibition from the product ethanol, the Cu ions, and the biomass degradation products that were present in the hydrolysate. The propagation of cell density was also modest, possibly due to the depletion of nutrients as well as the effect of the aforementioned inhibitors. Nevertheless, the ethanol concentration in the fermentation broth reached 20 g/L within the first 24 hours of fermentation, and the

metabolic yield[196] of ethanol was about 82%. The results suggest that Cu(bpy)-AHP pretreatment can be integrated with microbial fermentation for production of biofuel from hybrid poplar.

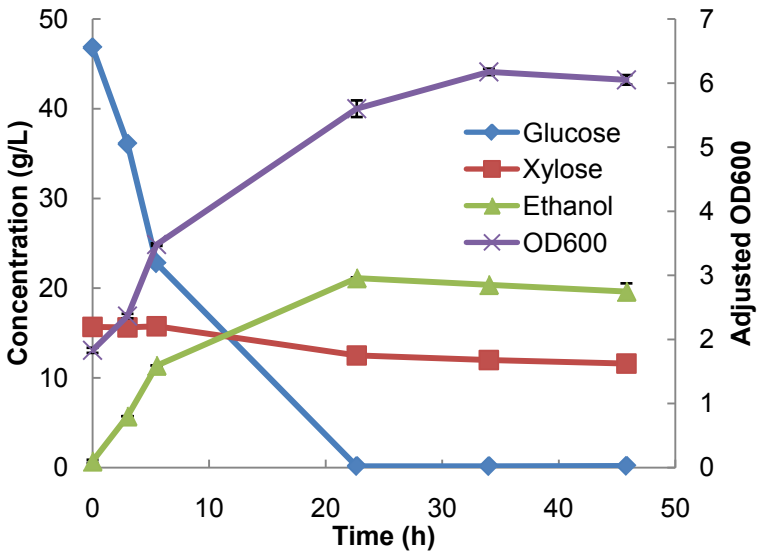

Figure 13. Fermentation of enzymatic hydrolysate from hybrid poplar pretreated with Cu(bpy)-AHP.

To access the impact of Cu toxicity on *S. cerevisiae* fermentation, cell growth was analyzed using three types of hybrid poplar hydrolysate prepared as described in Table 2. For "High Cu" hydrolysate, $CuSO_4$ and 2,2'-bipyridine was supplemented to the hydrolysate at a concentration of 4 mM and 20 mM, respectively (i.e. add 4 mmol of $CuSO_4$ and 20 mmol of 2,2'-bipyridine in 1 L of hydrolysate). "Low Cu" contains the lowest concentration of Cu due to the liquid-solid separation step following the pretreatment (see Section 3.5 for the discussion on Cu removal). "High Cu" hydrolysate has the highest Cu concentration because of the supplementation of catalyst following the hydrolysis. The aerobic growth of 105 *S. cerevisiae* strains in the hydrolysates was monitored using optical density measurements.

The maximum growth of 12 strains with the best growth in 48 hours is plotted in Figure 14. The growth in "Low Cu" was the least inhibited than the growth in "Medium Cu" for all 12 strains, suggesting that the inhibition was alleviated by the liquid replacement step (i.e. liquid-solid separation and addition of pH 5.5 deionized water) following the pretreatment. This reduction in hydrolysate toxicity is possibly

due to the removal of Cu and other toxic compounds from the hydrolysate. Growth of all 12 types of yeast in "High Cu" hydrolysate was strongly inhibited compared to other hydrolysates, suggesting that residual Cu(bpy) catalyst in the hydrolysate could play an important role in the toxicity of the hydrolysate from Cu(bpy)-AHP pretreated biomass, depending on the concentration of copper used during pretreatment.

Name of Hydrolysate	Pretreatment	After Pretreatment	Enzymatic Hydrolysis	After Hydrolysis
Low Cu	pH 11.5, 30°C 1 hr Cu(bpy)-AHP 4 mM $CuSO_4$ 20 mM 2,2'-bipyridine 10% w/w H_2O_2 loading 20% w/v solids loading	pH adjusted to 5.5, 33% of the liquid replaced with pH 5.5 deionized water	pH 11.5, 50°C 30 mg Ctec2 and 30 mg Htec2 protein for every g of glucan in untreated biomass	-
Medium Cu		pH adjusted to 5.5		-
High Cu		pH adjusted to 5.5, 33% of the liquid replaced with pH 5.5 deionized water		Add catalyst

Table 2. Procedure used to prepare hybrid poplar hydrolysate with different Cu concentrations.

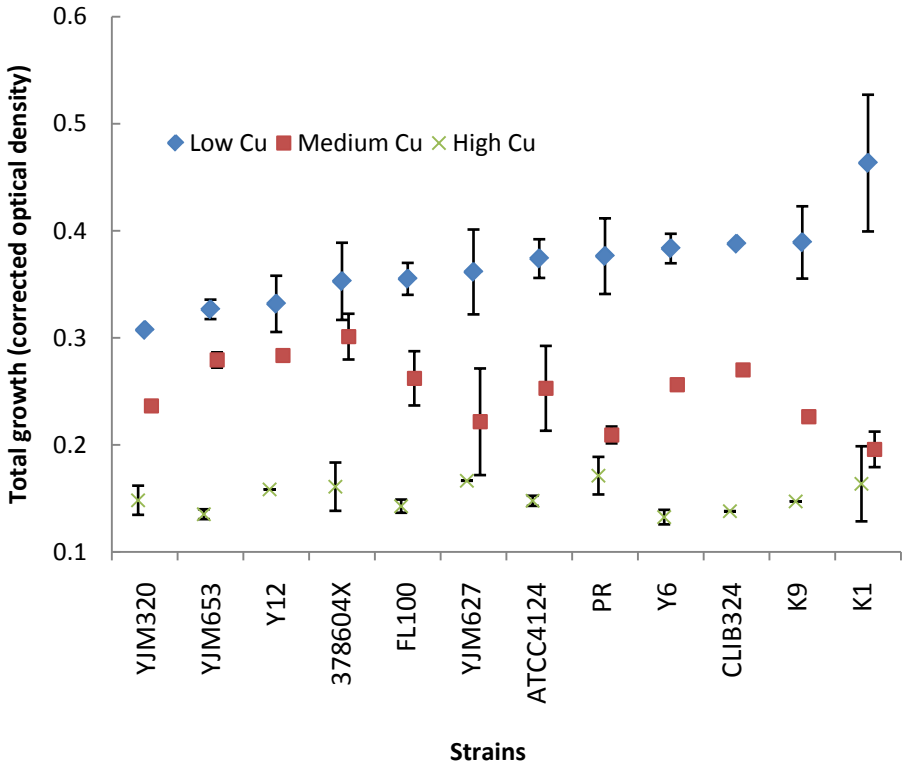

Figure 14. 48-hour aerobic growth of *S. cerevisiae* in hybrid poplar hydrolysates.

3.5 Catalyst Recovery and Process Integration

(This section was based on collaborative work with Mr. Charles Chen and Mr. Aditya Bhalla.)

Copper needs to be removed from pretreated biomass after pretreatment to reduce the Cu-induced toxicity in the biomass hydrolysate. Because the Cu(bpy) catalyst complex is soluble in water, a simple strategy for removing Cu is liquid-solid separation. Such a liquid-solid separation process is also an opportunity for the recovery and re-use of the Cu catalyst. During liquid-solid separation, the efficiency of catalyst recovery is affected by the adsorption of Cu on woody biomass. The adsorption of Cu ions on biomass surface is known to be correlated to the pH, as pH affects the ionic exchange properties of the biomass.[199] Under high pH, deprotonated functional groups (e.g. RO^-, $RCOO^-$) on the biomass surface are potential binding sites for cationic Cu ions and complexes. Cu adsorption behavior can also be affected by ligands, as ligand chelation affects the solubility and electronic properties of the Cu complex.[200]

To investigate the adsorption behavior of the Cu(bpy) complexes on biomass, adsorption studies were performed on raw hybrid poplar (mixture of heartwood and sapwood). $CuSO_4$ and 2,2'-bipyridine was added to a 10% (w/v) aqueous suspension of hybrid poplar at an L/M of 2. After complete mixing and 1 hour of incubation at 30 °C in an orbital shaker, the aqueous phase was sampled from the mixture and the Cu content in the sample was analyzed using atomic absorbance spectroscopy. The amount of Cu adsorbed to the biomass was calculated by subtracting the Cu present in the aqueous phase from the total amount of Cu added.

As seen from the results, the amount of Cu adsorbed to the biomass is associated with the loading of Cu and the pH of the aqueous solution (Figure 15). At pH 12 (the pH after 1 hour of Cu(bpy)-AHP pretreatment), over 90% of the Cu was adsorbed on the biomass. The high binding affinity of Cu(bpy) to biomass at high pH is possibly the result of ligand effects, as the π-acceptor 2,2'-bipyridine ligand potentially draws electron density from the d-orbital of Cu and increases the cationic charge on the metal center.[201] The biomass pretreated with Cu(bpy)-AHP might have higher binding affinity to Cu, as oxidative pretreatment introduces more functional groups with oxygen donors that are potential binding

sites. Strong Cu adsorption on biomass also facilitates oxidation catalysis in close vicinity of the biomass surface, thus possibly enhancing the spatial selectivity of the oxidation reactions.

At high Cu loadings (ca. 40 μmol/g biomass), about 30% of the Cu is not adsorbed to biomass at pH 10, and the percentage increases to 35% at pH 5. This suggests that the adsorption behavior of Cu(bpy) on biomass is strongly affected by pH, and that Cu possibly desorbs from pretreated biomass when the pH was lowered to 5.0 after the pretreatment. The adsorption of Cu on biomass is less affected in low concentration ranges of Cu that more closely resemble pretreatment conditions. It should be noted, however, that the data shown in Figure 15 only represent the Cu adsorption behavior on untreated biomass, and that pretreated biomass might have different Cu adsorption properties. Via pH adjustment and a liquid-solid separation process after Cu(bpy)-AHP pretreatment, Cu can be removed from the pretreated biomass and possibly be reused as catalyst for Cu(bpy)-AHP pretreatment. After Cu(bpy)-AHP pretreatment of alkali pre-washed hybrid poplar using recycled Cu catalyst, the enzymatic digestibility of hybrid poplar can be significantly improved (80% glucan conversion after 3 days of enzymatic hydrolysis, personal correspondence with Aditya Bhalla). A Cu(bpy)-AHP pretreatment process using recovered Cu catalyst is described in greater detail in Chapter 4.

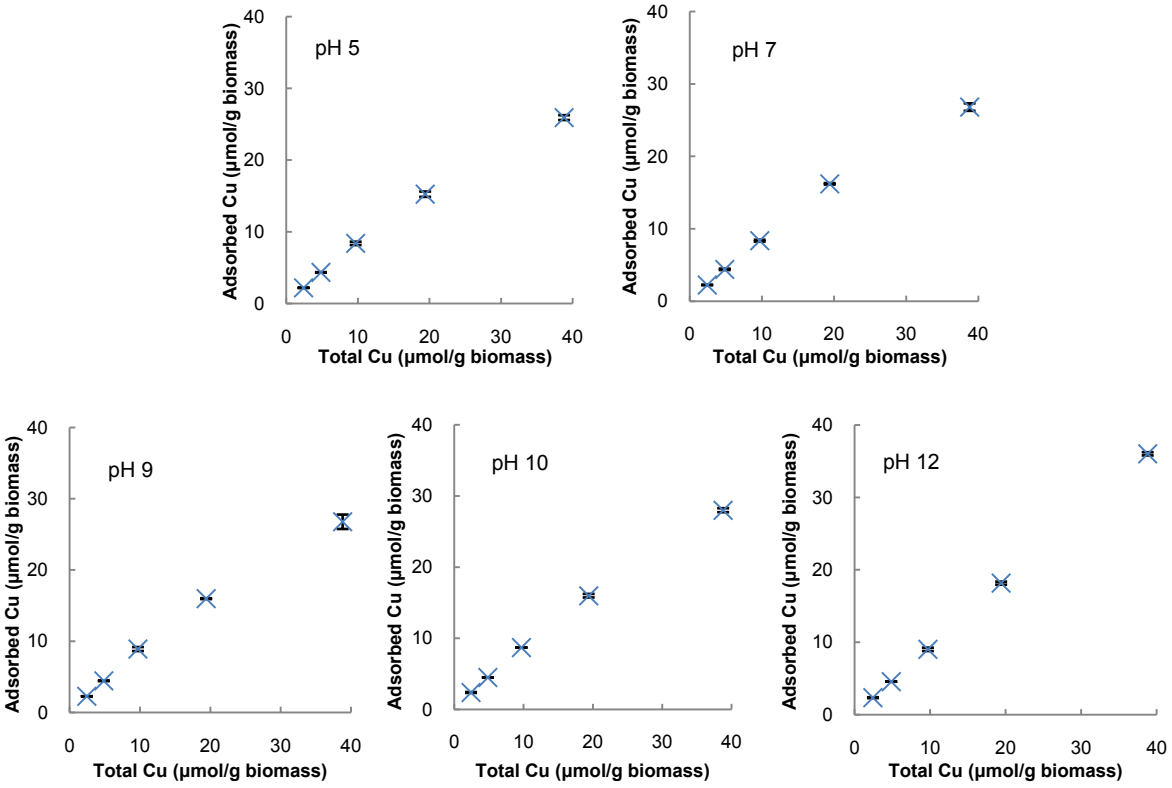

Figure 15. Adsorption of Cu on raw hybrid poplar at different pH.

CHAPTER 4

ANALYSIS OF COSTS IN SUGAR PRODUCTION

(This chapter was based on collaborative work with Dr. Aditya Bhalla.)

Ethanol produced from renewable feedstocks such as corn grain and sugarcane is being used as a partial replacement of petroleum-derived liquid-transportation fuel. Since 2010, the annual production of fuel ethanol in the U.S. has been over 13 billion U.S. gallons (EIA Monthly Energy Review, March 2014), and about 90% of the fuel ethanol produced is consumed as transportation fuel. Corn grain currently used to produce ethanol currently account for about 30-40% of the annually produced corn crop in the U.S., which raised concerns on competitive land use against food production. In addition, because of agricultural activities such as tillage and fertilizer use, the reduction in greenhouse gas emission associated with the adoption of corn ethanol biofuel is moderate.[202]

Utilization of cellulosic biomass has many ecological and agronomical advantages. Cellulosic bioenergy crops such as switchgrass and hybrid poplar can be produced on marginally productive land at high productivity with low input of energy and fertilizers.[203] Conversion to cellulosic corn fiber (a byproduct of dry corn grain milling process) to ethanol improves the productivity of the existing corn ethanol facilities, and at the same time reduces the carbon footprint of corn ethanol. Due to the resistance of cellulosic polysaccharides to enzymatic hydrolysis, however, the conversion process of cellulosic biomass to ethanol is costly and inefficient. The yield of sugars and ethanol during biomass conversion can be improved by biomass pretreatment, which renders biomass more susceptible to chemical and biological conversions. Although pretreatment process possibly requires high chemical and energy inputs, the process is necessary to achieve high conversion efficiency of recalcitrant feedstocks (e.g. woody biomass).[204]

High yield of sugars from Cu(bpy)-AHP pretreated hybrid poplar and the good fermentability of hybrid poplar hydrolysate suggest that conversion of hybrid poplar to ethanol may be practical. The economic feasibility of bioethanol production from hybrid poplar depends on the cost induced by the catalytic oxidative pretreatment, the efficiency of biomass conversion (enzymatic hydrolysis and ethanol

fermentation), as well as the energy input in the conversion process. Due to the lack of suitable fermentation organisms specifically engineered for hybrid poplar hydrolysate and the absence of poplar refinery demonstration facilities, the analysis in this chapter will be focused on the cost of chemicals required to produce fermentable sugars from hybrid poplar. The purpose of this analysis is to reveal the impact of process integration and catalyst recovery on the economic feasibility of hybrid poplar biorefinery.

4.1 Cu(bpy)-AHP Pretreatment Process: The Base Case

As described in Chapter 2 and Chapter 3, Cu(bpy)-AHP pretreatment involves the use of NaOH, H_2O_2 and Cu(bpy) catalyst. The process flow diagram of Cu(bpy)-AHP pretreatment and sugar conversion is shown in Figure 16. Under this base case, about 55% of the glucan and about 60% of the xylan in hybrid poplar (mixture of heartwood and sapwood) was recovered as monomeric sugars after 24 hours of Cu(bpy) pretreatment and 72 hours of enzymatic hydrolysis. The residual Cu catalyst remains in the biomass hydrolysate as well as in the hydrolysis residue after enzymatic hydrolysis.

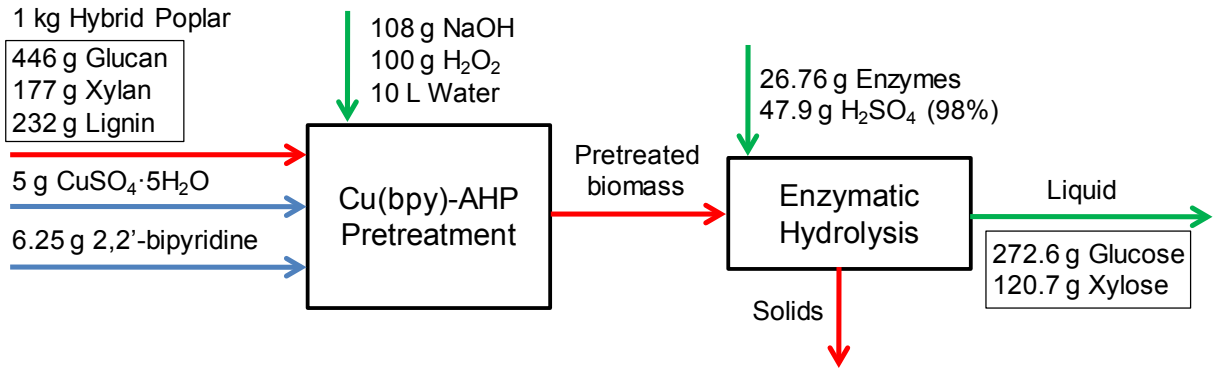

Figure 16. Process flow diagram showing the base case of Cu(bpy)-AHP pretreatment and the subsequent enzymatic hydrolysis yielding fermentable sugars.

Using the estimated material costs in Table 3, the cost of chemicals for producing fermentable sugars can be estimated (Figure 17). The cost of 2,2'-bipyridine ligand is a significant factor during the production of fermentable sugars from hybrid poplar. Apart from 2,2'-bipyridine, other major elements contributing to cost include feedstock (growth, harvest, transportation and handling),[205] cellulase and hemicellulase enzymes, hydrogen peroxide, and sodium hydroxide. As a process for producing fermentable sugars, the

Cu(bpy)-AHP base case as shown in Figure 14 is not ideal in many ways. Production cost of sugars is relatively high due to the low conversion yields (393.3 g of total monomeric sugars from 1 kg dry weight of biomass). The loading of cellulase and hemicellulase enzymes is not at the optimized loading under the specific pretreatment conditions, thus the amount of enzymes actually needed to achieve this sugar yield might be lower than the amount used in the base case.

Item	Cost ($/Mg)
Enzymes	4050
Hydrogen peroxide	530
Sodium hydroxide	380
Sulfuric acid	87
Copper sulfate pentahydrate	2500
2,2'-bipyridine	70000
Feedstock (hybrid poplar)	100

Table 3. Unit cost of raw materials used during conversion of hybrid poplar.

4.2 Enhanced Cu(bpy)-AHP Pretreatment Process

Alkaline pre-extraction of hybrid poplar under room temperature significantly improves the efficacy of the subsequent Cu(bpy)-AHP pretreatment (Aditya Bhalla, personal correspondence). The procedural scheme of this enhanced process is demonstrated in Figure 15. Alkaline pre-extraction is performed by soaking hybrid poplar in 10.8 g/L NaOH aqueous solution at the solids concentration of 10% w/v. After 1 hour of incubation at 30 °C, the pre-extracted biomass slurry is washed with deionized water of the same volume. The insoluble biomass was recovered and pretreated with Cu(bpy) catalyst and alkaline hydrogen peroxide. After the pretreatment, hybrid poplar becomes highly digestible by enzymes. The yields of monomeric glucose and xylose are about 80% on the basis of the glucan on the xylan content in the hybrid poplar before alkaline pre-extraction. This is possibly caused by the removal of alkali-soluble aromatic compounds that inhibit either the pretreatment or the fermentation. Due to the improvement in sugar productivity, the average cost for producing unit amount of sugars significantly decreases in spite of the increased consumption of NaOH (Figure 17). To reduce the cost in chemicals and water clean-up, the base in the spent washing liquid can potentially be reused for alkaline pre-extraction and Cu(bpy)-AHP

pretreatment. As shown in Figure 17, consumption of 2,2'-bipyridine has a strong influence on the overall production cost of sugars. Moreover, recovery of the catalyst is also necessitated by the toxicity of the catalyst to fermentation organisms.

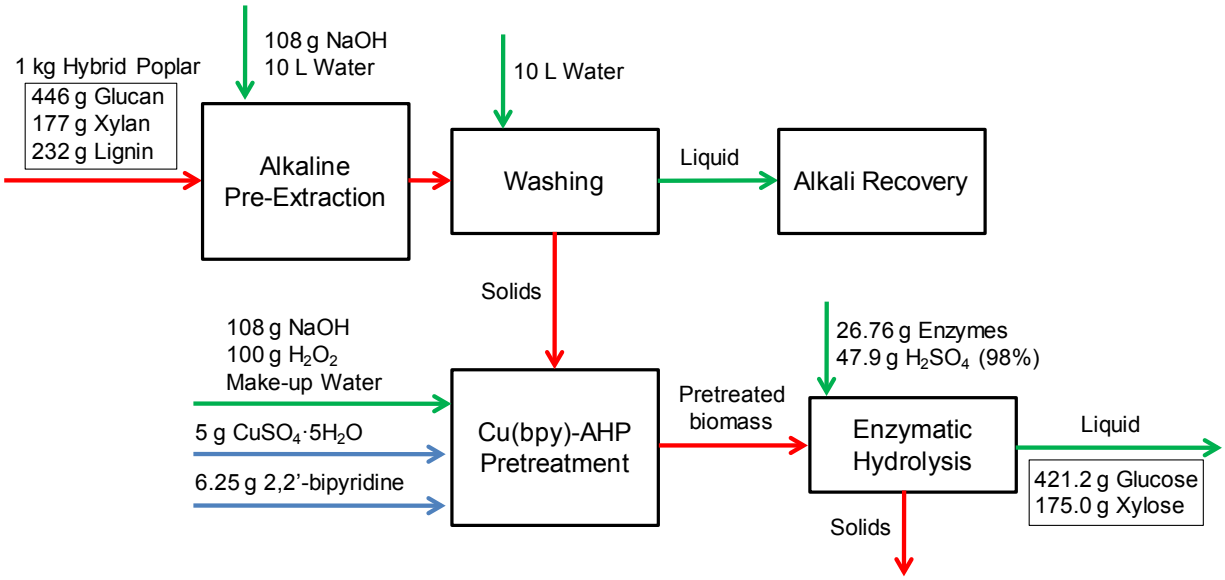

Figure 17. Process flow diagram of alkaline pre-extraction, Cu(bpy)-AHP pretreatment and hydrolysis.

4.3 Catalyst Recovery

As proposed in Section 3.5, the Cu catalyst may be recovered by pH adjustment and liquid-solid separation after the pretreatment. A scheme for catalyst recovery and reuse is proposed in Figure 16. After "standard" Cu(bpy)-AHP pretreatment (i.e. the base case), the pH of the reaction mixture is adjusted to 5.0 with sulfuric acid and the mixture is incubated at 30 °C for another hour. Next, half of the liquid volume is recovered via liquid-solid separation, and the recovered liquid (PTL) is mixed with alkali and untreated biomass for alkaline pre-extraction. During alkaline pre-extraction, the catalyst present in PTL is able to adsorb to the surface of untreated biomass and catalyze the pretreatment for a second time. After 1 hour of alkaline pre-extraction and catalyst impregnation, Cu(bpy)-AHP pretreatment with recovered catalyst is performed with no extra Cu or 2,2'-bipyridine added.

Enzymatic hydrolysis results suggest that recovered catalyst in PTL is effective in catalyzing the AHP pretreatment of hybrid poplar (63% glucose yield and 85% xylose yield). Such observation is possibly

interfered by the polysaccharides in PTL that are carried over from a previous round of pretreatment, but these soluble sugars might as well be washed away following the alkaline pre-extraction and do not affect the sugar yields. Under this scenario, the average cost for producing sugars is lower compared to the base case because of the improvement in sugar yields and the cost reduction via catalyst recovery. More importantly, the effectiveness of PTL as pretreatment catalyst suggests that the loading of catalyst in the base case is higher than actually needed. ICP-MS analysis of PTL revealed that PTL contains 0.9 mM of Cu ions, implying that 9 μmol of catalyst is enough for the pretreatment of 1 gram of hybrid poplar. Given this hypothesis, the amount of catalyst needed to pretreat 1 gram of hybrid poplar will be less than 9 μmol when catalyst recovery is performed.

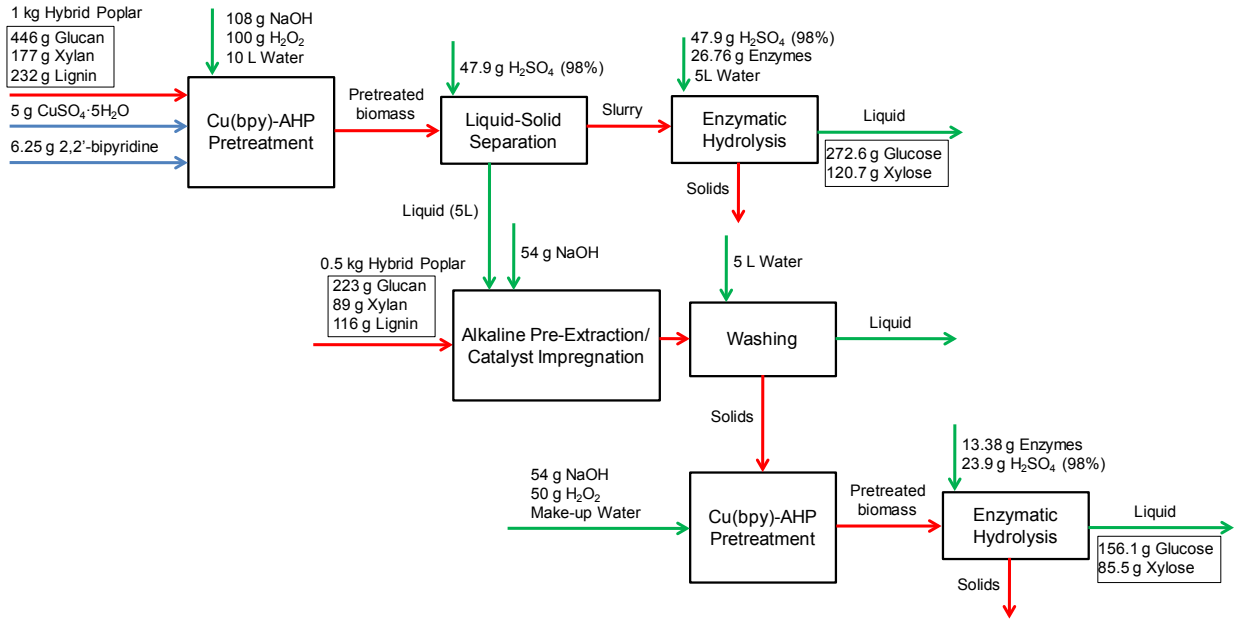

Figure 18. Process flow diagram of Cu(bpy)-AHP with reused catalyst.

4.4 Summary

At the current stage of development, Cu(bpy)-AHP pretreatment is not yet an economically viable option for production of ethanol from hybrid poplar due to the cost of chemicals and enzymes during pretreatment and enzymatic hydrolysis. Nevertheless, it should be pointed out that the pretreatment conditions and operation parameters are optimized for maximum monomeric sugar yield instead of minimum process cost. Further reduction in catalyst loading, H_2O_2 loading, and enzyme consumption is

still possible at the price of marginal decrease in sugar yields. Design of high-performance low-cost catalysts has already been accomplished via the use of cheap ligands (Namita Bansal, personal correspondence), and the application of catalyst recovery strategy will potentially further decrease the cost of catalyst during pretreatment. With a process that includes alkaline pre-extraction, the weight of NaOH consumed will be 1.6-2.2 times of the weight of H_2O_2 consumed. Therefore, a Cu(bpy)-AHP biorefinery can easily be integrated with a electrochemical generator of alkaline hydrogen peroxide which produces H_2O_2 via oxygen reduction in alkaline electrolyte.[151]

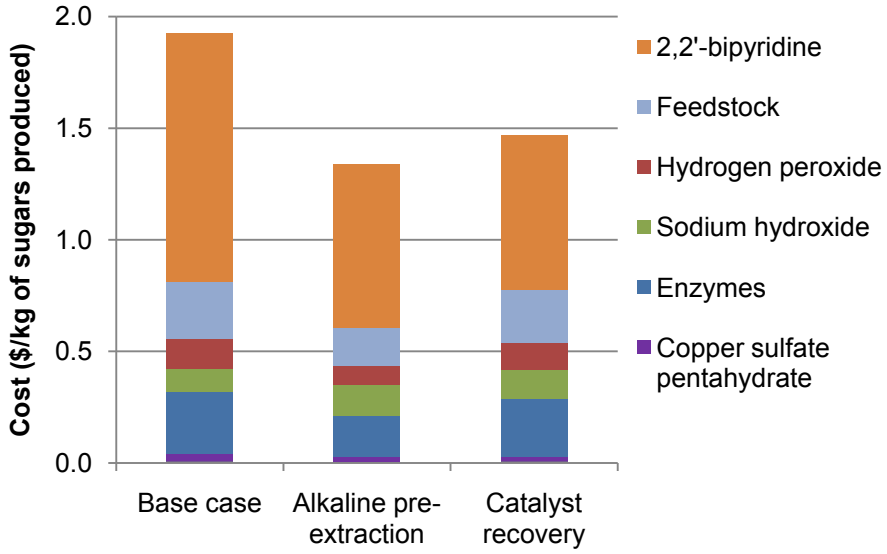

Figure 19. Cost of feedstock, chemicals and enzymes for production of fermentable sugars.

CHAPTER 5

STRUCTURAL AND CHEMICAL MODIFICATIONS OF PLANT CELL WALL

Although the efficacy of Cu(bpy)-AHP pretreatment on hybrid poplar has been proven and reported in literature,[185,206] the details of the structural and chemical modification of biomass during Cu-catalyzed H_2O_2 oxidation remain unclear. More specifically, the underlying mechanism of recalcitrance alleviation during Cu(bpy)-AHP pretreatment is not known. Using advanced microscopic and spectroscopic characterization techniques, some preliminary understanding of oxidation-induced cell wall modification has been obtained. Based on such knowledge, some inference can be made on the catalytic pathways and electron transfer mechanisms during Cu(bpy)-catalyzed oxidation.

5.1 Changes in Bulk Composition

Many biomass pretreatment methods fractionate plant cell wall components via dissolution. Hemicellulose can be dissolved and removed from solid biomass during dilute acid pretreatment,[207] liquid hot water pretreatment,[208] and many alkaline pretreatment methods.[79,209] Oxidative pretreatments are efficient in removing lignin from biomass.[83,91,93,152] Biomass delignification is also observed during SPORL pretreatment, organosolv pretreatment, and ionic liquid extraction.[183,207,210] Solubilization of hemicellulose and lignin removes the cell wall's physical barrier against enzymatic digestion and improves biomass digestibility. To investigate the impact of AHP and Cu(bpy)-AHP pretreatment on the solubility of biomass components, hybrid poplar heartwood was pretreated for 1 hour under 30 °C with 10% w/w loading of H_2O_2 at 10% w/v solids loading. For Cu(bpy)-AHP pretreatment, the concentration of CuSO4 and 2,2'-bipyridine was 2 mM and 10 mM, respectively. After pretreatment, the pretreated biomass was washed with a large volume of deionized water and dried in air. The total mass solubilized during pretreatment was quantified gravimetrically.[185] The mass of cell wall components solubilized during pretreatment was estimated using the gravimetric mass loss and the composition of untreated and pretreated biomass.

After 1 hour of uncatalyzed AHP pretreatment, 21% of the lignin (a.k.a. Klason lignin, which is quantified as the insoluble residue after two-step acidolysis of biomass[157]) in hybrid poplar is solubilized

into the aqueous phase (Figure 20). Cu(bpy)-AHP pretreatment solubilizes greater amount of lignin (44% of the total lignin in biomass) as well as 10% of the hemicellulosic polysaccharides. Assuming H_2O_2 oxidation as the cause of lignin solubilization during pretreatment, the oxidation efficiency during Cu(bpy)-AHP pretreatment is significantly higher than uncatalyzed AHP pretreatment. The solubilization and removal of lignin from cell wall matrix potentially reduces the effect of lignin inhibition during the enzymatic hydrolysis of pretreated biomass, thus resulting in higher yields of monomeric sugars during hydrolysis. Most of the cell wall polysaccharides are retained during Cu(bpy)-AHP pretreatment, suggesting that the catalytic oxidation has high specifity to lignin.

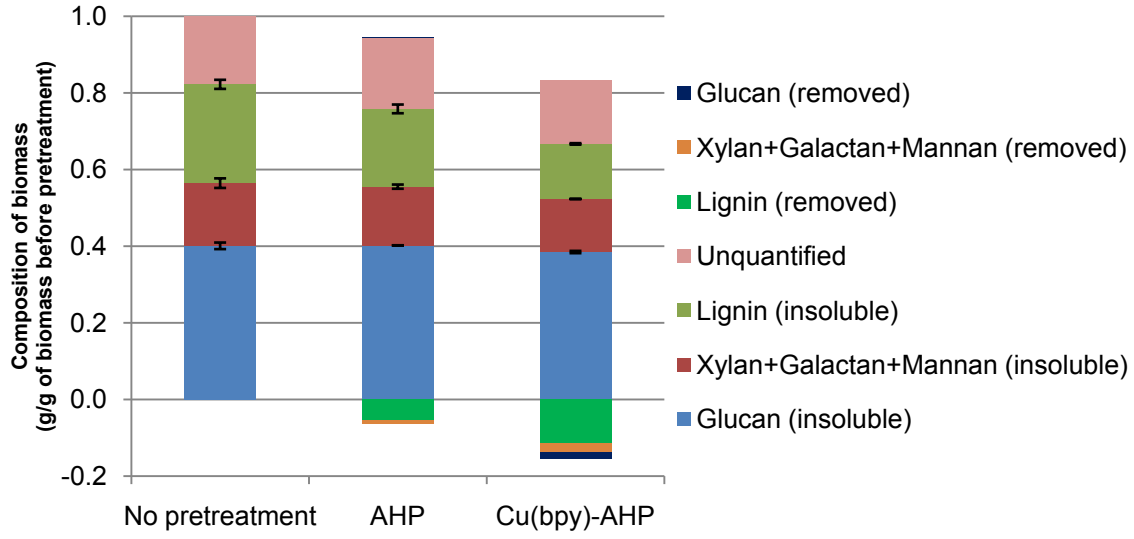

Figure 20. Mass balance of hybrid poplar heartwood before and after pretreatment.
Negative values represent cell wall components solubilized during pretreatment.

5.2 Disruption of Cell Wall Structure

Lignin distributes in the framework of cell wall carbohydrates and has important functions in maintaining the structural rigidity of the plant cell wall matrix. Lignin extrusion and solubilization increases the accessibility of cell wall polysaccharides, which is a possible mechanism of pretreatment-induced improvement in the enzymatic digestibility of biomass. Delignification also disrupts the fiber bundle structure in biomass, potentially creating more accessible area for hydrolytic enzymes.[207] To investigate the change in cell wall morphology during Cu(bpy)-AHP pretreatment and the effect of lignin removal on

cell wall structure, the structure modification of hybrid poplar (mixture of heartwood and sapwood) cell wall by pretreatment was studied using transmission electron microscopy (TEM) combined with X-ray energy dispersion spectroscopy (EDS) and electron energy loss spectroscopy (EELS). The protocol used for hybrid poplar pretreatment is identical to that used for composition analysis study discussed in Section 5.1. Cell wall samples of untreated hybrid poplar and hybrid poplar treated with AHP and Cu(bpy)-AHP for 24 hours was air dried and fixed in phosphate buffer containing 2.5% (w/w) glutaraldehyde and 2.5% (w/w) paraformaldehyde. The fixed cell wall samples were embedded in Spurr epoxy resin and sectioned by ultramicrotome. Thin sections are placed on 150 mesh gold grids with Formvar/carbon support film (Electron Microscopy Sciences, PA) and stained in 1% $KMnO_4$ solution for 60 seconds. The excess stain was rinsed off with deionized water after staining. Bright field TEM micrographs and EELS spectra were acquired with a JEOL (Peabody, MA) 2200FS 200kV field emission TEM with Gatan (Warrendale, PA) digital multi-scan camera. EDX spectra were acquired using an Oxford INCA system (Oxford Instruments, MA) coupled with the TEM.

Woody biomass mainly originates from the xylem of the stem which consists of tracheids and vessel elements. The cell walls in these structures consist of the middle lamella, the primary cell wall, and the lignified secondary cell wall that is particularly resistant to enzymatic digestion.[211-213] These multiple layers of the cell wall are shown in the TEM micrographs (Figure 21). The "dotted" line on the edge of the cell wall (pointed by arrows in Figure 21A) indicates the presence of the warty layer which is adjacent to the cell lumen.[214] The dark black stripes in the micrographs (Figures 21B and 21C) are artifacts introduced during ultramicrotome sectioning and are not native in cell wall.[215] After uncatalyzed AHP pretreatment, the structure of the cell wall remains similar to the untreated cell wall. The only notable change is some fissures formed between the middle lamella and the primary cell wall, possibly due to removal of lignin during pretreatment. The majority of the cell wall structures, however, remain unchanged after uncatalyzed AHP (Figure 22), and the improvement in enzymatic digestibility is accordingly small.

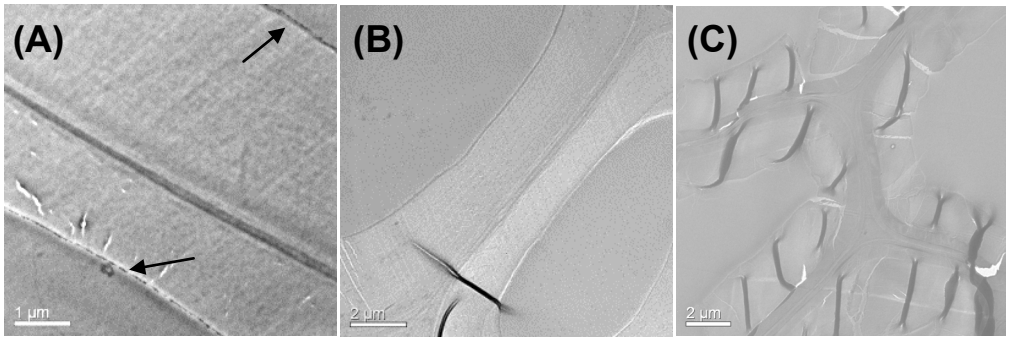

Figure 21. TEM images of untreated hybrid poplar cell wall.

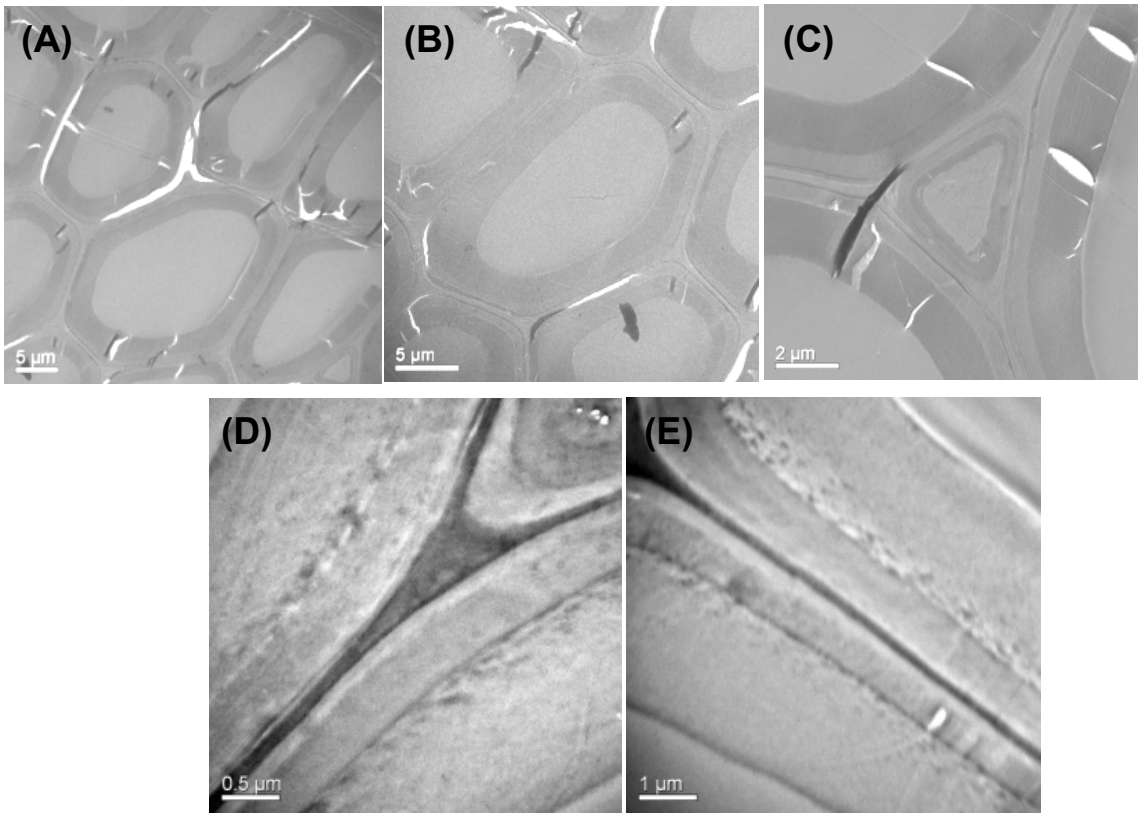

Figure 22. TEM images of hybrid poplar cell wall after uncatalyzed AHP pretreatment.

Cu(bpy)-AHP pretreated poplar has significantly lower lignin content and higher enzymatic digestibility than untreated poplar. Importantly, the structural changes in the cell wall are substantial (Figure 23). One obvious change is the delamination of the cell wall (Figure 23B) and the formation of fractures where the secondary cell wall (S1 and S2) are disintegrated (Figure 23A and 23C). Fractures and disruptions are also observed in other lignin-rich structures including cell corners (CC, Figure 23D) and compound middle lamella (CML, Figure 23F), suggesting that the structural changes may be caused by

lignin modification and removal. In addition, we observed many small aggregates with a diameter of approximately 20 to 100 nm scattered near the edges of modified regions of cell walls (Figures 23C, 23D and 23F). These aggregates are not found in untreated hybrid poplar or AHP pretreated poplar, and they are therefore very likely associated with the copper-catalyzed pretreatment.

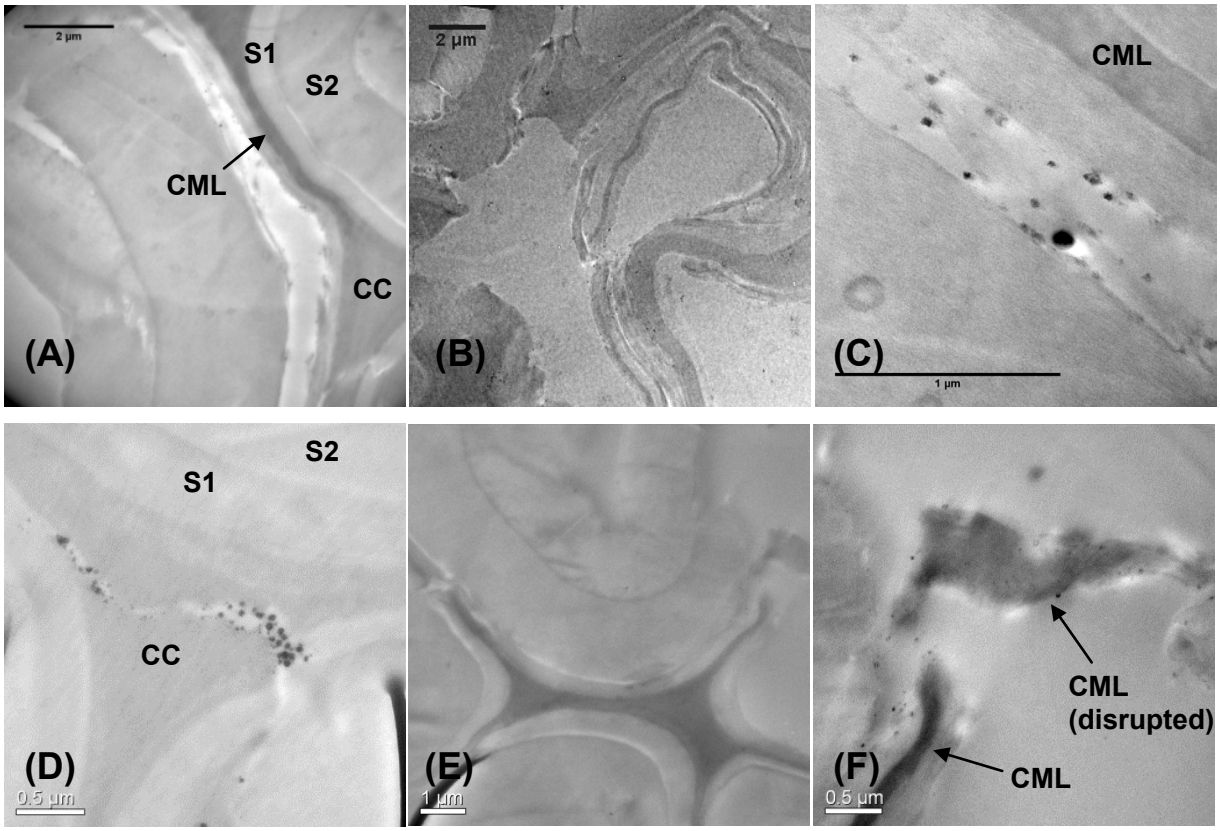

Figure 23. TEM images of hybrid poplar cell wall after Cu(bpy)-AHP pretreatment.

Energy-dispersive X-ray spectroscopy (EDS) is a powerful technique for elemental profiling as a stand-alone method or as an *in situ* analysis in combination with microscopy (SEM, ESEM, and TEM).[216] To characterize the elemental composition of the aggregates observed in the TEM images, EDS spectra were acquired at different locations in a TEM sample (Figure 24E and 24F) including at a cell corner, inside a secondary cell wall, and at the previously described aggregates. A comparison of the spectra from these locations reveal both similarities and differences in elemental composition (Figures 24A, 24B, 24C and 24D, corresponding to area A, B, C and D in Figure 24E). The Mn peaks in all four spectra results from the $KMnO_4$ staining, and the gold peaks correspond to the X-ray emissions from the gold grid that

supports the TEM sample. The EDS spectrum of the cell corner (Figure 24A) has a strong Ca L-edge peak indicating the presence of calcium ions, which are known to complex with pectin. Ca K-edge peaks (3.7 keV) are also present in the other cell wall areas. For area C and D where clusters of aggregates are analyzed, the EDS spectra feature characteristic peaks of Cu. In comparison, the Cu L-edge and K-edge peaks are not seen in the EDS spectra of either the contact cell corner (Figure 24A) or the secondary cell wall (Figure 24B). This spatial difference in Cu abundance suggests that the Cu catalyst accumulates at specific locations in the cell wall matrix where significant structural changes occur. Whether the penetration of copper into the cell wall matrix and the subsequent formation of Cu-containing aggregates occur during pretreatment or TEM sample preparation, however, is still unknown. One compelling interpretation of the spatial correlation between the Cu-containing aggregates and cell wall modification is that the Cu catalyst diffuses into the porous plant cell wall during pretreatment and accelerates the formation of localized oxidative radicals [e.g. hydroxyl radicals (•OH) and superoxide radicals ($\bullet O_2^-$)]. These radicals would then induce oxidative delignification and structural modification of the cell wall in the vicinity of the Cu catalyst.

To identify the nature of the Cu-containing aggregates, electron energy loss spectroscopy was employed to characterize the valence state of the Cu. Figure 25 shows the EELS spectrum of an aggregate with the pre-edge background subtracted. The sharp peak at the onset of the Cu $L_{2,3}$ edge indicates that the 3*d* orbital of Cu is oxidized,[217] and the relatively low intensity of this peak implies the presence of reduced Cu^I due to the involvement of copper in the redox reactions. It should be noted that Cu^I could also be formed when the Cu^{II} in the sample is reduced by the incident electrons in the TEM. High resolution TEM images at high magnification show that the aggregates are ~60 nm in diameter, and consist of crystalline nanoclusters ~2 nm in diameter. These nanoclusters are only present in biomass after catalytic pretreatment, and they are formed possibly during the pretreatment or the TEM sample preparation process.

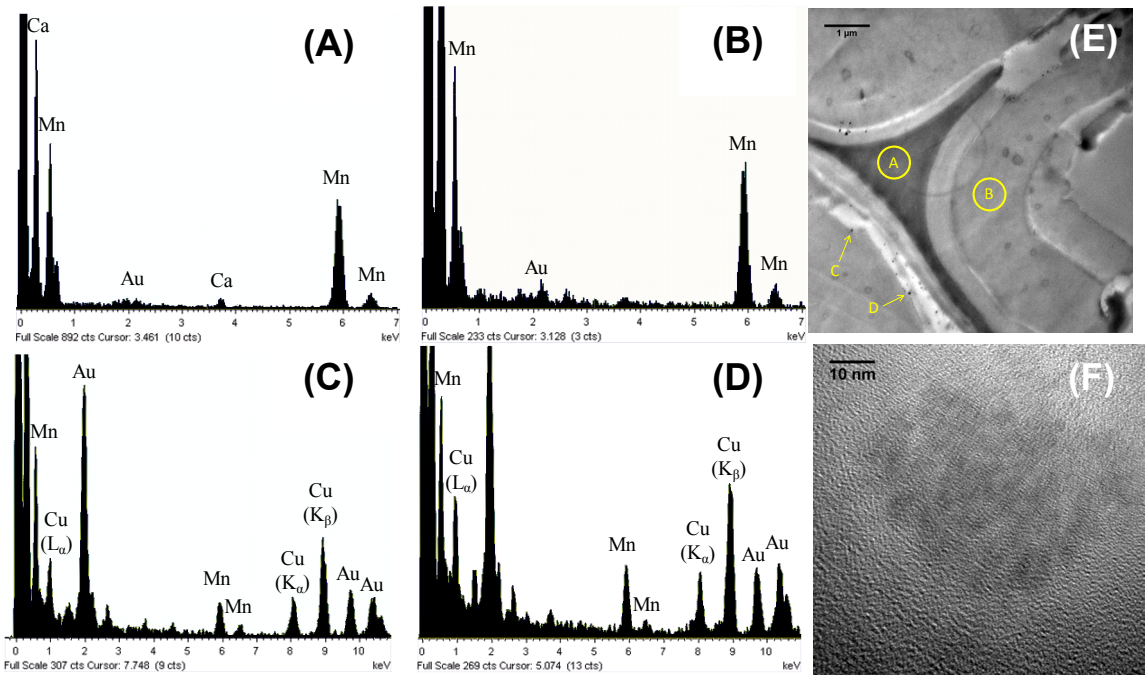

Figure 24. TEM images and X-ray EDS spectra of hybrid poplar cell wall.
(**F**) is an image of area D (containing an aggregate) in image (**E**) at high magnification.

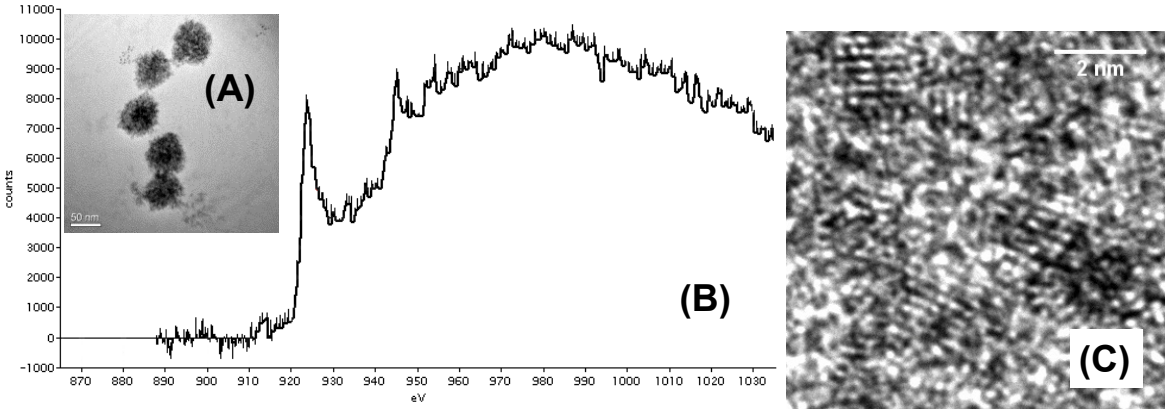

Figure 25. TEM images and EELS spectra of the aggregates.
(**A**) TEM image showing the nanoscale structure of the aggregates, (**B**) EELS spectrum of the aggregates, showing the Cu $L_{2,3}$ edge, and (**C**) TEM image of the aggregates showing the lattice fringes are shown.

5.3 Oxidative Fragmentation of Plant Cell Wall Constituents

About 44% of the lignin in hybrid poplar is dissolved during Cu(bpy)-AHP pretreatment, as evidenced by the mass loss during pretreatment as well as the change in Klason lignin content. One of the possible explanations for this removal is lignin oxidation/modification during the pretreatment process. To verify this hypothesis, the material dissolved during pretreatment was analyzed by size exclusion

chromatography. To obtain the solublilized biomass samples, hybrid poplar (0.5 gram) was pretreated in 5 mL aliquot of 0.01 g/L hydrogen peroxide at pH 11.5 and 30 °C. During Cu(bpy)-AHP pretreatment, 5 mM copper sulfate and 25 mM 2,2'-bipyridine was included in the 5 mL aliquot. Liquid samples from hybrid poplar after AHP and Cu(bpy)-AHP pretreatment were filtered through a 0.22 μm mixed cellulose ester membrane filter (EMD Millipore, MA) and analyzed using size exclusion chromatography (SEC) coupled to UV-Vis spectroscopy. Aromatic compounds in the samples were detected at 310 nm. A Waters (Milford, MA) Ultrahydrogel 250 column was used for SEC analysis following procedures described by Stoklosa and Hodge.[218] Aqueous solutions of sodium polystyrene sulfonate (Sigma-Aldrich, MO) with known number average molecular weight (4300, 6800, 10000, 32000) were used as calibration standards.

The elution profiles of the dissolved plant cell material differ by pretreatments conditions and pretreatment time (Figure 26). Such differences are possibly caused by multiple types of lignin modification occurring during pretreatment. The continuous change in the chromatogram during the uncatalyzed AHP pretreatment suggests that under these conditions the pretreatment progresses throughout the first 24 hours. In contrast, the high UV absorbance of biomass material solubilized after only 1 hour of Cu(bpy)-AHP pretreatment indicates significant lignin modification and solubilization during the initial hour of Cu(bpy)-AHP pretreatment, which is presumably the cause of the rapid increase in biomass digestibility within the same time frame. The difference in lignin modification kinetics between AHP and Cu(bpy)-AHP pretreatment implies that the two processes proceed via distinct mechanisms. Because these differences are potentially associated with the variance in enzymatic digestibility, it is compelling to obtain a better understanding of the lignin modifications that occur during Cu(bpy)-AHP pretreatment of hybrid poplar.

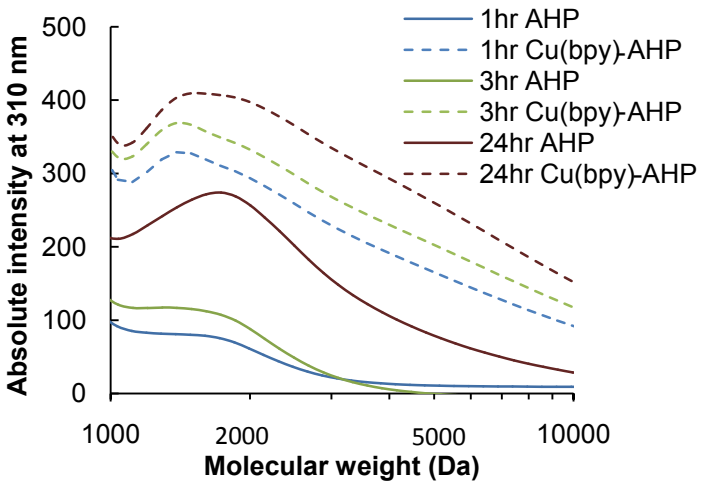

Figure 26. SEC chromatogram of plant cell wall dissolved during Cu(bpy)-AHP pretreatment.

To investigate the nature of the lignin modifications, the lignin that dissolved after 1 hour of catalytic pretreatment was recovered and analyzed with ^{1}H-^{13}C 2D HSQC NMR spectroscopy (HSQC is the abbreviation for Heteronuclear Single Quantum Coherence experiment). To prepare the lignin sample for NMR analyses, hybrid poplar (10 grams) was pretreated in 100 mL aliquot of 0.01 g/L hydrogen peroxide with 5 mM copper sulfate and 25 mM 2,2'-bipyridine at pH 11.5 and 30 °C for 1 hour. Following the pretreatment, the aqueous phase was separated from the solid phase (i.e. the insoluble portion of pretreated poplar) via filtration and the filtrate was acidified to pH 2.0 with sulfuric acid. The precipitate from the acidified filtrate was recovered via centrifugation and washed with a large volume of aqueous sulfuric acid (pH 2.0). The washed precipitate (denoted as "Cu(bpy)-APL") was collected by centrifugal solid-liquid separation and lyophilized. The 2D HSQC NMR spectra of three types of samples (untreated hybrid poplar, Cu(bpy)-APL and the insoluble portion of pretreated poplar) were acquired and analyzed as previously described by Kim et al.[219]

The crosspeaks in the two-dimensional spectra (Figure 27) represent covalently bonded hydrogen and carbon atoms, and the location of the crosspeaks on the spectra represents specific chemical shifts. Anomeric carbon atoms (δ_C=90~105 ppm) are characteristic of carbohydrates and are non-existent in lignin. The low abundance of anomeric carbon in Cu-APL indicates that Cu-APL is mostly pure lignin containing little polysaccharides. Significantly, the NMR spectra provide evidence for lignin oxidation.

Cu-APL contains guaiacyl carbon adjacent to a carbonyl group (guaiacone, guaiacyl aldehyde and vanillate), while such structures are not observed in the lignin from untreated hybrid poplar or the insoluble portion of pretreated poplar. The abundance of syringyl units containing a Cα carbonyl is also higher in Cu-APL compared with the insoluble lignin in biomass. Although the arene ring is inactivated toward oxidation due to carbonyl conjugation,[220] the aryl α-carbonyl structure is susceptible to alkaline depolymerization, e.g. attack of OH^- on Cα followed by cleavage of the Cα-Cβ bond on the propyl side chain of lignin.[221-223]

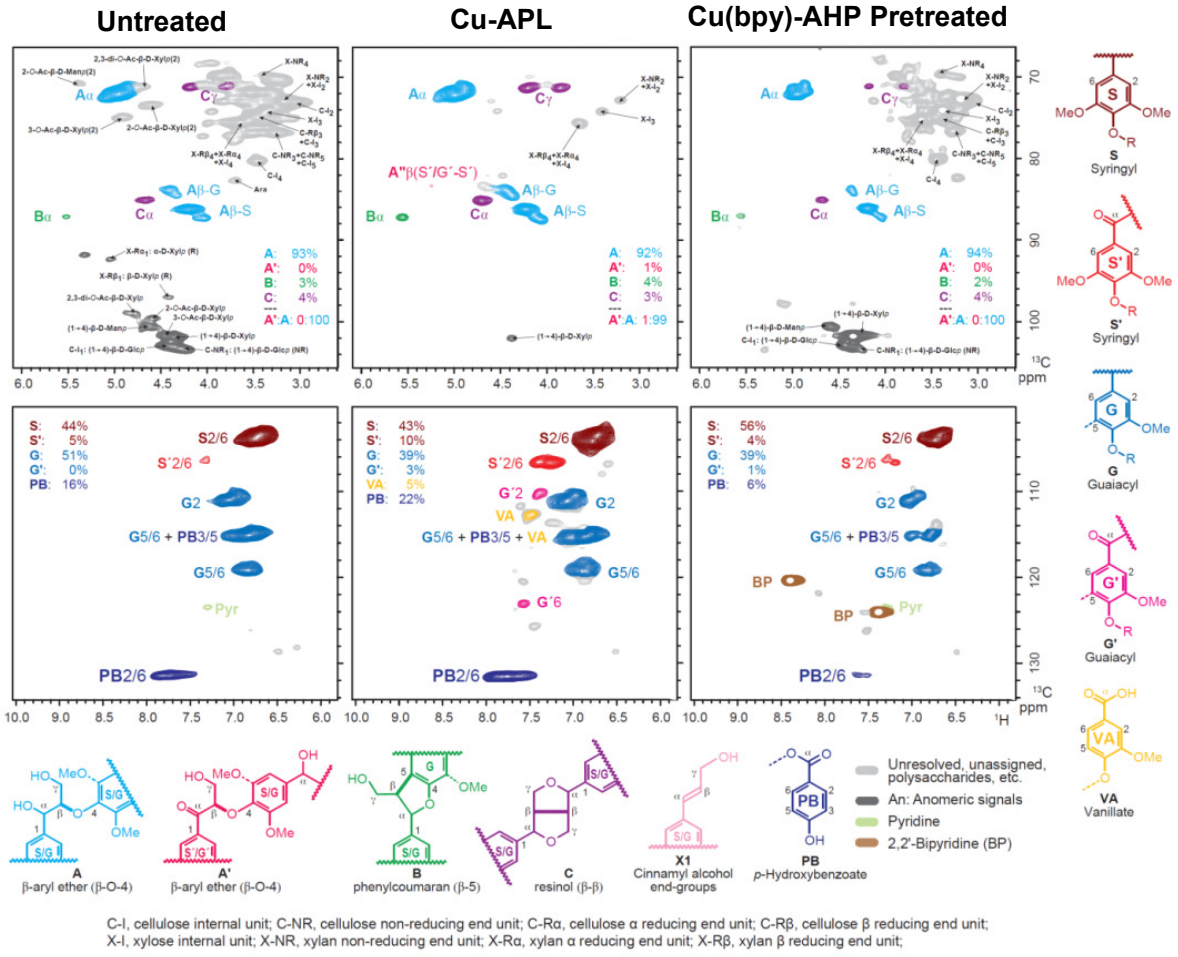

Figure 27. Partial HSQC NMR spectra of untreated poplar, Cu-APL and Cu(bpy)-AHP pretreated poplar.

Surprisingly, despite the importance of lignin oxidation during Cu(bpy)-AHP pretreatment to the subsequent lignin solubilization, it is interesting to note that the proportion of oxidized lignin structural

units observed in Cu-APL is relatively low. In fact, only 19% of the syringyl units and 7% of the guaiacyl units in Cu-APL are oxidized, suggesting that the change of lignin solubility under alkaline pH is the result of very limited lignin oxidization. In addition, lignin depolymerization is not extensive during 1 hour Cu(bpy)-AHP pretreatment, as the β-O-4, β-5 and β-β linkages are still present in the dissolved lignin. Therefore, with a composition and structure resembling native biomass lignin, Cu-APL may be a promising source of sulfur-free lignin for producing value-added products. By controlling the pretreatment time and the oxidation stoichiometry, the molecular weight and chemical properties of Cu-APL could possibly be fine tuned and customized for production of various types of functional materials and fine chemicals.[221]

CHAPTER 6

CONCLUDING REMARKS

At moderate chemical loadings under mild conditions, Cu(bpy)-AHP pretreatment of hybrid poplar has been shown to substantially improve the biomass hydrolysis yields relative to uncatalyzed AHP pretreatment. Following Cu(bpy)-AHP pretreatment and enzymatic hydrolysis, over 80% of the cell wall polysaccharides in hybrid poplar can be recovered as monomeric sugars. In fact, Cu(bpy)-AHP pretreatment is one of the most effective methods for preparing recalcitrant hybrid poplar for enzymatic hydrolysis. The Cu catalyst is highly active, and only 9 μmol of catalyst is needed for effective pretreatment of 1 gram hybrid poplar. After the pretreatment, the Cu catalyst can be easily removed from the biomass and reused in the next batch pretreatment. As the result of efficient Cu removal and recovery, the hydrolysate of Cu(bpy)-AHP pretreated hybrid poplar can be fermented to ethanol at high metabolic yield, and the hydrolysate does not need to be detoxified prior to fermentation.

Catalytic oxidation results in removal of lignin from biomass and potentially increases the accessibility of cell wall carbohydrates to enzymatic hydrolysis. As revealed by electron microscopy, Cu(bpy)-AHP pretreatment introduces biomass deconstruction which can possibly be associated with localized copper catalysis, lignin oxidation, and lignin solubilization. The adsorption of the Cu catalyst on biomass is possibly influenced by pH-dependent cell wall ionization. At higher pH, more Cu ions are adsorbed to the cell wall as the result of electrostatic interactions. The catalysis of hydrogen peroxide oxidation at close vicinity of lignocellulosic biomass determined the spatial selectivity of the oxidation and potentially improved the atomic efficiency of the hydrogen peroxide oxidant. Although the active catalytic complexes are not yet identified, it is possible that the oxidation reactions are accelerated by several copper complexes that catalyze the decomposition of hydrogen peroxide, activate hydrogen peroxide via formation of copper-peroxide complex, and activates oxygen via formation of adducts and radicals. Catalytic and non-catalytic oxidation induces lignin solubilization, and is possibly the cause of the plant cell wall disruption.

The presence of Cα carbonyl structure and the fragmentation of lignin are observed after the oxidation of lignin by brown rot *P. placenta* and white rot *P.chrysosporium*.[224,225] Monomeric and oligomeric fragments with aryl-aldehyde and aryl-acid structure have been indentified in the products of catalytic *in vitro* oxidation of lignin[221] and lignosulfonate.[226,227] Cu(bpy)-AHP pretreatment is yet another oxidation process that results in oxidative modification of lignin. As revealed by NMR spectroscopic characterization, Cu(bpy)-AHP pretreatment oxidizes lignin and introduces Cα carbonyl structures which are conjugated to the aromatic nuclei of lignin. Subsequent to Cα oxidation, hydrolytic cleavage of the oxidized lignin propyl side chain induces lignin fragmentation and increases lignin hydrophilicity. This oxidative change in lignin is possibly the cause of lignin solubilization and cell wall disruption during Cu(bpy)-AHP pretreatment.

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