ABSTRACT

Effect of Varying Feedstock-Pretreatment Chemistry Combinations on the Production of Potentially Inhibitory Degradation Products in Biomass Hydrolysates

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A variety of inhibitory degradation products are produced during pretreatment of lignocellulosic biomass. Production and release of these degradation products is highly affected by the pH and redox potential of pretreatment reactions. Qualitative and quantitative interrogation of hydrolysates is paramount to identifying potential correlations between pretreatment chemistries and microbial inhibition in downstream bioconversion processes. In the present study, corn stover, poplar, and pine wood were pretreated under eight different chemical conditions, which are representative of leading pretreatment processes that have been investigated in recent years. Pretreatment processes included: 0.7% H_2SO_4, 0.07% H_2SO_4, liquid hot water, wet oxidation, neutral buffer solution, aqueous ammonia, lime, and oxidative lime. Forty lignocellulosic degradation products resulting from pretreatment were analyzed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and ion chromatography (IC) in order to determine correlations between concentrations of inhibitory degradation products and pretreatment chemistry.

Effect of Varying Feedstock-Pretreatment Chemistry Combinations on the Production of Potentially Inhibitory Degradation Products in Biomass Hydrolysates

by

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CHAPTER ONE

Introduction

Energy Consumption Review

Since global industrialization in the middle of the nineteenth century, economic development and expansion has required a greater amount of energy and additional energy resources to meet rapidly increasing demand. Fossil fuels, including coal, petroleum, and natural gas, gradually replaced wood as a primary energy source due to their tremendous reserve. For a long period, fossil fuels served as the preeminent energy source through the late twentieth century, in which the population experienced an extreme expansion, and concurrently, usage and energy demands increased for industrial and transportation needs. As a result, there is an emerging need to obtain renewable energy resources. Accordingly, energy supply and demand is playing an increasingly significant role in national security and economic spending (Annual energy overview 2007, XIX). Geologists understand that oil is a finite resource in the earth's crust, and at some point in the future, world oil production will reach a peak, after which production will decline (Hirsch et al 2005). World oil demand is expected to increase by 50 percent by 2025 (U.S. Department of Energy 2004).

To meet that demand, ever-larger volumes of oil will have to be produced. The debate over peaking of oil resources is generally categorized in terms of pessimists and optimists (Greene et al. 2006, 515). Pessimists, who estimate that oil peaking is

imminent, such as Campbell and Laherrere (Campbell and Laherrere 1998, 78), used several different techniques to estimate the current known crude oil reserves and the reserves that remain undiscovered. These two studies concluded that the decline in worldwide crude oil production will begin before 2010. They also predicted that annual global oil production would decrease from the current 25 billion barrels to approximately 5 billion barrels in 2050.

On the other hand, optimists believe technological innovations and markets will make the limitation of oil resources irrelevant. The viewpoint of Greene et al. shows that it is neither so distant in time nor so gradual that negative impacts can be neglected. Understanding oil peaking and the consequent transition to alternative energy sources is a significant priority to guarantee a sufficient supply for continuous energy consumption (Greene et al. 2006, 515). However, no one is capable of correctly predicting when the fossil energy will be depleted and there is no certainty that alternative energy sources will successfully take the place of fossil energy in the near future without increasing effort towards the development of alternative fuel.

As the global need for energy grows, diverse renewable energy sources are under development to reduce dependence on fossil energy. Common renewable energy sources include wind, hydropower, solar energy, geothermal energy, hydrogen and biomass. Wind energy currently generates a large percentage of US national electricity. With a current annual growth rate of 27%, the US wind energy capacity increased from 2,500 megawatts (MW) in 1996 to more than 11,500 MW at the end of 2006 (Biomass program, Energy Efficiency and Renewable Energy). In addition

to its current use in lighting and heating, clean solar energy can also be applied to generate electricity by concentrating solar energy through photovoltaics. Geothermal energy, a vast and underused heat and power resource that is clean and reliable, is widespread in the U.S. This energy is used to heat homes and offices, commercial greenhouses, fish farms, and food processing facilities. Hydrogen fuel cells are an important technology and have the potential to offer cleaner, more-efficient alternatives to the combustion of gasoline and other fossil fuels. Fuel cells have the potential to replace the internal combustion engine in vehicles and provide power in stationary and portable applications (Biomass program, Energy Efficiency and Renewable Energy). Currently, total alternative energy resources are still less than 10 quadrillion Btu, which is about one tenth of fossil fuels consumption (Annual energy overview 2007, XX).

Oil is still the major source used to fuel the great variety of the world's automobiles, trucks, airplanes, trains, ships, farm equipment, and so on (Annual energy overview 2007). The total transportation energy consumption increased up to about 30 quadrillion Btu in 2007 from about 8 quadrillion Btu in 1950, with petroleum accounting for 95% of transportation energy (Annual energy overview 2007, XXI). Currently, no renewable energy could effectively and practically replace fossil fuels as the primary source for transportation fuel consumption on a cost-competitive scale. However, significant progress is being made through application of hydrogen fuel cells, which is still suffering from engine technologies on a per-mile basis such that full application of fuel cells to aircrafts or vehicles cannot

be realized in the near future (Biomass program, Energy Efficiency and Renewable Energy).

Biomass refers to living and recently living biological material. This material can be used as fuel or for other industrial products. Most commonly, biomass refers to plant matter grown for use as biofuel, but also includes plant or animal matter used for production of fibers, chemicals or heat. Use of liquid transportation fuels such as ethanol and biodiesel, currently derived primarily from agricultural crops, is increasing dramatically (Biomass program, Energy Efficiency and Renewable Energy). In 2003 ethanol production was 2.81 billion gallons per year. As of May 2006, production increased to 4.8 billion gallons per year. Use of biofuels reduces toxic air emissions, greenhouse gas buildup, and dependence on imported oil, while supporting agricultural and rural economies (Biomass program, Energy Efficiency and Renewable Energy). In this study, we focus on evaluating technologies for ethanol production from biomass resources.

Ethanol Production from Sugarcane and Corn

Ethanol can be produced from biomass and used either as an alternative fuel or as an octane-boosting, pollution-reducing additive to gasoline (Wyman 1996, 1). Current bioethanol research is driven by the need to reduce the cost of production. In the history of ethanol production, technologies have been developed to minimize the capital cost and to determine the optimal feedstock: the one with the most sugar.

The least complicated approach to producing fuel ethanol is to use biomass containing sucrose, which can be fermented directly to ethanol. Sugarcane and sugar beets are examples of biomass that contain substantial amounts of sucrose. In the mid-1980s, Brazil was the only country mass-producing ethanol from sugarcane for motor vehicles (Rosillo-Calle and Cortez 1998, 115). Techniques to improve ethanol production from sugarcane are still being developed. In 2006, Dawson et al. demonstrated that the post-harvest sugarcane residue could be used to produce fuel grade ethanol. A chemical pretreatment process using alkaline peroxide or acid hydrolysis was applied to remove lignin, which acts as a physical barrier to cellulolytic enzymes (Dawson and Boopathy 2007, 1695).

Sugarcane, the best sugar-rich feedstock for bioethanol production, is not feasible in countries other than Brazil. In the United States, bioethanol is mostly derived from the starch contained in corn. Starch, a biopolymer of glucose, consists of glucose molecules cross-linked by α-glycosidic linkages. In the process of starch fermentation, monosaccharides are usually released from biopolymers by chemical or physical pretreatments before they are fermented into ethanol (Energy Efficiency and Renewable Energy, Department of Energy; Lin and Tanaka 2006, 627).

On an industrial scale, high-temperature hydrolysis (140–180 ˚C) is an effective approach for breaking down starchy materials and increasing starch fermentation efficiency (Matsumoto et al. 1985, 291). However, capital costs on ethanol production remain high due to the high-energy consumption in pretreatment process and the requirement for large amounts of enzymes. In addition, much greater

production of ethanol from corn starch may conflict with food production needs and even lead to frequent fluctuations of feedstock prices not only in the US, but also globally.

Lignocellulosic Biomass

Without affecting the food market or any other domestic services, less expensive feedstocks and more advanced conversion technologies need to be investigated. Due to the poor geographical conditions for sugarcane growth and shortage of corn in terms of intensive competition with food consumption, the US and other countries are trying to utilize a larger amount of lignocellulosic materials, a cheaper biomass (Zaldivar et al. 2001, 17). Lignocellulosic materials include wheat straw, corn stover, hardwoods, softwoods, waste papers, and municipal solid waste. The preceding list represents the most abundant global sources of biomass, which have been widely unutilized (Polman 1994, 709). Lignocellulosic biomass is a complex material made up of three major organic fractions with representative compositions on a dry-weight basis as follows: 35%–50% cellulose, 20%–35% hemicellulose, and 12%–20% lignin (Wyman 1996, 424).

Cellulose, a structural compound composed exclusively of glucose linked via β-(1, 4)-glycosidic bonds, is highly crystalline and compact making it very resistant to biological degredation (Gray et al. 2006, 141; Wyman 2003, 254). Hemicellulose is a highly-branched chain of xylose and arabinose that also contains glucose, mannose and galactose (Weislogel et al. 1996, 105). The cellulose and hemicellulose are

tightly bound to lignin primarily via hydrogen bonding (Lee 1997, 1). Hemicellulose can also be covalently linked to lignin via ferulic acid ester linkages (Gray et al. 2006, 141). Thus, the compactness and complexity of lignocellulose makes it much more difficult than starch (as discussed above) to be enzymatically degraded to fermentable sugars. Hence, the cost of producing a gallon of ethanol from lignocellulosic biomass is higher than production from starch (Wyman 2003, 254). A primary research effort must be directed to reduce the capital cost of producing ethanol from lignocellulosic biomass.

Bioconversion of Lignocellulosic Ethanol

Conversion of lignocellulosics to ethanol consists of four major steps: 1) pretreatment, 2) hydrolysis, 3) fermentation, and 4) product separation/purification (Wyman, 1999, 189). Pretreatment is required to alter the biomass size and structure as well as its submicroscopic chemical composition and structure. Cellulose and hemicellulose are liberated from their complex with lignin through delignification so that hydrolysis of the carbohydrates to monomeric sugars can be achieved more efficiently (Lee 1997, 1). Hydrolysis includes the processing steps that convert the carbohydrate polymers into monomeric sugars.

The hexoses and pentoses released during hydrolysis are ready for fermentation (Leonard and Hajny 1945, 390; Clark and Mackie, 1984, 101; Beck 1986, 617; Frazer and McCaskey 1989, 31; Du Preez 1994, 944; Olsson and Hahn-Hagerdal 1996, 312). Fermentation technology is still being optimized to convert xylose to ethanol as well

as overcome the inhibitory effects of lignocellulose degradation products to make the overall conversion more cost-effective. Finally, ethanol is recovered by distillation to remove any residual lignin, unreacted cellulose and hemicellulose, ash, enzyme, organisms, and other components (Ladisch et al. 1984, 437).

Application of Promising Pretreatments

Lynd has summarized the prerequisites for an ideal lignocellulose pretreatment; it should: (1) produce reactive fibers; (2) yield pentoses in non-degraded form; (3) not lead to the release of compounds that significantly inhibit the fermentation; (4) require little or no size reduction; (5) be able to work in reactors of reasonable size and moderate cost; (6) produce no solid residues; (7) have a high degree of simplicity, and (8) be effective at low moisture contents (Lynd 1996, 403). Although no single pretreatment configuration can meet all of these requirements at the same time, several potentially cost effective pretreatment methods have been developed. Pretreatment methods include steam explosion, liquid hot water, dilute acid, alkali (lime), and ammonia pretreatments. Differences in the methods have been reviewed by Mosier et al. and are briefly outlined in the following discussion (Mosier et al. 2005, 673).

Uncatalyzed steam explosion (Saddler et al. 1993, 73) refers to a pretreatment technique in which lignocellulosic biomass is rapidly heated by high-pressure steam without addition of any chemicals (Avellar and Glasser 1998, 205; Glasser and Wright 1998, 219). Water, itself, acts as an acid at high temperatures (Weil et al. 1997, 21).

The process results in increased water solubilization of hemicelluloses by hydrolysis of glycosidic bonds in hemicellulose and bonds between hemicellulose and lignin (Li et al. 2007, 3061). In addition, hemicellulose is also thought to be hydrolyzed by the acetic and/or other acids generated during the steam explosion process, since acetic acid is released from hydrolysis of acetyl groups associated with the hemicellulose (Weil et al. 1997, 21).

Hydrolysis by hot, compressed, liquid water (LHW) involves contacting biomass with heated water for up to 15 min at temperatures of 200–230 ˚C. Between 40% and 60% of the total biomass is dissolved in the process, with 4–22% of the cellulose, 35–60% of the lignin and all of the hemicellulose removed from the solubilized fraction (Mok and Antal 1992, 1157; Mok and Antal 1994, 1572). Water pretreatment reduces the need for neutralization and conditioning chemicals since acid is not added. Size reduction of the incoming biomass is not needed because the lignocellulose particles break down when hydrolyzed in water (Kohlmann et al. 1995, 237; Weil et al. 1997, 21). Upon addition of a specific enzyme or enzymes, a highly digestible cellulose results (van Walsum et al. 1996, 157). LHW pretreatment can also be performed in the presence of oxygen and this technique is termed wet oxidation. Wet oxidation has been proven to be an efficient pretreatment method of wood and wheat straw, dissolving the hemicellulosic fraction and making the solid cellulose fraction susceptible to enzymatic hydrolysis and fermentation (McGinnis et al. 1983, 352; Bjerre et al. 1996, 568; Schmidt and Thomsen 1998, 139; Klinke et al. 2003, 738; Martin and Thomsen 2007, 174).

The most widely used and tested approaches are based on pretreatment with dilute sulfuric acid (Nguyen, et al. 2000, 561; Kim et al. 2000, 129). However, nitric acid (Brink 1993; Brink 1994), hydrochloric acid (Goldstein and Easter 1992, 135), and phosphoric acid (Israilides et al. 1978, 43) have also been tested. Dilute sulfuric acid has been added to cellulosic materials to commercially produce furfural (Zeitsch 2000). The acid is mixed or contacted with the biomass, and the mixture is held at temperatures of 160–220 ˚C for periods ranging from seconds to minutes. Addition of sulfuric acid is initially applied to release hemicellulose from lignin either in combination with breakdown of cellulose to glucose or prior to acid hydrolysis of cellulose and to hydrolyze hemicellulose to xylose, which enhances digestibility of cellulose in the residual solids (Converse and Grethlein 1985; Grous et al. 1985, 274).

Alkali pretreatment processes commonly utilize lower temperatures and pressures than other pretreatment methods. In alkali pretreatment, lime is often employed instead of sodium hydroxide or potassium hydroxide due to its lower cost. Alkali pretreatment may also be carried out at ambient conditions, but pretreatment time is measured in terms of hours or days rather than seconds or minutes. Unlike acid-catalyzed pretreatments, a limitation occurs because some of the alkali is converted to irrecoverable salts or incorporated as salts into the biomass. The major effect of alkaline pretreatment is removal of lignin from the biomass, thus improving the reactivity of remaining polysaccharides. When oxygen is added in lime pretreatment, biomass delignification is enhanced, especially in highly lignified materials such as poplar (Chang and Holtzapple 2000, 5).

Ammonia fiber expansion (AFEX) pretreatment, recognized as one of the leading pretreatment technologies (Lynd 1996, 403), yields optimal hydrolysis rates for pretreated lignocellulosics with close to theoretical yields at low enzyme loadings (<5 FPU/g of biomass or 20 FPU/g cellulose) (Holtzapple et al. 1991, 59; Dale et al. 1996, 111). Pretreatment with aqueous ammonia in a flow-through scheme involves loading an ammonia solution (5–15%) through a column reactor packed with biomass at elevated temperatures and most commonly a fluid velocity of 1 mL $cm^{-1}min^{-1}$ with specific residence times. This method is also known as the ammonia recycled percolation (ARP) process since ammonia is separated and recycled. During ARP, a considerable fraction of hemicellulose is removed along with lignin (Kim and Lee 2005, 2007). A large adjustable degree of delignification has been reported in tests with hardwood (Yoon et al. 1995, 5) and agricultural residues (Iyer et al. 1996, 121).

Potentially Inhibitory Degradation Products for Fermentation

Various pretreatment methods have been developed to hydrolyze lignocellulosic materials efficiently for fermentation. In addition to the pretreatment approach, other factors which affect the capital cost of ethanol include: optimization of feedstocks, identification of inhibitory compounds, and optimization of enzyme or yeast. Depending on the chosen pretreatment method, undesirable compounds originate, such as lignin residues, and various organic acids and aldehydes. It is necessary to identify and minimize the presence of such compounds, because they

may have an inhibitory effect on the activities of the microorganism utilized for the fermentation process (Zaldivar et al. 2001, 17).

In different processing schemes, variable amounts of lignin are hydrolyzed and other potentially inhibitory compounds are released from the carbohydrates during pretreatment. Aliphatic acids, such as acetic acid and fumaric acid, are released when the hemicellulose structure is degraded. The inhibitory effect of an acid is pH-dependent, because it is the undissociated acid that penetrates the yeast cell membrane and then dissociates in the cytoplasm where the pH is almost neutral (Palmqvist and Hahn-Hagerdal 2000, 25). In addition, furans, such as furfural and 5-hydroxymethylfurfural (5-HMF), and secondary inhibitors from furans, levulinic acid, formic acid, and humic substances (Clark and Mackie 1984, 101) are produced as by-products in prehydrolysis and hydrolysis due to the degradation of hexoses and pentoses (Olsson and Hahn-Hagerdal 1996, 312). Another group of potential inhibitors includes a broad range of aromatic and polyaromatic compounds with a variety of substituents, generated from partial breakdown of lignin and formed during carbohydrate degradation (Maiorella et al. 1983, 103). The low molecular weight phenolic compounds are considered by Clark and Mackie to be most toxic (Clark and Mackie 1984, 101; Buchert et al. 1989, 309) and exert a considerable inhibitory effect on the fermentation of lignocellulosic hydrolysates (Palmqvist and Hahn-Hagerdal 2000, 25). Lastly, products from the fermentation process such as ethanol, acetic acid, glycerol, and lactic acid, have an interactive inhibitory effect on the microorganism (Maiorella et al. 1983, 103; Olsson and Hahn-Hagerdal 1996, 312). Hence, a greater

understanding of the inhibitory mechanisms of individual compounds and their interactive effects, as well as the influence of parameters such as the type of chemical conditions, solid concentration of feedstocks, and kinetics, will facilitate progress in the development of more efficient fermentation processes.

Identification of Components in Hydrolysates

It is obvious from the above discussion that by-products produced during pretreatment have an inhibitory effect on microorganisms and compromise their enzymatic activities. The composition of inhibitory compounds depends upon the type of lignocellulosic material and the chemistry and nature of the pretreatment process (Martinez et al. 2001, 287). A great amount of effort and progress has been made on analysis of biomass hydrolysates since the first investigation of pretreatment-induced degradation products in 1945 (Leonard and Hajny 1945, 390).

Early in 1984, Bonn and Bobleter (Bonn and Bobleter 1984, 445) conducted HPLC analysis on plant biomass hydrolysates pretreated under hydrothermal conditions. The analytical separation of monomeric sugars, glucose and xylose, and their degradation and fermentation products, such as cellobiose, fructose, ethanol, hydroxymethylfurfural, and furfural was reported in this study. Burtscher et al. first reported an attempt at comprehensive analysis of the product distribution of sugars, lignin degradation products, and sugar decomposition products generated upon hydrothermal treatment of poplar wood at temperatures ranging from 160-300 ˚C in 1987 (Burtscher et al. 1987, 401). The major hydrolysate components were

analyzed as a function of reaction temperature, flow rate, and dimensions of the reaction vessel. Monosaccharides and 21 sugar degradation products, such as furfural, 5-hydroxymethylfurfural, phenol carbonic acids, phenol, aromatic aldehydes, and ketones, were analyzed by reversed-phase HPLC. Marko-Vargo and co-workers reported a similar analytical scheme for the analysis of carbohydrates in sulfite-spent liquor and an enzymatic hydrolysate in 1994 (Marko-Varga et al. 1994, 317). In this study, monomeric sugars, such as glucose, xylose, galactose, arabinose, and mannose were analyzed using both ligand-exchange chromatography and ion-exchange chromatography.

Several researchers have focused on the analysis of degradation products in dilute-acid hydrolysates. In 1998, Fenske et al. tentatively identified and quantified 14 compounds with structures indicative of lignin monomers or esterified phenolics by gas chromatography-mass spectrometry. The dilute-acid hydrolysates were prepared from three feedstocks, including two herbaceous feedstocks, corn stover and switchgrass, and one hardwood feedstock (poplar), in a 0.6-L stainless steel Parr reactor at 10% solids under the condition of 1.0% (w/w) dilute acid at a temperature of 180 ˚C for 1 min (Fenske et al. 1998, 364).

Larsson et al. (Larsson et al. 1999, 151) studied spruce, a softwood feedstock. Dry spruce chips, less than 30 mm in size, were mixed with sulfuric acid to final concentrations of 0.5%, 2.4%, or 4.4% (w/w) and incubated at room temperature overnight (Tengborg et al. 1998, 3). Treatment with saturated steam at 150, 180, 200, 210, 225, or 240 ˚C continued for 1, 5, 10, 15, 20 or 30 min. Sugars, such as

cellobiose, xylose, galactose, arabinose, and mannose, and inhibitory compounds, such as furfural and 5-HMF, were analyzed by HPLC with a refractive index detector. Formic, acetic, and levulinic acids were determined by capillary electrophoresis using a fused silica capillary. In 2002, Brink and co-workers (Luo et al. 2002, 125), who focused on a total analysis of major and minor hydrolysate components, successfully identified more than 35 potential fermentation inhibitors in hydrolysates generated using nitric acid pretreatment. Hybrid poplar wood chips were fed into the reactor and the hydrolysis reaction was carried out at a steady state condition by dilute nitric acid (0.25 g/L) at a temperature of 170.7 ˚C for 25.3 min and a pH of 1.45.

Pan et al. (Pan et al. 2006, 851) conducted an experiment to convert hybrid poplar to ethanol using an organosolv fractionation process. The organosolv process involves extraction with hot aqueous ethanol (180 ˚C, 60 min, 1.25% H_2SO_4, and 60% ethanol) and resulted in fractionation of poplar chips into a cellulose-rich solids fraction, an ethanol organosolv lignin fraction, and a water-soluble fraction containing hemicellulosic sugars, sugar breakdown products, degraded lignin, and other components. Monosaccharides were determined using a DX-500 HPLC system. Furfural and HMF were determined using a Dionex Summit HPLC system.

Also in 2006, Chen et al. (Chen et al. 2006, 54) quantitatively identified 32 potential inhibitors, including organic acids, phenols and aromatic aldehydes, with the utilization of an HPLC system with a UVD 170U multi-wavelength ultraviolet detector. In this experiment, corn stover was pretreated with 1% (v/v) sulfuric acid by preheating for 3 min at 200 ˚C, followed by 8 min of heating at 160 ˚C.

Project Objective

The objective of this study was to characterize accumulation trends of potential inhibitors during biomass pretreatment by mapping out degradation product concentrations with different feedstock-pretreatment chemistry combinations. Previously, bioethanol researchers either looked for methods to identify the concentrations of hydrolysate components of multi-feedstocks in one of the leading pretreatment processes mentioned above, or sought to determine the effects of different pretreatment chemistries on the composition of hydrolysates for a specific feedstock. Using previously successful pretreatments, we have monitored concentrations of 40 lignocellulosic degradation products for a combination of 8 pretreatment processes and 3 lignocellulosic feedstocks: corn stover, poplar, and pine, which are considered the leading representative sources from agricultural wastes, hardwoods, and softwoods, respectively. We performed eight pretreatment schemes similar to maturely developed pretreatment processes. Conditions were tested from 0.7% H_2SO_4 to lime; two of which were also tested in the presence of high pressure oxygen. Forty target compounds were determined simultaneously with optimal resolution using HPLC-PDA-MS/MS and IC. Monitored compounds are categorized into three groups based on their structure: aliphatic acids, aromatic acids, and aldehydes and ketones collectively. The availability of analytical information on this wide range of potentially inhibitory degradation products is expected to promote a more predictive understanding of pretreatment effects on the downstream enzymatic hydrolysis and fermentation in biomass-to-ethanol conversion.

CHAPTER TWO

Materials and Methods

Unless otherwise noted, all solvents and reagents were reagent grade or better, purchased from commercial sources, and used as received. Corn stover and poplar wood feedstocks were obtained from the National Renewable Energy Laboratory (NREL, Golden, CO, USA). A pine wood feedstock was provided by the Forest Bioproducts Research Initiative at the University of Maine (Orono, ME, USA).

Biomass Pretreatment Procedure

The chemical composition of the three feedstocks used in this study (corn stover, poplar, and pine) is presented in Table 2.1. Feedstock handling and biomass pretreatment were conducted prior to HPLC and IC analysis using a method similar to previously reported procedures (Yourchisin and van Walsum 2004, 1073; Chen et al. 2006, 54). First, all feedstocks were ground using a coffee grinder, sieved with a 16 mesh filter and dried at 105 ˚C for 20 hours to obtain approximately 0.8 g of material. The dry biomass was then pretreated with the following pretreatment processes: 0.7% (w/w) H_2SO_4, 0.07% (w/w) H_2SO_4, liquid hot water, wet oxidation, sodium phosphate buffer (pH 6.9), 0.1% (w/w) aqueous NH_4OH, lime, and oxidative lime. For oxidative lime and wet oxidation pretreatments, oxygen was injected at 174 psi into the pretreatment reactors prior to pretreatment. All pretreatment processes were conducted at a solids concentration of 10 g/L.

Pretreatment reaction conditions are presented in detail in Table 2.2. Briefly, mixtures were reacted in a 150-mL 316 stainless-steel pressure vessel (Swagelock, Solon, OH, USA). Desired reaction temperature was achieved by placing the reactor in a sand bath at 220 ˚C for 3 minutes. Then the reactor was immediately transferred to a separate sand bath at 180 ˚C for 8 min using a SBL-2D fluidized bath with a TC-8D temperature controller (Techne, Burlington, NJ, USA). After 8 minutes, pretreatment quenching was accomplished by immersing the reactor in an ice bath. Particulates were removed by filtration using Whatman glass-microfiber membrane filters (90 mm diameter; 0.45 μm pores; VWR Scientific, Suwanee, GA, USA). The resulting hydrolysate samples were collected as a clear filtrate and stored at 4 ˚C until processed for HPLC-PDA-MS/MS and IC-conductivity analysis.

Table 2.1. Chemical composition of corn stover, hybrid poplar, and pine.

feedstocks	chemical composition (%)					
	glucan	xylan	mannan	other polysaccharides	lignin	extractives and other minor components
corn stover	34.40	22.80	0.60	7.80	11.00	23.40
poplar	43.80	14.85	3.94	1.73	29.12	6.56
pine	40.00	8.90	16.00	3.60	27.70	3.50

Analytical Sample Preparation

Before HPLC-PDA-MS/MS and IC-Conductivity analysis, target analytes were extracted from the hydrolysate with methyl *tertiary*-butyl ether (MTBE) using a method similar to that reported by Chen et al. (Chen et al. 2006, 54). In brief, the

Table 2.2. Detailed pretreatment conditions.

pretreatment conditions	chemical environment	temperature (°C)	reaction time (min)	solids (g/L)
0.7% H_2SO_4	0.7% (w/w) aqueous sulfuric acid	180	8	10
0.07% H_2SO_4	0.07% (w/w) aqueous sulfuric acid	180	8	10
liquid hot water	deionized water	180	8	10
wet oxidation	deionized water saturated with $O_2(g)$ at 174 psi	180	8	10
buffer	pH 7 buffer solution ($H_2PO_4^-/HPO_4^{2-}$)	180	8	10
NH_3	0.1% aqueous NH_4OH	180	8	10
lime	water containing 0.1 g $Ca(OH)_2$ per g solid	180	8	10
oxidative lime	lime solution saturated with $O_2(g)$ at 174 psi	180	8	10

extraction was performed using 5-mL aliquots of acidified hydrolysate placed in contact with 45 mL of MTBE on a rotating wheel at 25 ˚C for approximately 15 minutes. After extraction, the two phases were separated with a centrifuge and the hydrolysate was re-extracted to ensure optimal recovery. The volume of both MTBE extracts was combined and reduced to 1–2 mL using a Turbovap concentrator (Zymark, Hopkinton, MA, USA). The concentrated extract was finally diluted to 5.00 mL with nano-pure water prior to analysis. It is relevant to note one significant deviation from the procedure developed by Chen and coworkers. In that work, a precipitation/filtration step was included, which resulted in the removal of a brown precipitate from the hydrolysate samples. This precipitation was required to maintain performance of the HPLC analytical column. If omitted, it was observed that column performance substantially declined over time. However, in the current work we found that the precipitation/filtration step was unnecessary for LC-MS/MS analysis as no negative effects were observed over time when precipitation/filtration was not included prior to extraction.

HPLC-PDA-MS/MS Analysis

The details of the HPLC-PDA-MS/MS system (Varian, Inc. Palo Alto, CA, USA) and its operation have been described elsewhere (Sharma et al. 2008). For HPLC-PDA-MS/MS analysis, 38 of the 40 standards (formic acid and acetic acid not included) were prepared in methanol to make a stock solution with a concentration of 500 μg/mL. Eight calibration standards over the concentration range 0.1 mg/L to 15 mg/L were made via serial dilution with distilled and deionized water (18 MΩ). Both internal standards, benzoic acid-D5 (0.02 g) and *para-tert*-butylphenoxyacetic acid (0.05 g), were dissolved in methanol to provide concentrations of 400 μg/mL and 1000 μg/mL, respectively.

Concentrations of the 38 analytes in hydrolysates were determined using external calibration. Briefly, calibration plots for each analyte were constructed by plotting the observed response factor (area of analyte peak divided by area of internal standard peak) versus analyte concentration for each calibration standard. These data were fit to a straight line using a linear least squares regression, and concentrations of target analytes in undiluted, pretreated hydrolysates were directly determined from the resulting calibration curves by inserting the response factor from the hydrolysate sample into the calibration-curve equation. If the concentration of an analyte exceeded the upper calibration limit, which typically ranges from 15 to 20 mg/L, extracts were diluted either 10 or 100 fold prior to extraction to obtain a resulting instrumental response that was in the linear range of the constructed calibration curves. For every hydrolysate analyzed, a replicate sample spiked with 5

mg/L of each target analyte (50µL of a 500 mg/L stock solution) was also extracted and analyzed. Quantitative data for the spiked extract was used to determine analyte recoveries as reported previously (Sharma et al. 2008). Where multiple dilutions (i.e., undiluted, 1:10, 1:100) returned concentrations that were within the linear range of the calibration curve, the dilution demonstrating the most quantitative recovery (i.e., recovery closest to 100%) was selected to report the concentration of a given analyte in the undiluted, unspiked extract. Note that where concentrations of diluted hydrolysates were measured, the actual values were reported after correcting with the specific dilution-factor.

IC Analysis

Acetic acid and formic acid were observed to have especially poor resolution in HPLC-PDA-MS/MS analysis, making it difficult to reliably determine their concentrations. To resolve this issue, concentrations for these two analytes were determined by ion chromatography using a suppressed-conductivity detector (ED40 electrochemical detector, Dionex Corp.). These analyses were conducted by Drs. Richard Mowery and Shou-Feng Chen. A brief description of the extraction and analysis follows. A 250-µL aliquot of pretreated, filtered hydrolysate was loaded onto a preconditioned Supelclean™ LC™-18 SPE cartridge. The cartridge was rinsed with slightly less than 5 mL of deionized water and the eluate was diluted to 5.00 mL in a volumetric flask. Chromatographic separation was carried out at 30 °C using a 50 mm × 4 mm IonPac AS11-HC guard column and a 250 mm × 4 mm

IonPac AS11-HC analytical column connected in series. This procedure allows monitoring of acetic and formic acids (with propionic acid as internal standard). Observed retention times for acetic, formic, and propionic acids were 18.87 min, 23.09 min, and 26.18 min, respectively (Chen et al. 2007, 5912).

Quantitation of target analytes was accomplished using a multipoint internal standard calibration curve. A constant amount of propionic acid (10 mg/L) was added as an internal standard. Response factors were determined for each analyte by dividing the peak area of the analyte by the peak area of the internal standard, and calibration curves were constructed by plotting a linear regression of the average response factor versus analyte concentration for all calibration standards analyzed. Calibration curves were then used to determine analyte concentrations in pretreated hydrolysate samples as discussed above.

CHAPTER THREE

Results and Discussion

In this project, 40 potential degradation compounds were quantified by HPLC-PDA-MS/MS and IC-conductivity analysis. This broad range was selected owing to the previous identification of these analytes as potential inhibitors which result from sugar degradation and lignin breakdown. The compounds monitored included aliphatic acids, aromatic acids, aldehydes and ketones.

Analytes were separated and accurately quantified by HPLC-PDA-MS/MS and IC-conductivity methods as discussed in Chapter 2. Hydrolysate of corn stover, treated with 0.7% H_2SO_4, was chosen as a representative sample to demonstrate the analytical separation. Chromatograms resulting from HPLC-PDA-MS/MS analysis of a corn stover hydrolysate are shown in Figures 3.1 and 3.2. It is important to note that MS/MS data in Figure 3.1 show a total ion chromatogram (TIC) of the hydrolysate, which sums the total MS response. However, as described in the work by Sharma et al. (Sharma et al., 2008), the mass spectrometer is operated in multiple-reaction-monitoring mode (MRM mode), and chromatographic run time is divided into segments in which only select MS/MS transitions are monitored. As shown in Figure 3.1, the HPLC separation enabled MS/MS analysis of 36 analytes in 55 minutes. Note that the response of 4-hydroxycoumaric acid (peak 33) was truncated in this chromatogram so that less abundant peaks could be observed.

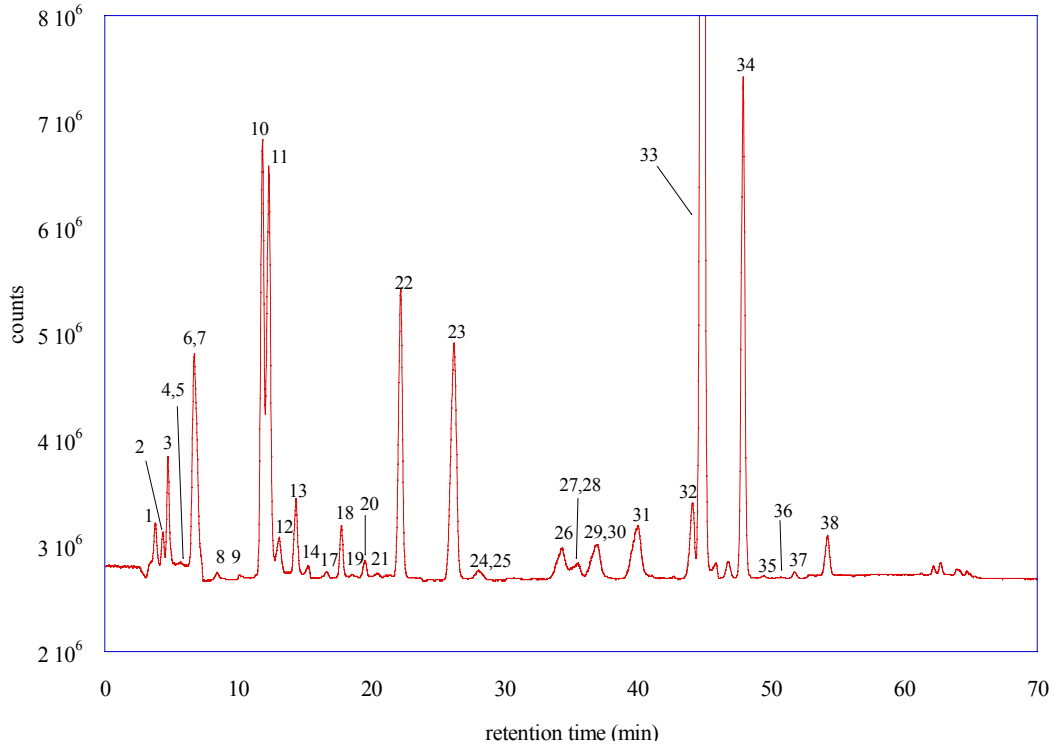

Figure 3.1. Representative mass spectrometry chromatogram of hydrolysate of corn stover treated with 0.7% (w/w) sulfuric acid. Peak identifications are as follows: (1) malonic acid; (2) maleic acid; (3) lactic acid; (4) *cis*-aconitic acid; (5) methylmalonic acid; (6) succinic acid; (7) fumaric acid; (8) trans-aconitic acid; (9) levulinic acid; (10) glutaric acid; (11) itaconic acid; (12) 2-hydroxy-2-methylbutyric acid; (13) 2-furoic acid; (14) gallic acid; (17) adipic acid; (18) 3,4-dihydroxybenzoic acid; (19) 3,5-dihydroxybenzoic acid; (20) 2,5-dihydroxybenzoic acid; (21) 3,4-dihydroxybenzaldehyde; (22) salicylic acid; (23) 4-hydroxybenzaldehyde; (24) vanillic acid; (25) homovanillic acid; (26) 4-hydroxyacetophenone; (27) caffeic acid; (28) syringic acid; (29) vanillin; 4-hydroxy-3-methoxycinnamic acid; (30) 4-hydroxybenzoic acid; (31) benzoic acid; 4-hydroxycoumarin; (32) syringaldehyde; (33) 4-hydroxycoumaric acid; (34) ferulic acid; (35) 3-hydroxy-4-methoxycinnamic acid; (36) 4-hydroxycoumarin; (37) *ortho*-toluic acid; and (38) *para*-toluic acid.

The UV chromatogram at 290 nm (Figure3.2) was generated simultaneously with the MS chromatogram to monitor 5-HMF and furfural, which have low sensitivity in MS analysis and co-elute with gallic acid and adipic acid. However, the UV responses of gallic and adipic acids are low at 290 nm (Sharma et al. 2008). Due to the higher concentration of furans produced in strongly acidic conditions, the

responses of 5-HMF and furfural in this UV chromatogram are much larger than the other compounds. Analyte peaks in both mass spectrometry and UV chromatograms were automatically integrated by software, but manual verification was performed to make sure that all peaks were fully integrated prior to calculating analyte concentrations.

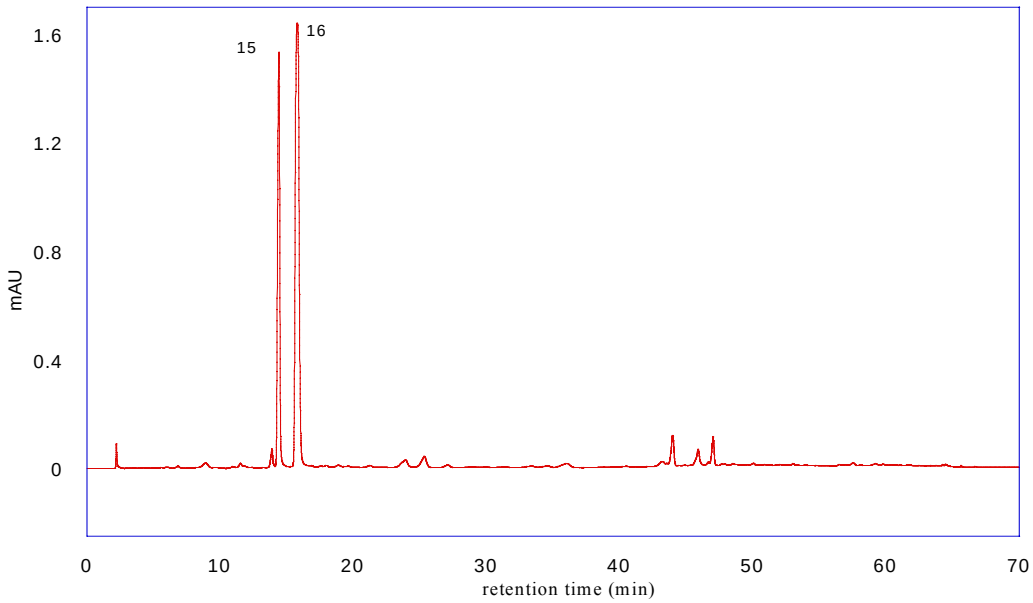

Figure 3.2. Representative UV chromatogram of the corn stover hydrolysate prepared by treatment with 0.7% (w/w) sulfuric acid. Identified peaks: (15) 5-HMF and (16) furfural.

Data Quality

In an effort to understand the variation in sample recoveries, 3 replicate corn stover hydrolysates were prepared using 0.7% H_2SO_4 according to the procedure outlined in Chapter 2. Resulting mean concentrations, mean recoveries, and the relative standard deviation (RSD) of concentrations determined in triplicate are contained in Table 3.1. Method precision was evaluated by comparing the RSDs and

recoveries across the range of analytes. Through careful inspection of the data in Table 3.1, it is observed, in general, that RSDs range from 3.7% to 29.5% and recoveries range from approximately 70% to 128%. These data suggest that determined concentrations of degradation products quantified from a pretreated feedstock are accurate to within 30%. Accordingly, only differences greater than 30% (i.e., relative differences in analyte concentrations observed for dissimilar pretreatments) were considered to be meaningful when assessing accumulation trends as a function of feedstock and/or pretreatment chemistry. It is noted that the recovery of malonic acid (57%) is an exception to the generalization; that is, malonic acid recoveries do not fall within a 30% window. It is also apparent that the RSD for triplicate determinations of malonic-acid concentration is among the highest in the data set. The observed decrease in recovery and high RSD for malonic acid may be due to the fact that it is not well-retained and elutes very close to the solvent front. Owing to poor retention characteristics, malonic acid is considered an outlier for this data (Table 3.1) and special attention is given when assessing variable trends for malonic acid.

The primary benefit of identifying only variations in concentration greater than 30% is that it allows qualitative assessment of accumulation trends without the necessity of analyzing every sample in triplicate. Although such repetition is preferable, triplicate analysis of each sample would be costly and time consuming (considering the magnitude of the data set), preventing the completion of this project within a reasonable period of time. Thus, assessment of accumulation trends in this

Table 3.1. RSDs and recoveries of analytes measured in the hydrolysates of corn stover pretreated with 0.7% H_2SO_4.

	analyte	concentration (mg/L)	recovery %	RSD%
1	malonic acid	1.5	57	30
2	lactic acid	20	78	4.1
3	maleic acid	1.3	115	19
4	*cis*-aconitic acid	1.6	127	19
5	methylmalonic acid	0.023	87	14
6	succinic acid	2.9	76	4.2
7	fumaric acid	3.7	79	3.7
8	*trans*-aconitic acid	0.31	93	18
9	levulinic acid	41	71	15
10	glutaric acid	0.57	78	17
11	itaconic acid	7.2	70	9.7
12	2-hydroxy-2-methylbutyric acid	0.008	73	13
13	2-furoic acid	2.4	85	6.2
14	gallic acid	0.027	103	22
15	5-hydroxymethylfurfural	44	95	23
16	furfural	220	78	4.7
17	adipic acid	0.11	73	9.1
18	3,4-dihydroxybenzoic acid	0.44	93	11
19	3,5-dihydroxybenzoic acid	0.022	84	13
20	2,5-dihydroxybenzoic acid	0.23	99	24
21	3,4-dihydroxybenzaldehyde	0.38	87	4.1
22	salicylic acid	1.9	88	29
23	4-hydroxybenzaldehyde	3.6	87	7.8
24	vanillic acid	3.3	99	10
25	homovanillic acid	0.29	77	13
26	4-hydroxyacetophenone	0.84	82	19
27	caffeic acid	0.11	93	27
28	syringic acid	2.0	73	18
29	vanillin	4.0	81	9.6
30	4-hydroxybenzoic acid	0.028	80	15
31	benzoic acid	1.5	94	7.2
32	syringaldehyde	1.8	82	8.3
33	4-hydroxy-coumaric acid	5.6	93	14
34	ferulic acid	6.6	93	12
35	3-hydroxy-4-methoxycinnamic acid	0.043	92	13
36	4-hydroxycoumarin	0.033	99	17
37	*ortho*-toluic acid	0.024	108	29
38	*para*-toluic acid	0.59	109	9.6

study assumes that variation of analyte concentrations for other tested pretreatment chemistry-feedstock combinations do not vary by more than 30%.

Effects of Substrate Type

Differences in concentrations of various degradation products were observed and compared across three feedstocks and eight chemical conditions. In general, it was observed that the presence and type of compounds produced during pretreatment is more dependant on feedstock than the pretreatment chemistry employed. However, select compounds exhibited dependency on both feedstock and pretreatment chemistry while others demonstrated no obvious trends. Concentrations of all monitored analytes in hydrolysates resulting from investigated pretreatment chemistry-feedstock combinations (three feedstocks and eight pretreatment conditions) and the plant origins of monitored degradation products are listed in Tables 3.2, 3.3, and 3.4 for corn stover, hybrid poplar, and pine feedstocks.

Data in figures 3.3 to 3.21 highlight the predominant variations in analyte concentrations that were observed during these experiments. In these figures, pretreatment method or feedstock type is typically plotted along the x-axis and concentration of specific analytes is shown along the y-axis. Results from the three feedstocks are generally represented in each plot so that relative differences can be readily compared.

The most obvious differences were observed when concentrations of certain compounds were dominant for one feedstock regardless of the pretreatment schemes

Table 3.2. Degradation product concentrations observed in corn stover hydrolysates.

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aliphatic acids									
malonic acid	sugar/lignin	1.5	2.6	0.32	1.1	2.6	1.7	1.8	2.2
lactic acid	sugar/lignin	20	17.8	5.5	24	45	38	27	24.2
maleic acid	sugar/lignin	1.3	0.80	2.3	3.1	1.1	0.83	0.82	2.3
cis-aconitic acid	sugar/lignin	1.6	0.22	0.055	0.90	1.8	2.6	4.7	2.0
methylmalonic acid	sugar/lignin	0.023	0.011	0.007	0.055	0.070	0.025	0.028	0.040
succinic acid	sugar/lignin	2.9	1.7	2.2	5.2	11	6.5	9.8	5.9
fumaric acid	sugar/lignin	3.7	3.9	3.9	1.8	3.2	5.9	5.4	1.9
trans-aconitic acid	sugar/lignin	0.31	0.063	0.036	0.23	1.0	0.95	0.94	0.29
levulinic acid	sugar	41	1.5	0.48	1.9	1.5	1.7	1.1	1.6
glutaric acid	sugar/lignin	0.57	0.24	0.23	0.65	1.1	1.2	0.89	0.94
itaconic acid	sugar/lignin	7.2	2.0	1.2	2.1	3.9	3.2	5.1	6.2
2-hydroxy-2-methylbutyric acid	sugar/lignin	0.008	0.008	0.006	0.010	0.030	0.011	0.008	0.033
gallic acid	lignin	0.027	0.040	0.020	0.033	0.070	0.003	0.014	0.021
adipic acid	sugar/lignin	0.11	0.14	0.15	0.20	0.19	0.18	0.17	0.25
acetic acid	sugar/lignin	170	83	34	58	240	180	120	110
formic acid	sugar	120	76	55	79	110	250	43	92

Table 3.2. Degradation product concentrations observed in corn stover hydrolysates. (Cont.)

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aromatic acids									
2-furoic acid	sugar	2.4	1.1	0.88	1.2	1.1	1.1	0.77	1.2
3,4-dihydroxybenzoic acid	lignin	0.44	0.34	0.43	0.73	0.53	0.031	0.24	0.32
3,5-dihydroxybenzoic acid	lignin	0.022	0.006	0.011	0.03	0.035	0.048	0.023	0.028
2,5-dihydroxybenzoic acid	lignin	0.23	0.15	0.14	0.04	0.12	0.015	0.029	0.073
salicylic acid	lignin	1.92	0.78	1.3	2.0	1.4	1.0	1.1	2.8
vanillic acid	lignin	3.3	1.5	2.6	4.3	4.6	3.2	3.3	5.1
homovanillic acid	lignin	0.29	0.11	0.19	0.17	0.47	0.19	0.30	0.26
caffeic acid	lignin	0.11	0.14	0.14	0.20	0.073	0.070	0.094	0.038
syringic acid	lignin	2.0	1.5	1.8	2.17	2.3	1.54	1.7	1.4
4-hydroxybenzoic acid	lignin	0.028	0.028	0.059	0.07	0.080	0.065	0.048	0.087
benzoic acid	lignin	1.5	1.3	0.16	0.31	0.36	1.3	1.7	1.3
4-hydroxycoumaric acid	lignin	5.6	14	11	11	14	11	17	8.1
ferulic acid	lignin	6.6	2.6	2.2	1.0	5.1	4.2	6.6	0.76
3-hydroxy-4-methoxycinnamic acid	lignin	0.043	0.019	0.039	0.050	0.22	0.098	0.11	0.045
ortho-toluic acid	lignin	0.024	0.018	0.016	0.030	0.023	0.028	0.028	0.021
para-toluic acid	lignin	0.59	0.46	0.36	0.98	0.56	0.45	0.80	0.45

Table 3.2. Degradation product concentrations observed in corn stover hydrolysates. (Cont.)

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aldehydes and ketones									
5-hydroxymethylfurfural (5-HMF)	sugar	44	11	2.3	2.8	4.3	0.89	2.3	3.8
Furfural	sugar	220	26	8.0	6.5	3.8	0.40	1.5	3.2
4-hydroxybenzaldehyde	lignin	3.6	2.4	2.7	4.4	3.2	1.5	2.2	2.1
3,4-dihydroxybenzaldehyde	lignin	0.38	0.25	0.31	0.82	0.76	0.057	0.27	0.061
4-hydroxyacetophenone	lignin	0.84	0.28	0.41	0.48	0.50	0.37	0.35	0.46
Vanillin	lignin	4.0	2.8	2.6	6.7	5.5	2.6	3.6	1.7
syringaldehyde	lignin	1.8	0.60	1.0	2.0	1.7	0.31	1.0	0.084
4-hydroxycoumarin	lignin	0.033	0.007	0.035	0.006	0.030	0.007	0.009	0.008

Table 3.3. Degradation product concentrations observed in hybrid poplar hydrolysates.

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aliphatic acids									
malonic acid	sugar/lignin	1.0	0.49	0.15	1.2	2.1	1.5	1.3	2.7
lactic acid	sugar/lignin	29	19	1.8	22	26	26	26	22
maleic acid	sugar/lignin	0.27	0.25	0.25	3.7	0.58	0.43	0.35	1.7
cis-aconitic acid	sugar/lignin	0.90	0.60	0.075	0.70	0.31	0.12	0.20	0.64
methylmalonic acid	sugar/lignin	0.028	0.025	0.005	0.042	0.034	0.017	0.035	0.045
succinic acid	sugar/lignin	2.5	0.93	2.3	2.4	4.1	1.7	6.4	3.1
fumaric acid	sugar/lignin	0.22	0.13	0.11	1.0	0.56	0.19	0.42	1.7
trans-aconitic acid	sugar/lignin	0.070	0.018	0.028	0.13	0.16	0.027	0.039	0.16
levulinic acid	sugar	45	1.3	0.29	0.83	0.75	1.2	0.93	1.5
glutaric acid	sugar/lignin	0.61	0.26	0.23	0.25	1.1	0.35	1.1	0.49
itaconic acid	sugar/lignin	0.11	0.13	0.093	0.17	0.22	0.088	0.26	0.55
2-hydroxy-2-methylbutyric acid	sugar/lignin	0.010	0.012	0	0.050	0.004	0.020	0.011	0.030
gallic acid	lignin	0.052	0.029	0.018	0.021	0.016	0.020	0.007	0.002
adipic acid	sugar/lignin	0.057	0.10	0.048	0.14	0.64	0.13	0.19	0.20
acetic acid	sugar/lignin	310	160	57	61	270	120	180	210
formic acid	sugar	210	150	82	110	310	52	65	120

Table 3.3. Degradation product concentrations observed in hybrid poplar hydrolysates. (Cont.)

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aromatic acids									
2-furoic acid	sugar	3.1	1.7	0.94	0.76	0.30	0.49	0.40	0.52
3,4-dihydroxybenzoic acid	lignin	0.38	0.45	0.78	1.7	0.36	0.018	0.16	0.17
3,5-dihydroxybenzoic acid	lignin	0.007	0	0	0.005	0.011	0.019	0.013	0.022
2,5-dihydroxybenzoic acid	lignin	0.070	0.017	0.070	0.012	0.020	0.060	0.019	0.007
salicylic acid	lignin	19	44	19	26	31	28	29	26
vanillic acid	lignin	5.9	5.7	4.1	5.3	2.5	2.5	5.4	4.5
homovanillic acid	lignin	0.13	0.18	0.12	0.049	0.11	0.12	0.20	0.11
caffeic acid	lignin	0.003	0.013	0.019	0.003	0.053	0.003	0.016	0.003
syringic acid	lignin	2.4	3.1	2.4	3.2	2.6	1.7	3.5	1.6
4-hydroxybenzoic acid	lignin	0.016	0.017	0.031	0.12	0.020	0.028	0.038	0.070
benzoic acid	lignin	1.7	1.8	0.78	1.1	1.6	2.6	2.6	2.4
4-hydroxycoumaric acid	lignin	0.15	0.93	0.19	0.050	0.26	0.070	0.60	0.018
ferulic acid	lignin	0.19	0.46	0.23	0.070	0.18	0.13	0.20	0.031
3-hydroxy-4-methoxycinnamic acid	lignin	0.020	0.005	0.020	0.030	0.040	0.017	0.019	0.014
ortho-toluic acid	lignin	0.017	0.016	0.013	0.024	0.032	0.041	0.040	0.025
para-toluic acid	lignin	0.53	0.55	0.37	0.72	0.53	0.91	0.93	0.61

Table 3.3. Degradation product concentrations observed in hybrid poplar hydrolysates. (Cont.)

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aldehydes and ketones									
5-hydroxymethylfurfural (5-HMF)	sugar	64	4.0	0.45	0.39	0.17	0.079	0.36	0.20
Furfural	sugar	220	31	2.6	2.1	0.85	0.50	1.8	1.1
4-hydroxybenzaldehyde	lignin	0.17	0.49	0.23	0.47	0.17	0.093	0.24	0.32
3,4-dihydroxybenzaldehyde	lignin	0.25	0.24	0.25	0.50	0.28	0.038	0.18	0.082
4-hydroxyacetophenone	lignin	0.030	0.070	0.036	0.070	0.015	0.026	0.065	0.036
vanillin	lignin	5.5	5.6	3.1	9.1	2.6	2.8	4.4	4.9
Syringaldehyde	lignin	7.4	4.1	1.8	5.8	2.4	0.93	3.9	1.9
4-hydroxycoumarin	lignin	0.020	0.004	0.020	0.001	0.010	0.001	0.002	0.002

Table 3.4. Degradation product concentrations observed in pine hydrolysates.

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aliphatic acids									
malonic acid	sugar/lignin	0.54	0.39	0.21	1.7	1.1	0.97	1.9	3.1
lactic acid	sugar/lignin	3.7	4.5	8.7	18	28	36	39.6	32
maleic acid	sugar/lignin	0.13	0.11	0.19	1.4	0.35	0.27	0.19	2.2
cis-aconitic acid	sugar/lignin	0.80	0.50	0.26	1.1	0.31	0.15	0.048	0.92
methylmalonic acid	sugar/lignin	0.026	0.018	0.011	0.025	0.054	0.016	0.012	0.031
succinic acid	sugar/lignin	0.73	0.34	0.75	1.8	4.4	2.39	4.1	3.0
fumaric acid	sugar/lignin	0.054	0.057	0.084	0.31	0.38	0.19	0.24	1.5
trans-aconitic acid	sugar/lignin	0	0.005	0.027	0.026	0.068	0.027	0.050	0.081
levulinic acid	sugar	30	1.7	0.63	0.48	0.40	0.64	1.2	1.1
glutaric acid	sugar/lignin	0.37	0.18	0.16	0.31	0.76	0.66	1.2	0.56
itaconic acid	sugar/lignin	0.070	0.032	0.090	0.24	0.25	0.099	0.28	0.65
2-hydroxy-2-methylbutyric acid	sugar/lignin	0.020	0.009	0.040	0.030	0.017	0.010	0.044	0.050
gallic acid	lignin	0.20	0.13	0.11	0.13	0.68	0.020	0.028	0.005
adipic acid	sugar/lignin	0.076	0.090	0.054	0.18	0.15	0.13	0.22	0.22
acetic acid	sugar/lignin	120	69	14	24	94	65	120	110
formic acid	sugar	110	70	42	66	210	41	67	120

Table 3.4. Degradation product concentrations observed in pine hydrolysates.. (Cont.)

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aromatic acids									
2-furoic acid	sugar	1.1	0.80	0.83	0.91	0.23	0.55	0.45	0.86
3,4-dihydroxybenzoic acid	lignin	0.46	0.62	0.82	0.68	0.81	0.021	0.34	0.21
3,5-dihydroxybenzoic acid	lignin	0	0.026	0	0.040	0.018	0.026	0.012	0.017
2,5-dihydroxybenzoic acid	lignin	0.020	0.023	0.030	0.007	0.020	0.035	0.005	0.011
salicylic acid	lignin	0.31	0.24	0.54	0.66	0.50	0.44	0.68	0.98
vanillic acid	lignin	5.2	3.6	2.3	4.8	3.8	4.8	8.7	6.6
homovanillic acid	lignin	0.22	0.090	0.15	0.10	0.17	0.15	0.31	0.17
caffeic acid	lignin	0.009	0.012	0.019	0.009	0.028	0.007	0.005	0.005
syringic acid	lignin	1.0	0.75	0.64	0.59	0.49	0.60	1.0	0.40
4-hydroxybenzoic acid	lignin	0	0.007	0.024	0.14	0.010	0.030	0.033	0.18
benzoic acid	lignin	1.8	2.2	1.9	1.7	2.0	1.9	2.3	3.8
4-hydroxycoumaric acid	lignin	0.020	0.050	0.20	0.080	0.049	0.12	0.33	0.037
ferulic acid	lignin	0.12	0.22	0.31	0.14	0.088	0.16	0.19	0.083
3-hydroxy-4-methoxycinnamic acid	lignin	0.003	0.005	0.030	0.014	0.014	0.022	0.020	0.019
ortho-toluic acid	lignin	0.026	0.021	0.016	0.023	0.024	0.031	0.015	0.022
para-toluic acid	lignin	0.61	0.64	0.43	0.58	0.52	0.90	0.44	0.63

Table 3.4. Degradation product concentrations observed in pine hydrolysates.. (Cont.)

Analyte	plant origin	concentration (mg/L)							
		0.7% H_2SO_4	0.07% H_2SO_4	LHW	wet oxidation	buffer	NH_3	lime	oxidative lime
aldehydes and ketones									
5-hydroxymethylfurfural (5-HMF)	sugar	170	9.5	1.3	0.64	0.95	0.16	0.63	0.76
Furfural	sugar	190	13	2.5	1.9	1.4	0.65	5.4	2.2
4-hydroxybenzaldehyde	lignin	0.45	0.45	0.43	0.68	0.34	0.24	0.52	0.70
3,4-dihydroxybenzaldehyde	lignin	0.31	0.45	0.33	0.66	0.63	0.041	0.45	0.15
4-hydroxyacetophenone	lignin	0.040	0.020	0.040	0.090	0.033	0.061	0.048	0.073
Vanillin	lignin	4.6	5.8	2.4	7.1	2.6	3.2	7.0	5.3
Syringaldehyde	lignin	1.1	0.60	0.26	0.83	0.39	0.25	0.71	0.20
4-hydroxycoumarin	lignin	0.020	0.002	0.020	0.005	0.010	0.002	0.003	0.005

applied. Such a result implies that production of certain compounds is specific to a particular feedstock composition and not solely the result of chemical decomposition of ubiquitous plant components. For example, salicylic acid was detected at much higher concentrations in hydrolysates of poplar than in hydrolysates of corn stover and pine. The relative magnitude of this difference is easily identified from the bar graph in Figure 3.3. As is the case with salicylic acid, it is often difficult to relate the observed feedstock/inhibitor specificity to feedstock chemical composition, as the need to fully understand biomass composition in is only currently emerging. In this work, apparent feedstock-dependent degradation products are identified and discussed in light of what is currently known about specific biomass composition.

Aliphatic Acids and Aldehydes

One of the more relevant aliphatic acids produced during pretreatment is acetic acid. This relevance arises as a result of its high economic value as a useful

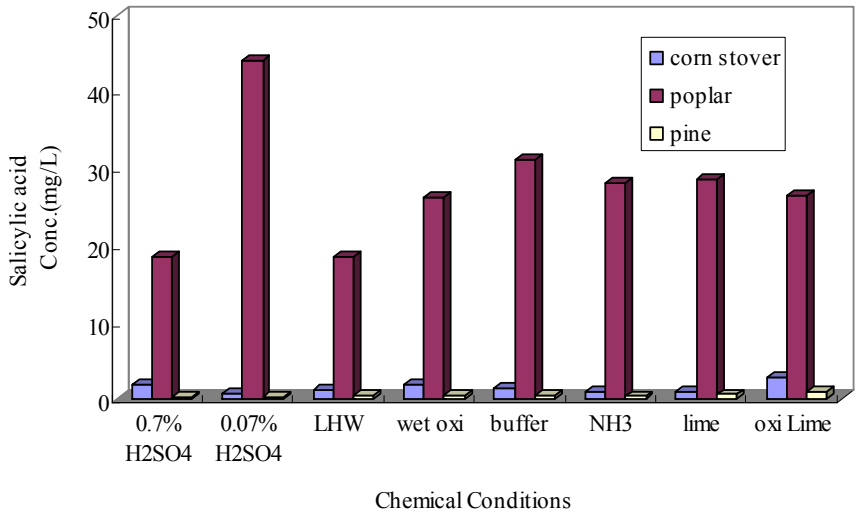

Figure 3.3. Comparison of salicylic acid among corn stover, hybrid poplar, and pine.

commercial product, and its tendency to be a strong inhibitor of microbial ethanol production. For example, acetic acid has been identified as a stronger inhibitor of yeast (*Saccharomyces Cerevisiae)* during ethanol fermentation by many groups (Brown et al. 1981, 151; Maiorella et al. 1983, 103; Ando et al. 1986, 567; Baugh et al. 1988 62; Pampulha and Loureiro-Dias 1989, 269; Luo et al. 2002, 125). In this study, acetic acid was investigated for obvious production differences depending on substrate type. From data in Tables 3.2 to 3.4, it is clear that acetic acid is produced in significant quantities (10s-100s of mg/L) for each feedstock. Although some variation is noted across different pretreatment types, there is no clear pretreatment-dependant trend.

Fengel and Wegener have previously observed that acetic acid is produced in higher quantities for hardwood feedstocks than for softwood feedstocks (Fengel and Wegener 1989). As an explanation, Fengel and Wegener note that hemicellulose in softwood has more mannose and glucose units than hemicellulose in hardwood, which usually contain higher amounts of xylose units. Moreover, hemicelluloses are more acetylated in hardwoods than in softwoods. When biomass is degraded under thermal and chemical pretreatments, acetyl functional groups are cleaved from the hemicellulose units resulting in the release of acetic acid. In the current study, higher concentrations of acetic acid resulting from pretreatment of hardwood feedstocks can also be observed. Figure 3.4 contains a plot of acetic acid concentrations in hydrolysates resulting from poplar and pine feedstocks (hard and soft wood, respectively) across all pretreatment conditions. From this data (Figure

3.4) it is apparent that for each pretreatment, higher acetic acid concentrations are observed in the hydrolysate of poplar than in hydrolosates of pine samples, which is consistent with observations made by Fengel et al.

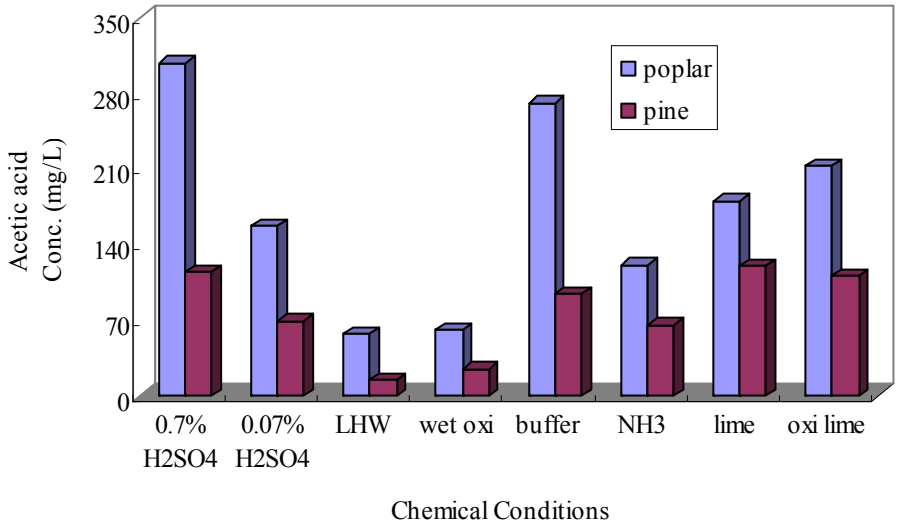

Figure 3.4. Comparison of acetic acid between hybrid poplar and pine.

Itaconic acid and 4-hydroxybenzaldehyde also exhibited strong feedstock dependency, and plots of their concentration versus pretreatment (for each feedstock) are contained in Figures 3.5 and 3.6. Both Itaconic acid and 4-hydroxybenzaldehyde are observed at higher concentration in hydrolysates of corn stover than in hydrolysates of poplar and pine. In fact, for most pretreatments, itaconic acid was produced in corn stover hydrolysate at concentrations approximately 10-fold greater than in poplar and pine (i.e., an order of magnitude). Although such differences pose an interesting question for investigation, no specific rationale is currently apparent to explain the observed specificity.

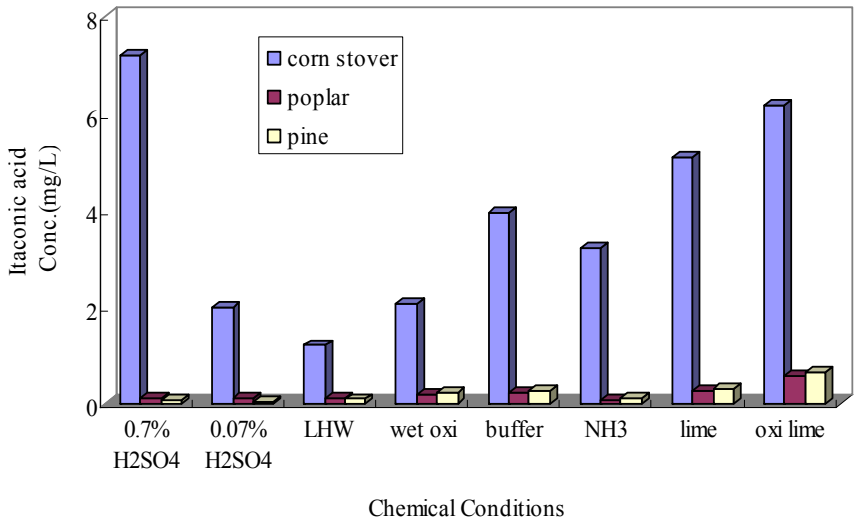

Figure 3.5. Comparison of itaconic acid among corn stover, hybrid poplar, and pine.

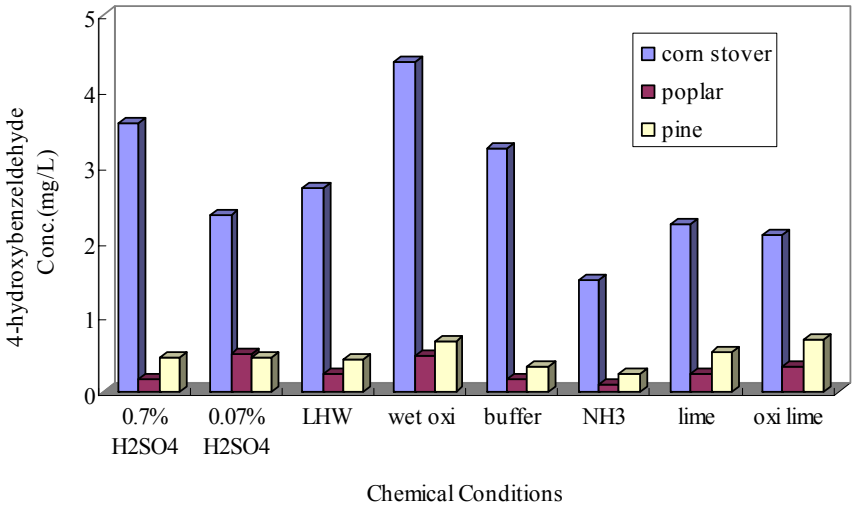

Figure 3.6. Comparison of 4-hydroxybenzaldehyde among corn stover, hybrid poplar, and pine.

Aromatic Acids and Phenols

The incorporation of aromatic rings in monomer residues (i.e., lignin) is ubiquitous in biomass feedstocks; however, the specific type of aromatic moiety differs from one feedstock to the next. The terms p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) are used to denote the three most common types of aromatic-ring

structures in lignin (Klinke et al. 2004, 10). Softwood biomass almost exclusively generates G phenols, while hardwoods and herbaceous materials produce H, G and S phenols according to the biomass composition (Klinke et al. 2004, 10). Softwood lignin is mainly composed of coniferyl alcohol (G), together with small proportions of p-coumaryl alcohol (H). Hardwood lignin is generally composed of coniferyl (G) and sinapyl alcohols (S) in roughly equal amounts as well as small quantities of p-coumaryl alcohol (H). Apart from the monolignols (H, G and S), herbaceous plants also contain p-hydroxycinnamic acids integrated into their lignin (Campbell and Sederoff 1996, 3; Lawther and Sun 1996, 87). Moreover, Tran et al. and Jonsson et al. also reported that in hardwood hydrolysates, syringaldehyde and syringic acid formed from the degradation of syringyl propane units (S) (Tran and Chambers 1985, 841; Jonsson et al. 1998, 691).

In this work, we observed that syringic acid (Figure 3.7) and syringaldehyde (Figure 3.8), both of which are degraded from sinapyl alcohols (S), were present in the highest concentrations in hydrolysates of poplar, but not always dominantly. The reason for this is likely due to the ratio of H/G/S units in the lignin, which is highly dependent on plant type. More sinapyl alcohols (S) are identified in hardwood lignin than the other two feedstocks, which is anticipated considering hardwood is known to contain larger S:G ratios than softwood (Klinke et al. 2004, 10).

4-hydroxycoumaric acid and ferulic acid are derived from H and G residues, respectively. Figures 3.9 (4-hydroxycoumaric acid) and 3.10 (ferulic acid) contain plots of concentration versus pretreatment for the three different feedstocks. These

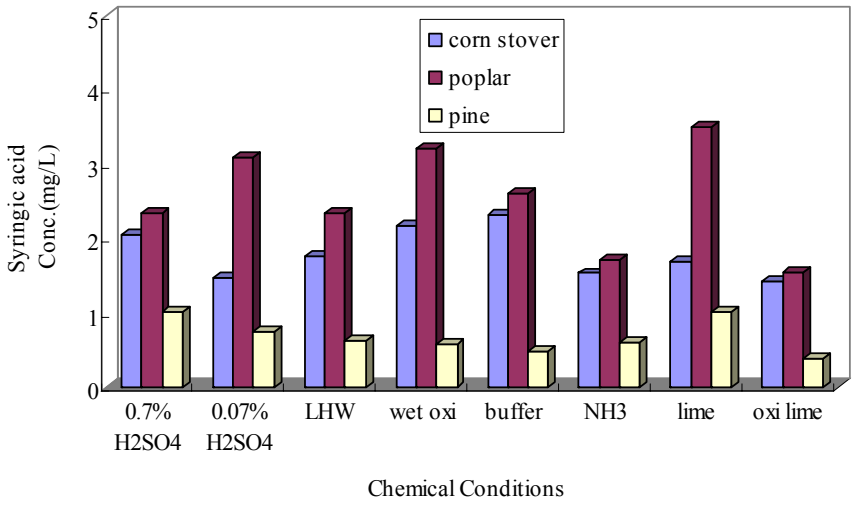

Figure 3.7. Comparison of syringic acid among corn stover, hybrid poplar, and pine.

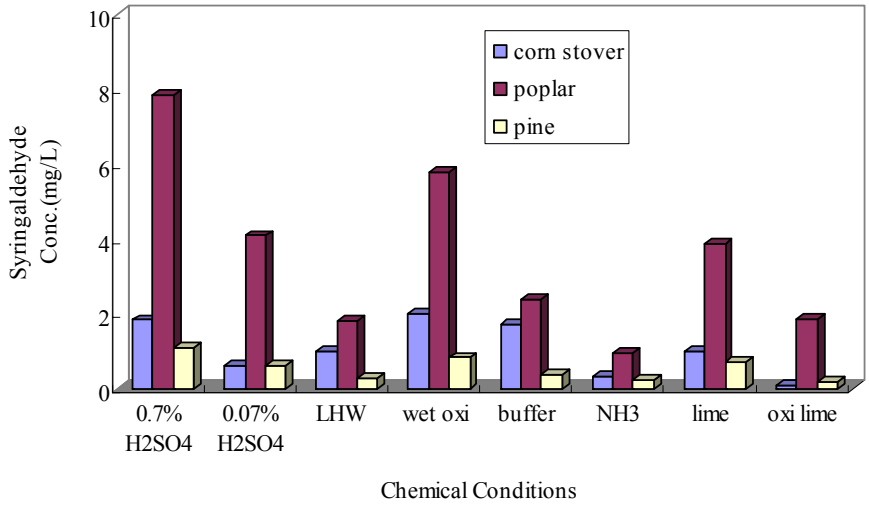

Figure 3.8. Comparison of syringaldehyde among corn stover, hybrid poplar, and pine.

data show that both aromatic acids are produced most abundantly from corn stover feedstocks, indicating that corn stover lignin is potentially composed primarily of H and G residues. In addition, note that the corn-stover specificity observed for 4-hydroxycoumaric acid and ferulic acid is similar to the trends for itaconic acid and 4-hydroxybenzaldehyde, which were also identified at higher concentrations in corn

stover hydrolysate. In particular, 4-hydroxybenzaldehyde is also derived from H residues.

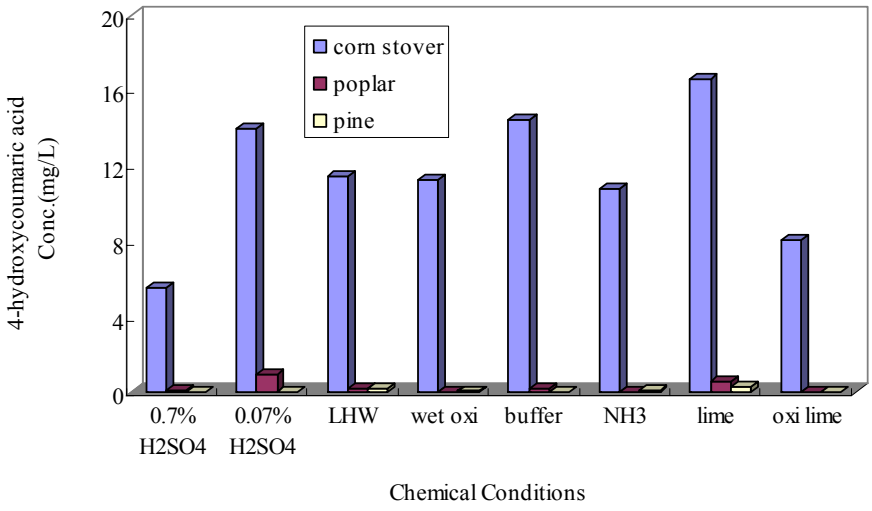

Figure 3.9. Comparison of 4-hydroxycoumaric acid among corn stover, hybrid poplar, and pine.

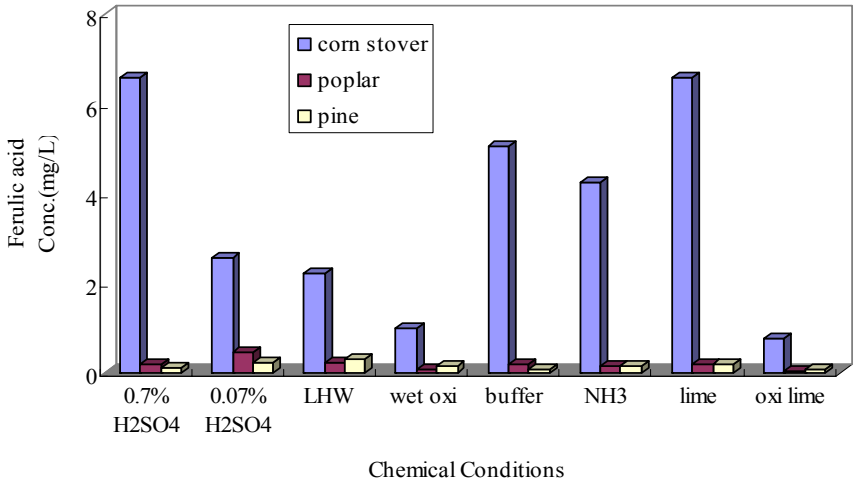

Figure 3.10. Comparison of ferulic acid among corn stover, hybrid poplar, and pine.

Effects of pH Variation

Careful analysis of the data in Tables 3.2-3.4 also reveals that the production of certain degradation products are not substrate specific, but rather a result of the

pretreatment process. For example, furfural and 5-hydroxymethylfurfural showed higher concentrations for all three feedstocks at low pH (i.e. H_2SO_4 pretreatments) and essentially negligible concentrations at higher pH (i.e. LHW-lime). These data are consistent with previous observations of Dunlop and Taherzadeh (Dunlop 1948, 204; Taherzadeh et al. 1997, 2653). When hemicellulose is degraded, multiple sugars are liberated (e.g. xylose, mannose, galactose, glucose, etc.). During severe acidic pretreatment conditions, furfural and 5-hydroxymethylfurfural are formed in high concentrations directly from degradation of pentose and hexose, respectively, especially at high temperature and pressure. Presumably under neutral and basic conditions, degradation of these sugars occurs through an alternate (although currently unknown) pathway.

Understanding the various accumulation trends of furfurals is particularly relevant as these compounds are known to have high inhibitory effects. For example, Banergee et al. (Banergee et al. 1981, 226) found that furfural inhibited ethanol production by *Saccharomyces cerevisiae* at concentrations as low as 0.5 g/L and complete inhibition occurred at 4 g/L. 5-HMF inhibits the organisms in the same manner with a slightly higher threshold concentration (Tran 1986). The accumulation trends for furfural and 5-hydroxymethylfurfural are shown in Figure 3.11 and Figure 3.12, respectively. In these figures, pretreatment conditions are arranged in order of increasing pH (left to right) along the x-axis. The pH ranged from 0.58 to 13.40 for conditions 0.7% H_2SO_4 to lime, respectively. From these data, it is clear that furfurals are produced most abundantly at low pH, suggesting that

higher pH pretreatments might be more amenable pretreatment procedures to limit the production of furans.

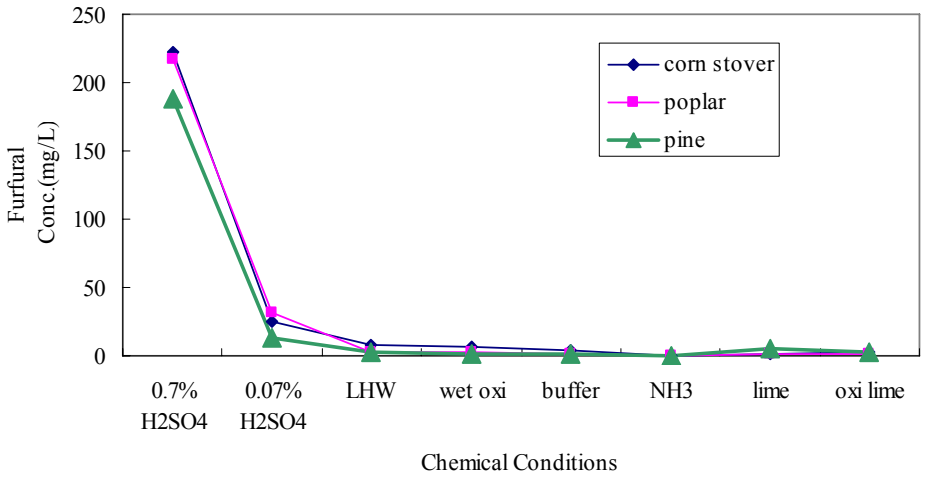

Figure 3.11. Comparison of furfural from low pH (0.7% H_2SO_4) to high pH (oxidative lime) for all three feedstocks.

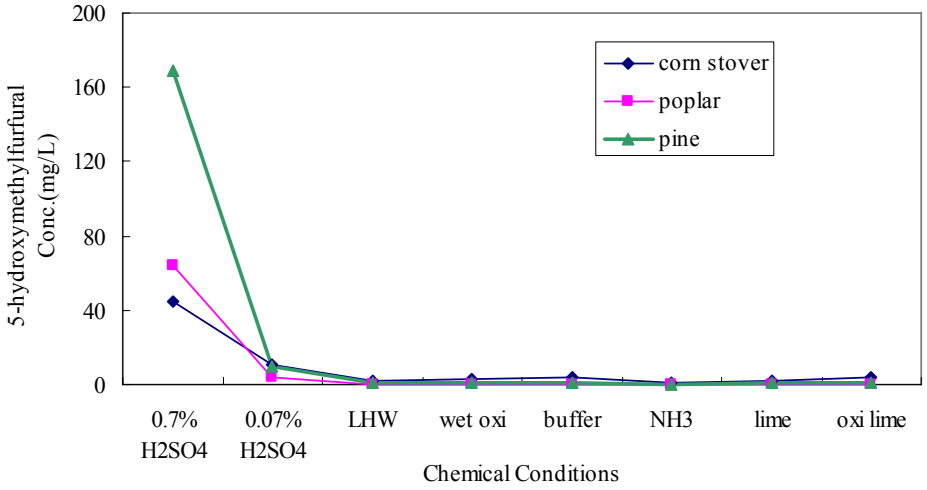

Figure 3.12. Comparison of 5-hydroxymethylfurfural from low pH (0.7% H_2SO_4) to high pH (oxidative lime) for all three feedstocks.

Levulinic acid was also observed to be produced at much higher concentrations during strongly acidic pretreatment conditions and essentially negligible amounts

were produced at higher pH. This trend (Figure 3.13) is similar to the trends exhibited by 5-hydroxymethylfurfural and furfural. The most probable explanation of this trend is that levulinic acid is formed from 5-hydroxymethylfurfural degradation (Ulbricht et al. 1984, 843; Palmqvist and Hahn-Hagerdal 2000, 25), which occurs under strongly acidic conditions. Other analytes in this study did not exhibit similar differences in production as a function of pH.

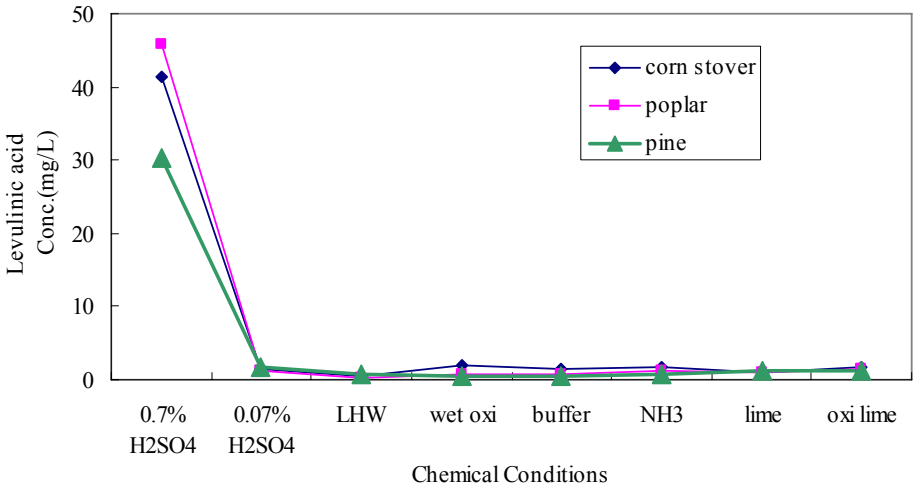

Figure 3.13. Comparison of levulinic acid from low pH (0.7% H_2SO_4) to high pH (oxidative lime) for all three feedstocks.

Effects of Oxygen Addition

LHW and lime pretreatments with the addition of oxygen (i.e. wet oxidation and oxidative lime, respectively) have been proven as effective pretreatment schemes that make the solid cellulose fraction of feedstocks more accessible for enzymatic hydrolysis and fermentation (McGinnis et al. 1983, 352; Bjerre et al. 1996, 568; Schmidt and Thomsen 1998, 139; Klinke et al. 2003, 738; Kim and Holtzapple, 2005). For many target analytes, production is not affected to a large degree by the addition

of oxygen. These include syringic acid, 4-hydroxyacetophenone and homovanillic acid in corn stover hydrolysates, 2-furoic acid and vanillic acid in poplar hydrolysates, and levulinic acid in pine hydrolysates. However, pretreatments with oxygen were found to affect the production of some compounds. For example, maleic acid (Figure 3.14), malonic acid (Figure 3.15), and itaconic acid (Figure 3.16), were all identified at higher concentrations in the presence of oxygen than when LHW and lime pretreatments were employed without oxygen. Conversely, some compounds exhibit increased production under wet oxidation, but decreased production when oxidative lime was used. For example, it is clear that concentrations of lactic acid (Figure 3.17), succinic acid (Figure 3.18), and syringaldehyde (Figure 3.19) increased to different degrees under wet oxidation pretreatment, but decreased slightly using oxidative lime pretreatment (decreased relative to lime).

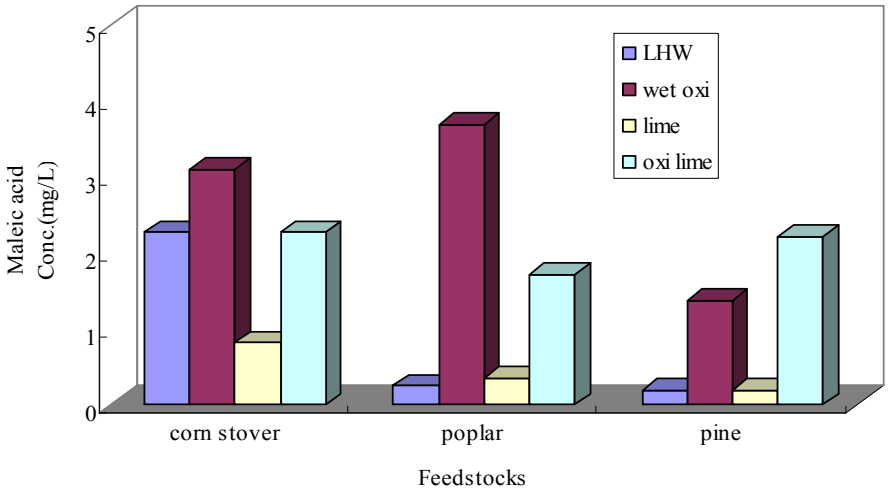

Figure 3.14. Effect of oxygen on production of maleic acid under LHW and lime.

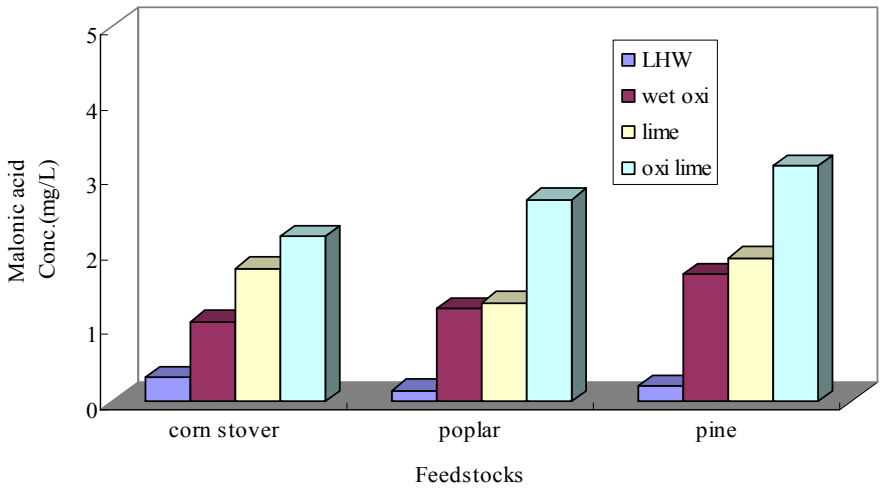

Figure 3.15. Effect of oxygen on production of malonic acid under LHW and lime.

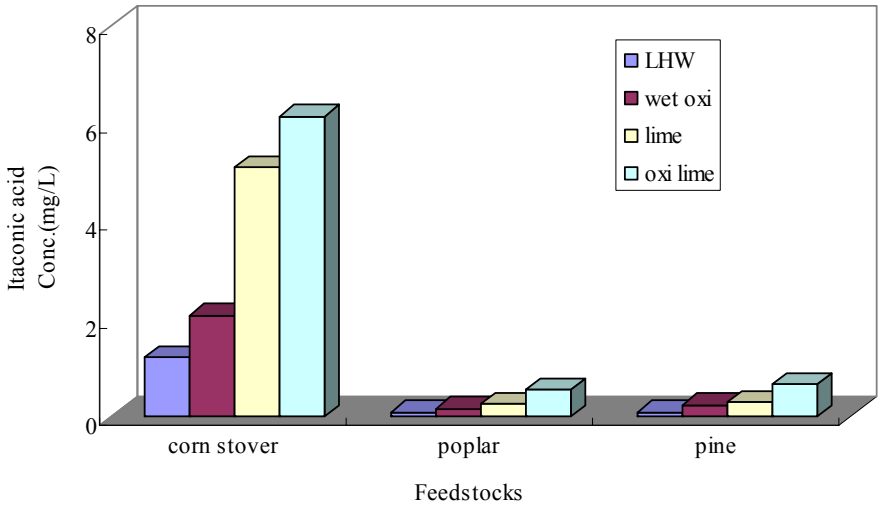

Figure 3.16. Effect of oxygen on production of itaconic acid under LHW and lime.

Vanillin also exhibited a variation in production dependant on the presence of oxidative conditions. Vanillin is formed by the degradation of the guaiacyl propane units of lignin, and has previously been detected in hydrolysates from willow (Jonsson et al. 1998, 691), poplar (Ando et al. 1986, 567), red oak (Tran and Chambers 1985, 841), and pine (Clark and Mackie 1984, 101). In this study, vanillin was found to be present in all three feedstocks, regardless of pretreatment (Tables 3.2,-3.4).

However, the concentration of vanillin was found to vary appreciably only with oxidation versus non-oxidation conditions. Specifically, vanillin production was identified to increase under wet oxidation; however, when lime pretreatments were used (i.e., lime and oxidative lime), there were no parallel variations for all of the feedstocks (Figure 3.20). That is, oxidative lime pretreatment decreased vanillin production when compared to lime pretreatment for a corn stover and pine feedstock. On the other hand, oxidative lime showed little variation in vanillin production from a poplar feedstock.

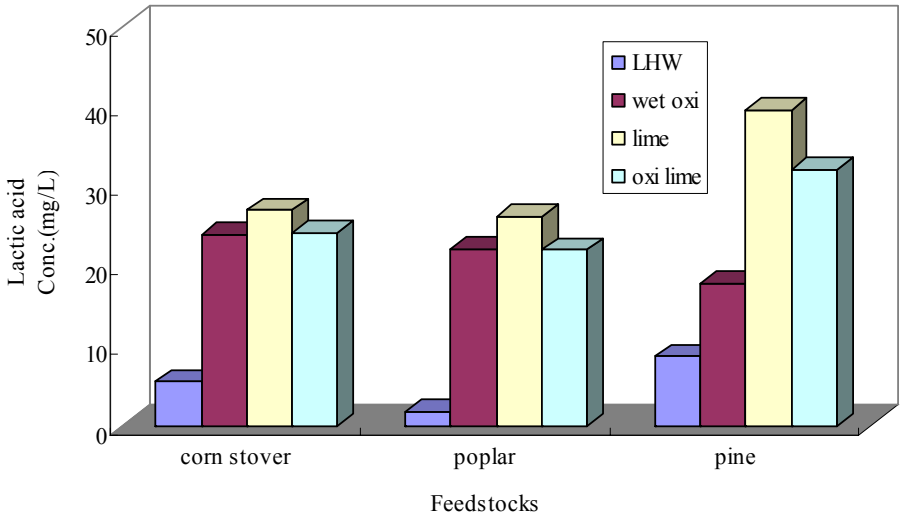

Figure 3.17. Effect of oxygen on production of lactic acid under LHW and lime.

Results of oxidative conditions versus non-oxidative conditions for fumaric acid are also lacking a general trend which includes all three feedstocks. A graph comparing the concentrations of fumaric acid across the three feedstocks under oxidative and non-oxidative conditions is contained in Figure 3.21. These data indicate that the addition of oxygen decreases fumaric-acid production from corn

stover feedstocks, but increases production from both poplar and pine. These results (Figure 3.20 and 3.21) suggest that there may be mechanistic interplay between the type of feedstock and type of pretreatment used which regulates the production of certain inhibitors. Currently, a rationale for the observed trends (and variation in trends) in degradation product accumulation under wet-oxidation and oxidative-lime conditions is not apparent.

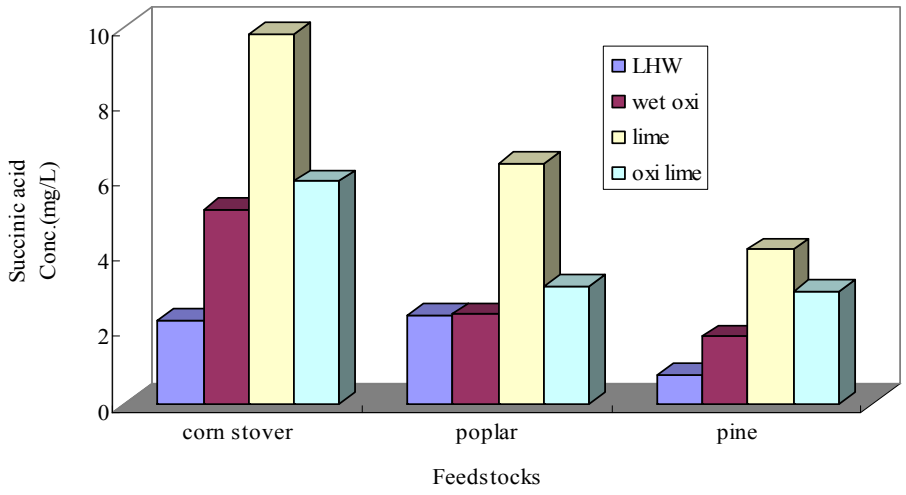

Figure 3.18. Effect of oxygen on production of succinic acid under LHW and lime.

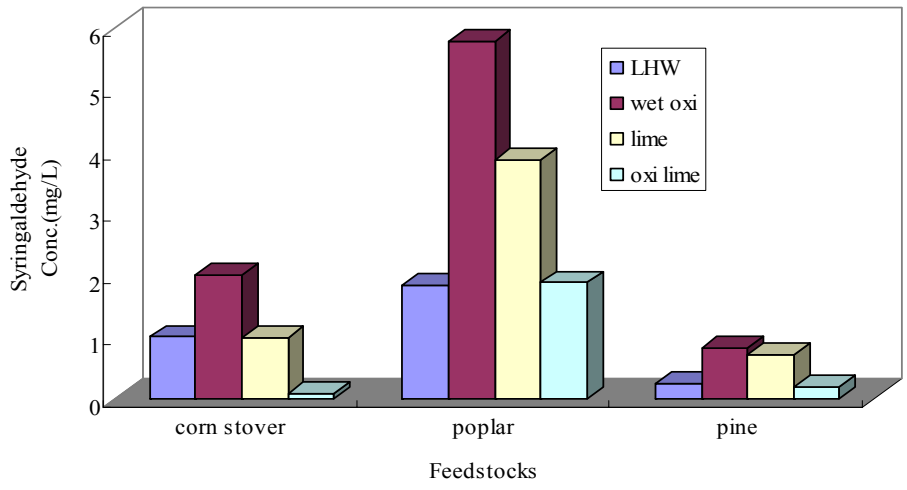

Figure 3.19. Effect of oxygen on production of syringaldehyde under LHW and lime.

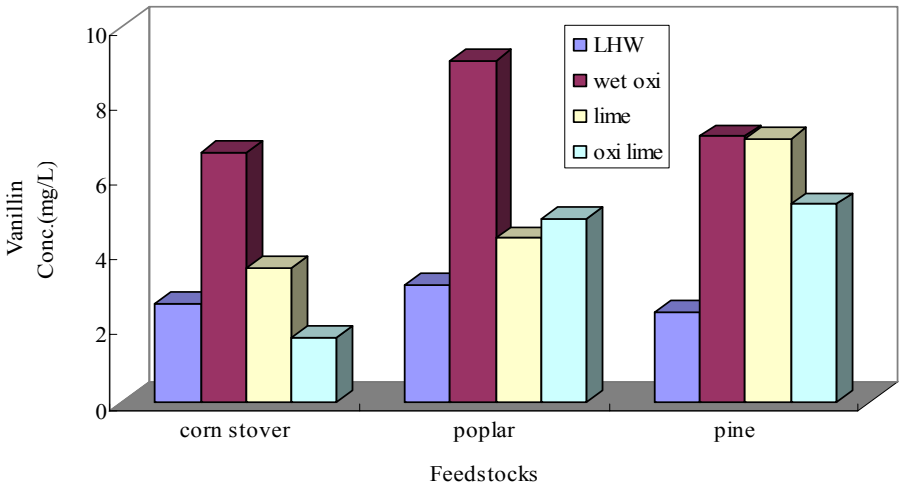

Figure 3.20. Effect of oxygen on production of vanillin under LHW and lime.

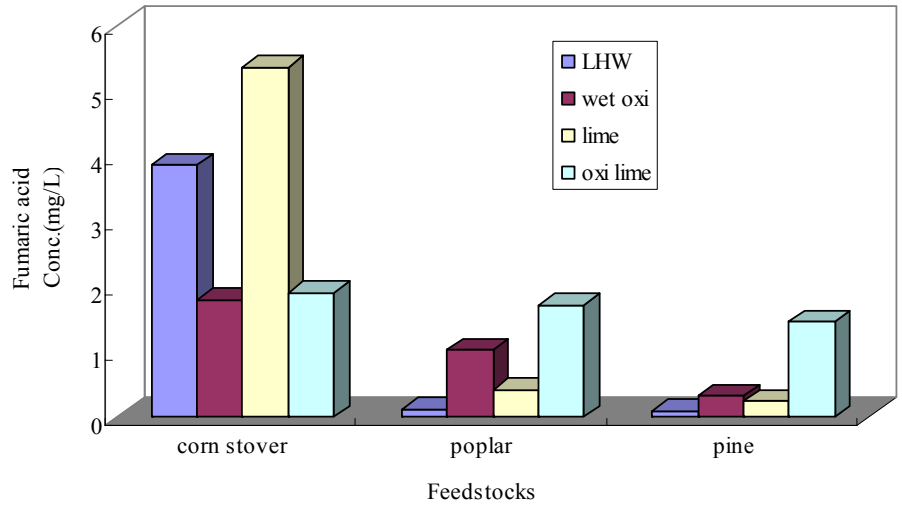

Figure 3.21. Effect of oxygen on production of fumaric acid under LHW and lime.

Summary and Future Direction

Thirty-eight potential degradation compounds derived from pretreated lignocellulosic biomass samples were both identified and quantified using a HPLC-PDA-MS/MS method. Acetic acid and formic acid were quantified by ion chromatography-conductivity separately due to their co-elution with other target compounds and their low resolution using HPLC-PDA-MS/MS. The concentration

of degradation products not only varies substantially with the type of feedstock (agricultural wastes, hardwood, and softwood), but also shows specific accumulation trends across pH variations and in the presence of oxygen. The data in this work clearly indicates that the production of degradation products during pretreatment is complex and requires careful consideration when developing pretreatment schemes. Ongoing work in our group seeks to determine a mechanistic understanding for the observed accumulation trends identified in this study. Other ongoing work involves further testing of the inhibitory effects of degradation products and identifying a predictive understanding of the relationship between one or multiple degradation products and inhibition of enzymatic hydrolysis and microbial fermentation.

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