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entitled

Characterization and Saccharification of Ionic Liquid Pretreated Lignocellulosic Biomass

by

Indira Priya Samayam

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Engineering

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An Abstract of
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The goal of this dissertation research is to gain a more fundamental understanding of the impact of ionic liquid (IL) pretreatment of lignocellulosic biomass in production of ethanol and other chemicals through a sugar platform and biochemical conversion of polysaccharides to monomeric sugars. Recalcitrance of cellulose hydrolysis is a primary roadblock for efficient enzymatic conversion of lignocellulosic biomass to monomeric sugars that are fermented to ethanol or other products. The structure of lignocellulosic biomass is rendered amenable to enzymatic saccharification upon IL pretreatment. Changes in the structure of cellulose in relation to the biomass digestibility with varying ionic liquid (IL) pretreatment were examined. Conversion of native cellulose I to amorphous cellulose or cellulose II improved the susceptibility of cellulose to enzymatic hydrolysis.

The performance of commercial enzyme mixtures were evaluated through saccharification of IL pretreated biomass substrates (poplar and switchgrass) at low biomass slurry concentrations. Poplar and switchgrass hydrolysis with commercial
cellulase, Spezyme CP, and Multifect Xylanase showed synergism between the two enzymes. However, switchgrass hydrolysis was less complete than that of poplar, likely due to differences in hemicellulose structural features.

Hydrolysis of polysaccharides in lignocellulose at high biomass slurry concentrations is essential in economical fermentation of hydrolyzates to ethanol and other products. Commercial enzyme mixtures were varied for simultaneous or sequential hydrolysis and fermentation of pentose and hexose sugars with *Pichia stipitis* for IL treated poplar. In sequential hydrolysis of poplar at modest enzyme loadings of 9 mg/g xylan Multifect Xylanase, 1.8 mg/g xylan SXA (β-xylosidase) at 50°C in the first step and 10 FPU/g glucan Spezyme CP, and 40 CBU/g glucan Novozyme 188 at 25°C in the second step, the yields of glucose and xylose at 15% (w/v) solid loadings were 61% and 83%, respectively. The incomplete hydrolysis may be due to the accumulation of cellobiose and low activity of enzymes at 25°C. Further increase in Novozyme 188 loadings or hydrolysis may improve hydrolysis yields. A maximum of 66% theoretical ethanol yield based on initial sugar analysis of poplar was expected from these hydrolyzates if all the released sugars were fermented to ethanol. In sequential hydrolysis and fermentation with *P. stipitis*, 56% theoretical ethanol yields based on the initial sugar composition of poplar respectively was achieved.
I dedicate my dissertation to my Beloved Father, Krishna Prasad Samayam; Beloved Mother, Vindhya Vali Samayam; Ever supportive and loving boy friend-to be fiancé-to be husband, Santhosh Konathala; Lovely Sister, Dr. Rinda Priya Samayam; Lovely Brother, Kiran Samayam; Supportive brother-in-law, Dr. Venkateswara Rao and to our cute little charm, Baby Nidhi. Their unconditional love and blessings has always been my pillar of strength and motivation. Without these people this dissertation would not have been attainable.
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Chapter 1

Introduction

1.1. Background

In the United States coal was the leading source of energy from 1885 to 1950. Coal was replaced by natural gas and crude oil in 1951 due to major political and safety issues in coal mining [1, 2]. Production and consumption of natural gas and crude oil quadrupled from 1950 to the 1970’s. The striking imbalance in US production and consumption of petroleum in 1980’s led to a rising dependence on foreign crude oil. With the oil embargo in 1970, concerns were raised about the energy security in the United States [3]. US import of crude oil and other petroleum products has steadily increased from six million barrels per day in 19th century to eleven million barrels per day in the 21st century [4].

The United States imports 60% of its total petroleum consumption and this rate is considered to be steadily increasing [5]. The alarming crude oil import and consumption could be a threat to the country’s economic security. Release of CO$_2$ from combustion of
crude oil is a major environmental concern. The US produces about 25% of the total CO$_2$ from burning fossil fuel and is projected to lower the carbon content by 25% from 2001-2025 [6]. Fossil fuels are CO$_2$ emitters due to their high carbon content. Only half of the CO$_2$ emitted from fossil fuels are utilized by natural processes [7]. The other half impacts the environment resulting in global warming, regional reduction in precipitation and melting of polar zones [7, 8]. The CO$_2$ released from biomass consumption is assumed to be balanced by the CO$_2$ removed from the atmosphere to grow biomass [9]. A reduction in the CO$_2$ emissions is possible only if the fossil fuel is substituted by renewable energy resources [9].

The renewable energy resources are the energy sources that are continuously replenished. These include the energy from sun, wind, water, biomass and geothermal sources. The biomass feedstocks such as crops, grassy and woody plants, algae, agriculture and forest residue and organic component of industrial and municipal residue is the second most important source of renewable energy in the United States due to wide availability. In particular, conversion of lignocellulosic material (biomass) to fuels provides a renewable non-food based feedstock which can provide for energy security in the USA [10].

1.2. **Biofuel from biomass**

Biomass is commonly plant material that can generate heat and electricity. Conversion technologies release energy from biomass in the form of heat, electricity, biofuel or biogas [11]. Bio-ethanol is a form of renewable energy produced from carbon
based sources (biomass). Ethanol is produced in large quantities from natural resources like corn grain, wheat, sugarcane, barley, oats, sorghum and rice. In 2001, about 62% of the global annual bio-ethanol production was from sugarcane in Brazil and from corn grain in the USA [12]. Ethanol production from food crops results in an imbalance in the crops for food versus crops for energy. Therefore, there is a need to find an inexpensive alternative source to avoid competition with world food resources/prices. An alternative renewable and sustainable source is lignocellulosic biomass [13]. It is widely available at low cost and has the potential to be carbon neutral or carbon negative source of bio-energy when coupled with carbon sequestration [14]. Ethanol produced from lignocellulosic biomass has desirable features as a petroleum substitute and could help make a smoother transition from a petro to a bio-based chemical industry [15]. The components of biomass can serve as a source of carbon based feedstock for fuel and chemical production in much the same way that crude oil serves as the carbon feedstock in petrochemical refineries [16-19].

1.3. **Lignocellulosic ethanol viability**

The Energy Independence Security Act of 2007 (EISA) established a mandate for annual U.S. production of 36 billion gallons of ethanol by 2022 from renewable resources out which 15 billion gallons may come from corn based technologies and 16 billion gallons may come from lignocellulose based technologies [20]. The President’s 2006 Advanced Energy Initiative (AEI) seeks to replace 30% of the nation’s dependence on foreign oil with domestic resources by 2030. The Department of Energy (DOE) joint
research agenda 2006 [5], predicted that by the year 2030, 30% of the US gasoline consumption will be displaced by ethanol. In 2004, 11% of US corn harvest yielded ~ 1.7% of the transportation fuel demand [5]. The energy requirements cannot be met even with 100% of corn harvest that could, at best, yield only 15% of fuel demand. The alternative approach to meet the US energy initiatives is cellulosic ethanol. To meet 30% of the US gasoline consumption, one billion dry tons of biomass per year must be available. The United States Department of Agriculture (USDA) estimates that 1.4 billion tons of biomass could be available by the middle of 21st century that could reduce the dependence on foreign oil [21].

1.4. Ethanol from corn and lignocellulose residues

Ethanol produced from corn is called a first generation biofuel and ethanol produced from lignocellulosic biomass is called a second generation biofuel. Corn is a plant material rich in starch whereas lignocellulosic biomass includes plant material left behind after harvest of food grains [12]. The energy used to produce ethanol and the energy produced from ethanol is greatest for corn compared to the lignocellulosic biomass residues. The economics are favorable due to the high ethanol yields from corn. The trade off’s in the ethanol production from corn are greater soil erosion and nitrogen leaching due to the very high nutrient requirements compared to lignocellulosic crops [22]. Despite the technical complexities involved in production of ethanol from plant derived residues, they are particularly attractive due to the low cost raw material and wide availability [23]. Sustainable technologies are needed to convert this non-starch
based feedstock not only into ethanol but also to other bio-based products and energy. It was estimated that the global total potential bio-ethanol production from crop residues and waste crops is 491 billion liters per year, about 16 times higher than the current world ethanol production. Lignocellulosic biomass has the potential to replace 32% of global gasoline consumption when used as E85 fuel (Ethanol blend of 85% in gasoline) in midsized motor vehicles [12].

1.5. Availability of lignocellulosic biomass residues

Lignocellulosic biomass feedstock is widely available in the form of agricultural and forest residues, newspaper waste, biological urban wastes residues such as municipal solid waste and hospital generated waste. The energy crops (crops dedicated for biofuel production) have the potential to grow on abandoned and degraded crop land of low quality. 320-702 million hectares of such lands are available in countries Africa, China, Europe, India, South America, and the United States[24]. Planting the second generation of biofuel feedstocks on abandoned and degraded cropland or grassland with marginal productivity may fulfill 26-55% of the current world liquid fuel consumption, without affecting the use of land with regular productivity for conventional crops and without affecting the current pasture land [24].

1.6. Processing of biomass to bio-ethanol

Terrestrial biomass (lignocellulose) is composed of three major components; cellulose (~30-45%), a highly crystalline polymer of glucose; hemicellulose (~20-30%), a
complex polymer of xylose and other sugar derivatives; and lignin (5-20%), a polyphenyl propanoid macromolecular assembly that is covalently cross-linked to hemicellulose [25]. The processing of lignocellulosic biomass to fuels can be done via a biochemical (sugar) platform or a thermochemical platform. In a biochemical platform, the biomass is hydrolyzed into its component sugars by acid or enzymes and the sugars are fermented by micro-organisms to ethanol or converted to other value added products. The thermochemical platform uses heat and pressure based conversions through pyrolysis or gasification to produce ethanol, other alcohols, hydrogen, hydrocarbon fuels, chemicals and power.

1.7. **Biochemical conversion of biomass to sugars**

Ethanol fuel is produced from biomass through a biochemical platform consisting of sequential or simultaneous steps. These include (i) pretreatment to produce a more easily hydrolyzable substrate; (ii) enzyme hydrolysis to convert cellulose and hemicellulose components to their sugar monomers; and (iii) fermentation of sugars to ethanol [26]. The enzymatic route is a viable strategy because of minimal by-product formation, low energy requirements and a potentially environmentally friendly process. Although currently high cost, it has better long term potential for cost reductions than other processes such as concentrated acid or two stage dilute acid process [27-29].

The enzymatic conversion of biomass to sugars involves a core barrier, the recalcitrance of highly crystalline cellulose to enzymatic degradation. Pretreatment is required to alter the structure and chemical composition of biomass to facilitate
hydrolysis of sugars by the enzymes [29, 30]. Numerous pretreatment technologies were developed over a decade to efficiently hydrolyzed the biomass to sugars and ferment the sugars to ethanol and other chemicals [31-34]. The pretreatment technology used in this dissertation research is based on ionic liquids.

1.8. Ionic liquid pretreatment

The ionic liquid (IL) 1-ethyl-3-methyl imidazolium acetate, EmimOAc, was used in the studies described in this dissertation due, in part, to its low melting temperature (~ -20°C) and low viscosity, leading to ease of handling. The molecular structure of IL is as shown below.

![Molecular structure of EmimOAc IL](Source: Sigma Aldrich)

Figure 1-1 Molecular structure of 1-ethyl, 3-methyl imidazolium acetate IL.

The imidazolium group is substituted with relatively short alkyl chains. Imidazolium based ILs with shorter alkyl chains have been found to be less toxic than those with longer alkyl side groups [35]. Additionally, the acetate anion renders the IL less corrosive than ILs with halide anions that are also effective in cellulose dissolution.
Ionic liquid (IL) pretreatment has been found as a promising technology in converting lignocellulosic biomass to ethanol fuel. However, the cost associated with the IL’s currently prohibits its use on commercial scale. The recyclability and reuse of IL without degrading its solvent properties is a challenge. Ionic liquids can be used effectively only if processes are developed to recover IL completely. The recovery of IL and reuse is possible but often involves high energy and cost.

IL pretreatment has the potential to enhance the enzymatic digestibility of cellulose by a factor of three compared to the non IL treated lignocellulose substrates due to the increase in porosity of the substrate and solubilization and redistribution of lignin components [26, 36-38]. This enhancement in digestibility of cellulose is attributed to the decrease in the crystallinity of cellulose [37]. During and after the IL treatment the structure of cellulose undergoes polymorphic transformations. The changes in the structure of cellulose alter the accessibility of the substrate to the enzymes [39, 40].

The process schematic involving the production of ethanol from biomass pretreated with ionic liquid is shown (Figure 1-2).
Figure 1-2. Overall process flow diagram of production of ethanol from IL pretreated lignocellulosic biomass.

1.9. Challenges in ethanol production from lignocellulosic biomass

The major challenge involved in the first step of the sugar platform is to produce a substrate that is readily degradable by enzymes. Choice of an effective pretreatment process is essential. Ideally, the pretreatment process should be done in such a way that it can alter the cellulose structure to enhance its digestibility without losing the sugars to the pretreatment liquor. Ionic liquid pretreatment is one such process.

Enzymatic hydrolysis can be expensive due to the high cost of enzymes. Technical strategies are required for optimization of the enzyme loadings and reuse of
enzymes to make the process economical. A crucial challenge here is to completely hydrolyze both cellulose and hemicellulose fractions at high biomass concentrations with minimal enzyme use. Unlike starch hydrolysis from corn which requires a single enzyme, lignocellulosic biomass requires synergistic action of a multiple enzymes. Identification of optimum enzymes mixtures for efficient hydrolysis of the complex components of lignocellulosic biomass involves in depth knowledge of the specific enzyme activities.

A major hurdle in biomass-to-ethanol conversion process is the fermentation of mixed sugars (glucose and xylose) by native micro-organisms. Corn based hydrolyzates are relatively easy to handle due to the existence of single sugar, glucose. Most industrial strains can readily metabolize glucose. Xylose cannot be metabolized unless alternative strategies are developed. Lack of suitable micro-organism in metabolizing both glucose and xylose sugars, research efforts were diverted in developing genetically modified strains (GMO’s) that can withstand robust industrial conditions. The long term viability of such strains is ambiguous and this dissertation addresses alternative strategies that can be used in metabolizing IL pretreated mixed sugar hydrolyzates with native yeast strain *P. stipitis*.

*P. stipitis* is a xylose fermenting yeast and experiences diauxic lag during mixed sugar fermentations. The diauxic lag can be avoided by priming the yeast cells with xylose rich streams in addition to growing the cells on xylose as opposed to glucose. A sequential or simultaneous saccharification with xylanase and cellulase and fermentation
with yeast is developed to successfully ferment both glucose and xylose without diauxic lag.

1.10. Organization of the dissertation

A goal of this dissertation is to produce ethanol through the ‘sugar platform’ from lignocellulosic biomass using an ionic liquid (IL) pretreatment step and biochemical conversion of polysaccharides to monomeric sugars. The research objectives involved in this dissertation focused on addressing the effect of IL treatment on cellulose structure and composition of biomass and induced changes in enzymatic digestibility. Evaluation was made of the performance of commercial enzyme mixtures through saccharification of different IL pretreated substrates at low and high biomass slurry concentrations. IL pretreated hydrolyzates were fermented with *P. stipitis* to produce ethanol through a sequential or simultaneous saccharification with xylanase and cellulase.

Literature pertaining to the major focus of each chapter is outlined in the introduction section and conclusions for the work were provided at the end of all the chapters.

Chapter 2 describes changes in the cellulose structure of various lignocellulosic biomasses with ionic liquid pretreatment. These changes were tracked with X-ray fiber and powder diffraction. A comparative enzymatic hydrolysis was performed to link the changes in the enzymatic hydrolysis with respect to the cellulose structure.

Chapter 3 also deals with the similar research objective as chapter 2. Raman spectroscopy was used to track the structural changes to corroborate the X-ray diffraction
results. A similar comparative enzymatic hydrolysis was performed in relation to the cellulose structural changes.

Chapter 4 briefly outlines the performance of different commercial enzyme mixtures through saccharification of various IL pretreated biomass at low biomass loadings and specific enzyme activity data.

Chapter 5 identifies the sugar concentrations and enzyme mixtures required in the fermentation step with *P. stipitis* through the sequential saccharification of IL pretreated poplar at high biomass loadings with xylanase and cellulase.

Chapter 6 examines approaches for fermenting IL pretreated poplar with *P. stipitis*. Sequential or simultaneous saccharification with xylanase and cellulase and fermentation was performed in this chapter. Pure sugar fermentation was also carried out to assess the maximum theoretical ethanol yields.

Chapter 7 briefly outlines possible future challenges of investigation.
Chapter 2

Ionic Liquid Induced Changes in Cellulose Structure Associated with Enhanced Biomass Hydrolysis

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2.1. Introduction

Terrestrial lignocellulosic biomass is composed of three major components; cellulose, a linear polymer of the hexose sugar, glucose; hemicellulose, a complex branched polymer of the pentose sugar, xylose, and other sugar derivatives; and lignin, a polyphenyl propanoid macromolecular assembly that is covalently cross-linked to
hemicellulose. The cellulosic component has a crystalline and fibrillar structure, which is partially oriented in the thick secondary cell wall; whereas the hemicellulose and lignin components have less ordered structures that encrust the cellulose fibers. These components can serve as a source of carbon based feedstock for fuel and chemical production in much the same way that crude oil serves as the carbon feedstock in petrochemical refineries [41]. In particular, the sugars derived from the cellulosic and hemicellulosic portions of biomass can be converted to platform chemicals and products through fermentation or chemical conversion of hexose and pentose sugars. Lignocellulosic biomass is a non-food based renewable resource that includes agricultural and forestry residues (e.g. wheat straw, corn cobs and stover), municipal waste (paper products), herbaceous (e.g. switchgrass), hardwood (e.g. poplar trees) and softwood (e.g. pine) crops [42]. Growth of biomass crops, such as wild grasses in mixed species plots, can provide a haven for wildlife, promoting biodiversity exceeding the monoculture plots needed for grain production [43].

The deconstruction of lignocellulosic biomass into simple sugars constitutes a core barrier for producing value added products from the sugar platform [44]. Several approaches are being developed to pretreat lignocellulosic biomass so that this deconstruction can be achieved more efficiently, including dilute acid, steam explosion, hydrothermal processes, organic solvents in aqueous media, biological and enzymatic processes, ammonia fiber [45-47] explosion (AFEX), strong alkali processes, and highly-concentrated acid treatment. The choice of pretreatment technology has a major impact
on the physiochemical properties of biomass. These properties in turn profoundly affect downstream processing including enzyme selection for saccharification, ease of microbial or chemical processing, sequencing of conversion and separation steps and by-product and waste formation.

Pretreatment can affect hemicellulose hydrolysis, lignin dissolution or redistribution and increase porosity of constituent polysaccharides (cellulose and hemicellulose) providing access for hydrolytic enzymes. The crystalline structure of native cellulose fibers, called cellulose I (and referring collectively to two naturally occurring allomorphs, Iα and Iβ) [48, 49] can be a major impediment to its hydrolysis to monomeric sugars [44]. Conversion of cellulose fibers to other crystal forms, such as cellulose II [50, 51], cellulose III1 [52] and cellulose III1 [53], or amorphous forms can greatly improve their susceptibility to hydrolysis [54].

Ionic liquid (IL) pretreatment is an emerging technology in which biomass is incubated in an IL, followed by IL displacement with anti-solvent, forming what is thought to be a largely amorphous cellulose substrate that is rapidly hydrolyzed into its glucose and xylose subunits [26, 55, 56]. On drying, amorphous cellulose can partially re-crystallize into various allomorphs. The improvement in hydrolysis is hypothesized to be primarily due to structural changes in the cellulose constituent [57]. In the studies presented here, X-ray powder diffraction (XRD) and X-ray fiber diffraction on biomass samples with unoriented and partially (fiber) orientated cellulosic components, respectively, is used to investigate the effects of varying IL pretreatment parameters
(incubation time, temperature and substrate) on the cellulosic structure. The fiber samples corresponded to intact fragments of naturally occurring biomass which have intrinsic texture, whereas the unoriented samples were ground and sieved to remove any naturally occurring texture. The advantage of collecting fiber diffraction data in addition to XRD data is that the diffraction is more resolved and; therefore, weaker features can be more easily detected. After collection of powder and fiber data, comparative enzymatic hydrolysis and sugar analysis were used to relate the observed changes in cellulose structure to biomass digestibility.

2.2. Materials and methods

2.2.1 Materials

The IL 1-ethyl, 3-methyl imidazolium acetate (EMIM-Ac) was purchased from Sigma Aldrich, (St. Louis, MO, USA). Three different biomass sources were investigated: poplar, switchgrass and cornstover. Poplar samples were provided by the National Renewable Energy Laboratory (Golden, CO, USA) and cornstover and switchgrass samples were provided by the United States Department of Agriculture (USDA, NCUAR Laboratory, Peoria, IL, USA). Two model cellulose samples were also examined to help with interpretation of the X-ray data collected from the biomass samples; a highly crystalline, oriented, fiber sample of almost pure cellulose prepared from dewaxed ramie fibers, purchased from a textile dealer; and a highly crystalline, unoriented, polycrystalline powder of almost pure cellulose, Avicel, (PH-101, from Fluka
chemicals, MO, USA). Commercial enzymes, Spezyme CP and Novozyme 188 were obtained from Genencor, (Rochester, NY, USA) and Sigma Aldrich, (St. Louis, MO, USA), respectively.

2.2.2 Fiber diffraction

Partial (fiber) orientation of cellulose occurs naturally in lignocellulosic biomass. Thin fragments of intact poplar, switchgrass and corn stover biomass suitable for fiber diffraction were incubated in IL at 50°C for 12 to 14 hours; and 120°C for one hour (by immersing a glass vial in a temperature controlled oil bath). After incubation, samples were rinsed with water to displace IL and dried at 50°C. Fiber diffraction data were collected from the pretreated samples at room temperature using an in-house Rigaku FR-E with R-Axis IV++ detector. In order to help with interpretation of the X-ray fiber data collected from the biomass samples, fiber diffraction data were collected from the model ramie cellulose fibers in their naturally occurring cellulose I phase, and after mercerization with NaOH in their cellulose II phase. Some of these data were collected at the Advanced Photon Source at Argonne National Laboratory, BioCARS BM-14-C, with an ADSC Q316 Detector. The ramie fibers were mounted tautly around a frame in order to preserve their orientation during mercerization and data collection using previously described methods [50].
2.2.3 **X-ray powder diffraction**

Poplar, switchgrass, and corn stover biomass samples were ground and sieved (−20/+80 sieve cut) and then incubated with IL at 5% (w/w) for 12-14 hours at 50°C, and for an hour at 120°C, followed by washing with water to displace IL and drying at 50°C. The purpose of the grinding and sieving process is to remove natural texture and, therefore, partial orientation from the samples. X-ray powder diffraction (XRD) data were collected at 25°C with an X’PERT PRO powder diffractometer PAN 188 analytical with X’celerator detector using Nickel filtered Cu Kα radiation. Samples were scanned over the range of 5–35° (2θ), with a step size of 0.05 and step time of 10 seconds. Crystallinity indices, CrI, were calculated using previously described methods [58].

In order to help with interpretation of the XRD data from the biomass samples, XRD data were collected from the model Avicel cellulose powder in its naturally occurring cellulose I phase, and after mercerization with sodium hydroxide (NaOH) in its cellulose II phase. Avicel was mercerized by incubation in 4.25 N NaOH solution at 65°C for 24 hours. The solution was neutralized with hydrochloric acid, and then thoroughly washed with water.

2.2.4 **Enzymatic hydrolysis and compositional analysis**

Enzymatic hydrolysis followed NREL standard LAP 009 protocol [59] with enzyme loadings of 15 FPU/ g glucan of Spezyme CP and 30 CBU/g glucan of Novozyme 188. The hydrolysis was run at 1% (w/v) solid loadings at 50°C in 50mM
sodium citrate buffer for 24 hours in a New Brunswick Scientific rotary water bath shaker (Thermo Fischer Scientific, PA, USA) at 200rpm. The compositional analysis for the biomass samples before and after the pretreatment was carried out using NREL standard LAP 002 protocol [60].

2.2.5 HPLC analysis

The released sugars in enzymatic and compositional analysis were analyzed by HPLC with refractive index detection on a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate analysis column. The mobile phase was HPLC grade water at a flow rate of 0.6 ml/min with a column temperature of 80-85°C. Mixed sugar standards of known concentrations were used to generate standard curves in order to calculate the concentration of released sugars. Glucose and xylose released from glucan and xylan, respectively, were reported as a percentage of theoretical yields of monomeric sugars based on glucan and xylan analysis of untreated substrates.

2.3. Results and Discussion

2.3.1 Identification of cellulose I and cellulose II

The X-ray fiber diffraction and XRD data collected from the cellulose I and cellulose II phases of the model cellulose fiber (ramie) and powder (Avicel) samples provide a key to interpret the data collected from biomass samples, Figure 2-1. One of the largest differences between diffraction from the two phases can be seen on the equators (Miller index l = 0) of the fiber diffraction images. In diffraction from cellulose I, there is
a doublet of reflections close to the beam center and a single dominant reflection at higher angle. This pattern is reversed in cellulose II, consisting of a single reflection on the equator closer to the beam center and a doublet at higher resolution. This arrangement can also be seen in the XRD scans from the two phases, although the equatorial diffraction is complicated by the superposition of diffraction features from higher layer-lines. Representative Miller indices for these reflections ((1-10), (110) and (200) for cellulose I and (1-10), (110) and (020) for cellulose II) are labeled in Figure 2-1, although there are overlapping contributions from others. The arrangement of these equatorial reflections serves to identify the presence of the two phases in both powder and fiber diffraction data.
Figure 2-1  Identification of cellulose I and cellulose II: Panels A and B are X-ray fiber diffraction images of ramie (cellulose I) and mercerized ramie (cellulose II) controls. Panel C is X-ray powder diffraction of Avicel (cellulose I) and mercerized Avicel (cellulose II) controls. The reflections have been labeled with their Miller indices to highlight distinct differences between cellulose I and cellulose II.

2.3.2 Biomass pretreatment at 50 and 120°C

X-ray fiber diffraction and XRD data collected from fiber and powdered samples of all three types of biomass after IL pretreatment at 50°C and 120°C are shown in Figure 2-2. After pretreatment at 50°C, all biomass samples have re-crystallized in the cellulose I phase with the fiber samples retaining a significant amount of cellulose fiber orientation. On the other hand, all biomass samples pretreated at 120°C re-crystallized in a form that most closely resembled cellulose II, with only the switchgrass fiber sample
retaining a significant amount of cellulose fiber orientation and evidence of residual cellulose I.
2.3.3 Intermediate structure of cellulose I

Weak fiber diffraction features were observed during the re-crystallization process with biomass samples after pretreatment at 50°C and washing with anti-solvent, but before the samples had completely dried. In particular, a low angle equatorial reflection corresponding to a real-space dimension of ~11.5 Å was clearly observed and is indicated by arrows in Figure 2-3. This feature was not detected in the XRD data from biomass samples.
Figure 2-3  Biomass intermediate structure formation at 25°C IL pretreatment: Panels A, B and C are the X-ray fiber diffraction images of an intermediate structure observed in re-crystallizing corn stover, poplar and switchgrass, respectively, after IL dissolution at room temperature followed by a water wash.

In order to investigate the possibility that these unexpected diffraction features correspond to an unknown intermediate ordered complex of cellulose and IL, further X-ray powder and fiber diffraction data were collected from the model ramie and Avicel cellulose samples after pretreatment with IL. XRD scans of Avicel powders pretreated with IL at 50°C (Figure 2-2) indicated that the cellulose recrystallized as cellulose II with no indication of the intermediate diffraction features. In contrast, fiber diffraction from ramie cellulose fibers treated at 23°C and 50°C reproduced the intermediate diffraction features, as shown in Figure 2-4. If these ramie fibers were kept under tension during the entire pretreatment process, the cellulose recrystallized in the cellulose I phase.
Figure 2-4  Ramie intermediate structure formation at 25 and 50°C IL pretreatment: Panels A and B are the X-ray fiber diffraction images of intermediate structures in re-crystallizing ramie samples after IL dissolution at room temperature and 50°C, respectively, followed by a water wash.

However, if the fibers lost tension during addition of the IL, they appeared to completely dissolve, resulting in a gelatinous mass following addition of an anti-solvent. When dried, this material showed no fiber orientation and resulted in a powder diffraction pattern (Figure 2-5) that most closely resembles cellulose II.
2.3.4 Comparative Enzymatic Hydrolysis

To evaluate the role of cellulose structure on substrate digestibility, the enzymatic hydrolysis of cellulose and biomass to monomeric sugars was assessed. The hydrolysis was more complete for Avicel samples of cellulose II (mercerized with NaOH) than for cellulose I as reported by others [40] (Table 2.1). The cellulose II sample from mercerized Avicel had a lower crystallinity index compared to cellulose I.

Table 2.1 Enzymatic hydrolysis of cellulose I and cellulose II: ‘Native’ Avicel, cellulose I and cellulose II (mercerized with NaOH) were enzymatically hydrolyzed with 15 FPU/ g glucan Spezyme CP and 30 CBU/g glucan Novozyme 188 at 1% (w/v) solid loading. All the measurements were an average of triplicates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Glucose Yield</th>
<th>% CrI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>Cellulose I</td>
<td>7±5</td>
<td>35±11</td>
</tr>
<tr>
<td>Cellulose II</td>
<td>39±4</td>
<td>87±9</td>
</tr>
</tbody>
</table>
Powdered biomass samples treated with IL at 50 or 120 °C were also hydrolyzed. Differences in digestibility of substrates for wet samples and those dried post IL expulsion were assessed (Table 2.2). Biomass samples treated with IL at 50°C (cellulose I) exhibited lower 24 hour hydrolysis yields of monomeric sugars compared to samples treated at 120°C. There appears to be no large differences in digestibility of wet and dry substrates. However, wet samples produced consistently higher monomeric sugar yields than dried samples.

Table 2.2  Enzymatic hydrolysis of wet and dry biomass treated with IL at 50 and 120°C: Biomass pretreated with IL at 50 or 120°C and enzymatically hydrolyzed by 15 FPU/g glucan Spezyme CP and 30 CBU/g glucan Novozyme 188 at 1 % (w/v) solid loading. Wet: Hydrolysis on wet substrate after IL treatment and displacement with water. Dry: Hydrolysis of substrates dried after IL treatment and displacement with water. Results are an average of duplicates with less than 8% deviation between samples.  

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wet % Glucose 24h</th>
<th>Wet % Xylose 24h</th>
<th>Dry % Glucose 24h</th>
<th>Dry % Xylose 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poplar 50°C</td>
<td>45</td>
<td>24</td>
<td>37</td>
<td>20</td>
</tr>
<tr>
<td>Poplar 120°C</td>
<td>95</td>
<td>81</td>
<td>87</td>
<td>73</td>
</tr>
<tr>
<td>Cornstover 50°C</td>
<td>51</td>
<td>30</td>
<td>45</td>
<td>27</td>
</tr>
<tr>
<td>Cornstover 120C</td>
<td>105</td>
<td>72</td>
<td>76</td>
<td>56</td>
</tr>
<tr>
<td>Switchgrass 50°C</td>
<td>29</td>
<td>12</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>Switchgrass 120°C</td>
<td>86</td>
<td>48</td>
<td>80</td>
<td>42</td>
</tr>
</tbody>
</table>

The 24 hour yields of monomeric sugars for IL pretreated biomass increased by a factor of about three or more at a treatment temperature of 50°C and by a factor of more than six at 120°C over that of untreated substrate. The yields of glucose and xylose after 24 hour hydrolysis were: both 6% for untreated poplar; 18% and 8% respectively for
untreated corn stover; and 13% and 3%, respectively, for untreated switchgrass. Increasing the incubation temperature of IL pretreatment resulted in nearly complete conversion of polysaccharides to monomeric sugars with negligible differences in lignin content (Table 2.3).

Table 2.3  Lignin contents and CrI of biomass treated with IL at 50 and 120°C: The weight percent of glucose, xylose and lignin in native and IL-treated (at 50°C and 120°C) poplar, switchgrass and corn stover are given below. Lignin is the sum of acid soluble and acid insoluble lignin. All the measurements are an average of triplicate samples except for switchgrass and corn stover measurements at 50°C. These are an average of duplicate samples with less than 6% deviation. The crystallinity indices were calculated on the dry substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Glucose</th>
<th>% Xylose</th>
<th>% Lignin</th>
<th>CrI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poplar</td>
<td>37±1.4</td>
<td>11±0.6</td>
<td>24±0.4</td>
<td>38</td>
</tr>
<tr>
<td>Poplar-50°C</td>
<td>30±3</td>
<td>11±1</td>
<td>23±6</td>
<td>19</td>
</tr>
<tr>
<td>Poplar-120°C</td>
<td>29±8</td>
<td>10±2</td>
<td>25±3</td>
<td>22</td>
</tr>
<tr>
<td>Cornstover</td>
<td>28±4</td>
<td>14±2</td>
<td>20±5</td>
<td>23</td>
</tr>
<tr>
<td>Cornstover-50°C</td>
<td>32</td>
<td>16</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Cornstover-120°C</td>
<td>25±1</td>
<td>11±1</td>
<td>19±3</td>
<td>29</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>28±3</td>
<td>15±3</td>
<td>21±6</td>
<td>21</td>
</tr>
<tr>
<td>Switchgrass-50°C</td>
<td>19</td>
<td>10</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>Switchgrass-120°C</td>
<td>20±2</td>
<td>12±1</td>
<td>22±1</td>
<td>26</td>
</tr>
</tbody>
</table>

2.4. Discussion

Both X-ray powder and fiber diffraction data show that, following IL displacement with water and drying, lignocellulosic biomass pretreated with IL re-crystallizes to cellulose I under mild conditions (50°C or less) and to cellulose II under
harsh conditions (120°C). Increasing the incubation temperature during pretreatment does not result in any major differences in the crystallinity indices of the cellulose I or cellulose II components or in the lignin content of biomass samples (Table 2.3). The hydrolysis yields are significantly greater (by a factor of about 2) for the pretreated biomass samples that contain cellulose II compared to those containing cellulose I. This observation is true regardless of whether the samples are wet or dry (Table 2.2). Therefore, one of the clearest factors associated with enhanced hydrolysis of the IL pretreated biomass observed in this study is the transformation of cellulose I to cellulose II.

Cellulose I in Avicel, which is lignin-free, is easily converted to cellulose II by mild IL pretreatment at lower temperature. Cellulose in ramie fibers (also having low lignin content (~1%) [61]) will recrystallize to cellulose I if the fibers are kept under mechanical tension. In comparison, this same substrate will be converted to cellulose II if the fibers lose tension and are allowed to fully dissolve during mild IL pretreatment at low temperature conditions. The biomass samples which contain relatively large amounts of lignin (~20%, Table 2.3) require higher temperature IL pretreatment in order to drive the transformation of cellulose I to cellulose II.

The dependence on lignification and mechanical tension of the IL induced conversion of cellulose I to cellulose II in biomass is remarkably similar to the behavior of cellulose in biomass during mercerization with NaOH. In the X-ray fiber diffraction studies of Revol & Goring [62] it was found that cellulose I in spruce wood (containing
~20% lignin) [63] treated with NaOH only partially converted to cellulose II, whereas complete conversion occurred in Kraft pulp, in which much of the lignin had been removed. Two possible contributing factors to this behavior were identified.

First, the presence of lignin may restrict the NaOH swelling of cellulose in wood so that it does not completely dissolve. The residual presence of some association of parallel oriented chains similar to that found in cellulose I, may promote nucleation of cellulose I rather than cellulose II when the alkali is washed out. Electron and X-ray fiber diffraction studies of cellulose mercerization with NaOH have also provided evidence for recrystallization to cellulose I rather than cellulose II in laterally compressed regions of ramie samples [64]. In their X-ray fiber diffraction studies of the conversion of cellulose I to cellulose II via the formation of Na-cellulose I, Nishiyama et al. noted that cellulose I could be regenerated from Na-cellulose I when swelling of cellulose in ramie fibers is restricted [65].

Secondly, in the model of the cell wall of wood proposed by Kerr & Goring (1975) [66], lignin encrusts and separates cellulose fibers that have opposite polarity. Within each cellulose I fibril the cellulose chains have parallel orientations, and therefore the fibrils themselves have an overall polarity. However within crystallites of cellulose II the chains are antiparallel with no overall polarity. Blackwell et al. proposed that conversion of cellulose I to cellulose II requires that chains migrate between fibers that have opposite polarities [67]. The presence of lignin in between fibers of opposite polarities prevents this from occurring.
If the above explanations for the behavior of cellulose during NaOH mercerization are also be relevant to the behavior of cellulose in biomass during treatment with IL at different temperatures then it seems clear that some type of change in lignin structure or distribution must occur at elevated temperatures. The experimental results presented here indicate that there is no loss of lignin during IL treatment (Table 2.3) but they provide no direct evidence for the nature of any structural change to lignin. Electron microscopy of biomass pretreated with dilute acid at high temperatures revealed the presence of droplets on the biomass surface attributed to a redistribution of lignin [68]. It was proposed that lignin coalesces and moves through the cell wall matrix and forming surface droplets at elevated temperatures. In recent imaging studies of biomass AFEX pretreatment it was shown that cell wall decomposition products, such as lignin-based phenolics, are redistributed to the outer cell wall surfaces, thus creating highly porous structures which may enhance enzyme access to cellulose [69]. However, neither AFEX nor dilute acid pretreatment promotes a conversion of cellulose I to cellulose II.

Although the same type of pore forming process may be occurring during IL treatment at elevated temperatures, it is also possible that more dramatic changes are taking place that allow complete and unrestricted cellulose swelling, including the melting of lignin. The glass transition temperature, $T_g$, of lignin has been found to vary from 100 to 160°C depending on the monolignol chemical composition and biomass source [68, 70-72]. In the fiber diffraction studies reported here, it was observed that the cellulose in switchgrass pretreated at higher temperature (120°C) retained a significant
amount of orientation during re-crystallization whereas the cellulose in corn stover and poplar did not. This difference may be due to a higher T$_g$ in switchgrass. Arora et al. report that switchgrass treated with IL under a range of temperatures from 100 to 160°C yielded the highest initial hydrolysis rates when treated with IL at 160°C pointing to a high T$_g$ of lignin in switchgrass [70].

The explanation that lignin is restricting the swelling of cellulose during IL pretreatment at low temperature, is in agreement with previous imaging studies, [39] and makes the observation of a low-temperature, intermediate ordered structure of cellulose and IL particularly interesting, because it suggests that IL mediates the recrystallization of the native cellulose to cellulose I or cellulose II.

Possible identification of the process of intermediate formation can be seen in the ramie re-crystallization diffraction data shown in Figure 2-4. In these diffraction images, there is a strong equatorial diffraction feature near the position of (200) reflection in cellulose I. This feature corresponds to the distance between hydrophobically stacked pyranose rings (~4.5 Å). Furthermore, there is a pronounced reduction or absence of diffraction at the positions of the (110) and (1-10) cellulose I reflections. There is also the appearance of strong diffraction corresponding to a spacing of ~11.5 Å. This spacing is much larger than the maximum equatorial unit cell parameters of cellulose I (cellulose I$_\beta$ has space group $P2_1$ with a reduced unit cell of $a = 7.784$ Å, $b = 8.201$ Å, $c = 10.38$ Å, and $\gamma = 96.5^\circ$) or cellulose II (space group $P2_1$ with a reduced unit cell of $a = 8.10$ Å, $b = 9.03$ Å, $c = 10.31$ Å, and $\gamma = 117.10^\circ$) [49, 50].
One interpretation of these new diffraction features is that the re-crystallizing cellulose orders with initial assembly of chains that are stacked with their glucose monomers face to face. These stacks of chains would appear to associate with each other, side by side, with a spacing of ~11.5Å, as shown in Figure 2-6. In between the stacks of chains, it is possible that IL solvent molecules are present and interact with the hydroxyl groups displayed at the edges of the stacks. This presence of IL solvent and interaction with hydroxyl groups is similar to the incorporation of solvent molecules between stacks of cellulose chains in cellulose swollen by liquid ammonia [73, 74] or EDA [75]. The low resolution reflection corresponding to the spacing of ~11.5 Å tends to be smeared towards the beam center, indicating a large range of side by side distances between the hydrophobic stacks of chains possibly caused by IL solvent. It is interesting to note that diffraction with low resolution features can also be found during the mercerization of cellulose as it adopts various complexes with Na and water, as most recently reported by Kobayashi et al., (2011) [76].
Figure 2-6  Schematic representation of IL penetration, IL displacement with water and recrystallization of cellulose fibers: The ellipses represent pyranose rings and pentagons represent the IL. The figure depicts the formation of IL-cellulose complex upon application of IL to the cellulose fibers and the formation of disordered cellulose upon further incubation in IL. After cellulose incubation in IL for a desired period of time and expulsion with water, an intermediate structure is formed with expansion of cellulose fibrils and IL intercalation between the cellulose fibrils. Then upon further washing and drying, the cellulose recrystallizes to either cellulose Iβ or cellulose II depending on the severity of the IL treatment.
2.5. Conclusion

In summary, conversion of cellulose I to cellulose II is an attribute that increases biomass digestibility. This conversion is enhanced during IL pretreatments at higher temperatures. One explanation for this behavior is that lignin is restricting the swelling of cellulose during IL pretreatment at low temperature. At higher temperatures, there would appear to be a change in lignin that allows for complete cellulose dissolution. These results suggest that selection of biomass feedstocks with lower lignin glass transition temperatures and may promote the conversion of cellulose fibers to cellulose II and improve pretreatment efficiency and biomass digestibility.

2.6. References


Chapter 3

Comparative Structural and Hydrolysis Studies of Ionic Liquid Treated Cellulose

3.1. Introduction

The structural changes in cellulose and lignocellulose with ionic liquid pretreatment were studied using X-ray fiber and powder diffraction techniques (Chapter 2). In this chapter, structural studies of IL treated cellulose were performed on pure cellulose substrates, ramie and Avicel, using Raman Spectroscopy in addition to assessment of enzymatic digestibility, to corroborate the X-ray fiber diffraction data. Comparative enzyme hydrolysis for cellulose Iβ and cellulose II was also performed to assess the differences in digestibility of these substrates.
Ramie and Avicel were used in our studies. The composition of both are approximately 80% (w/w) cellulose and less than 2% (w/w) lignin [77]. Cellulose in ramie and Avicel derived from plants is in the form of cellulose Iβ. With alkali treatment cellulose is ‘mercerized’ to form the more stable crystalline cellulose II. Mercerized ramie and mercerized Avicel were used as cellulose II substrates.

Ramie samples in tension showed no transformation from cellulose Iβ to II after IL treatment at 50°C. However, with complete dissolution in IL at 25°C followed by IL displacement with water, ramie did appear to transform from cellulose Iβ to II as seen in Chapter 2. Mercerized ramie (cellulose II) re-crystallized to cellulose II after IL treatment, IL displacement and drying. In contrast, Avicel, a highly crystalline cellulose I of lower degree of polymerization (DP ~225) [78], exhibits transformation from cellulose Iβ to cellulose II after IL treatment, IL displacement and drying. The lack of transformation of cellulose Iβ to II in ramie samples held in tension is likely due to the presence of residual crystallinity in the cellulose fibers serving as a seed for cellulose I recrystallization. Cellulose II hydrolysis was observed to be much more rapid than cellulose I due to its expanded structure as observed previously by Wada et al., [40].
3.2. **Materials and Methods**

3.2.1 **Materials**

The materials used in this chapter are described in Chapter 2.

3.2.2 **Mercerization**

The mercerization process for Avicel was replicated from Bradley Yaniga’s Master’s thesis research work [79]. Mercerization of Avicel samples was carried out in a glass vial immersed in a silicon oil bath on a Barnstead Thermolyne heating plate (ThermoFisher Scientific, PA, USA) and stirred at 800 rpm with a magnetic stir bar. Avicel was treated with alkaline 4.25N sodium hydroxide (NaOH) solution at 65°C for about 24 hours. The mercerization process was stopped by neutralizing the solution with concentrated hydrochloric acid. The neutralization was done in a cold water bath due to the exothermic neutralization reaction. After neutralization the sample was washed with water to remove salt. The mercerized Avicel sample was then dried in a dessicator at room temperature for about three days.

The mercerization for ramie fibers was carried out in the following manner. Dewaxed ramie fibers were mounted on a stainless steel sample frame in order to preserve orientation during mercerization. Ramie fibers were mercerized with 20% NaOH solution at room temperature for 36 hours, then washed with DI water. The fibers were then successively washed with 12% NaOH solution for 24 hours, followed by soaking in ice cold water. After about six hours the water was changed to 80°C water that
was heated on a hot plate to remove the residual salt bound to the fibers. After heating, sample was cooled in water to room temperature. Samples were left in water until mercerization was verified by fiber diffraction. The swollen fibers were then stretched in their stainless steel frame [50].

Verification of conversion to cellulose II was confirmed by either X-ray fiber (ramie) or powder (Avicel) diffraction and raman spectroscopy.

### 3.2.3 IL dissolution and precipitation

The 50°C IL treatment of ramie *in situ* was performed in a similar fashion to the Fiber diffraction experiments described in Chapter 2. Ramie and mercerized ramie was held tautly on a stainless steel sample frame and IL was applied on the samples placed on the goniometer of the X-ray diffraction machine. The samples were bathed in a dry nitrogen stream from an Oxford Industries Cryojet cryostat (at APS) held at 50°C. The samples were incubated in IL for about 40 to 180 minutes by applying the IL dropwise. The pretreated ramie samples were soaked in water to displace the IL from the samples and the wet samples were dried prior to Raman spectroscopy experiments.

The 25°C IL treatment on ramie and the 50 and 120°C IL treatment on Avicel were described in Chapter 2.

### 3.2.4 Raman spectroscopy studies

Room temperature Raman spectroscopy data for ramie and mercerized ramie samples were obtained with a Jobin Horiba Raman Spectrometer with a 633 nm incident
laser beam using a charge coupled detector (CCD). Spectra were collected using a 50X objective and recorded at 200-1600 cm\(^{-1}\) for 120 seconds and averaged over three scans.

Data were collected on the pretreated and washed ramie samples that were held on the sample pin by placing them directly on the sample stage with no microscopic glass slide. After data collection on the wet samples, the samples were dried at 50°C and data were again collected on the recrystallized samples in similar manner. The IL treated and washed ramie and Avicel samples formed a gel and were placed on a microscopic glass slide with wells to keep the samples wet with water and data were collected. Then the samples were dried at 50°C and data were again collected in similar manner. Raman spectra of the microscopic glass slide and water revealed no overlapping peaks with cellulose.

3.3. Results and Discussion

3.3.1 Raman Spectroscopy

Raman spectroscopy is a technique in which the vibration, rotation and lower frequency modes are studied in a system. The vibrational motion is specific to discreet chemical bonds and molecular arrangement. The Raman effect occurs when a laser light illuminates a solid, liquid or gaseous sample, and the photons from the illumination interact with the electron cloud of the molecule.
3.3.2 Identification of cellulose Iβ and cellulose II

Cellulose Iβ and cellulose II are distinguished not only by differences in lattice parameters observed by X-ray diffraction but are also distinguished through differences in their vibrational spectra (Figures 3-1, 3-2). These differences in spectra arise from the conformation of the anhydroglucopyranose skeletons defining the secondary structure of cellulose [80].

The raman spectra and X-ray diffraction data were collected for two cellulose Iβ substrates, ramie and Avicel, and cellulose II, mercerized ramie and mercerized Avicel (Figures 3-1 and 3-2). The peak assignments and approximate vibrational modes for both cellulose crystal polymorphs, cellulose Iβ and cellulose II, are tabulated in Table 3.1. The spectra for Avicel and ramie show similar features for both polymorphs.

Significant differences between cellulose Iβ and cellulose II are observed from 300 cm⁻¹ to 1500 cm⁻¹ wave numbers. The wave numbers that uniquely correspond to the cellulose Iβ polymorph are 339, 379, 457 and 966 cm⁻¹. The wave numbers that uniquely correspond to the cellulose II polymorph are 348, 364, 424, 1260 and 1291 cm⁻¹. The wave numbers that correspond to both cellulose I and cellulose II are 379, 457, 493, 515, 962, 1095, 1120, 1335, 1375 cm⁻¹. There are some peaks that overlap for native cellulose samples around 380, 457, 702, 900, 966, 1096, 1118, 1260, 1332, 1384 and 1454 cm⁻¹ wave numbers with IL. IL has strong Raman signal. The IL spectrum was also measured as one of the controls. The wave numbers that correspond to IL alone are 238, 304, 397, 454, 599, 636, 700, 902, 960, 1022, 1091, 1117, 1252, 1335, 1384, 1419, and 1451 cm⁻¹.
Table 3.1  **Raman frequencies and vibrational assignments of cellulose I and II:**
The spectra were collected in the frequency region below 1500 cm\(^{-1}\) [80].

<table>
<thead>
<tr>
<th>Cellulose I</th>
<th>Polymorphic phases</th>
<th>Cellulose II</th>
<th>Approximate Vibrational modes</th>
</tr>
</thead>
<tbody>
<tr>
<td>339</td>
<td>I</td>
<td></td>
<td>(\delta (\text{CCC}), \delta (\text{CO}), \delta (\text{CCO}), \text{ring deformation})</td>
</tr>
<tr>
<td>II</td>
<td>348</td>
<td></td>
<td>(\delta (\text{CCC}), \delta (\text{CO}), \delta (\text{CCO}), \text{ring deformation})</td>
</tr>
<tr>
<td>II</td>
<td>364</td>
<td></td>
<td>(\delta (\text{CCC}), \delta (\text{CO}), \delta (\text{CCO}), \text{ring deformation})</td>
</tr>
<tr>
<td>379</td>
<td>I</td>
<td></td>
<td>(\delta (\text{CCC}), \delta (\text{CO}), \delta (\text{CCO}), \text{ring deformation})</td>
</tr>
<tr>
<td>II</td>
<td>424</td>
<td></td>
<td>(\delta (\text{CCC}), \delta (\text{CO}), \text{ring deformation})</td>
</tr>
<tr>
<td>433</td>
<td>I &amp; II</td>
<td>437</td>
<td>(\delta (\text{CCC}), \delta (\text{CCO}), \text{ring deformation})</td>
</tr>
<tr>
<td>457</td>
<td>I</td>
<td></td>
<td>(\delta (\text{CCC}), \delta (\text{CCO}), \text{ring deformation})</td>
</tr>
<tr>
<td>491</td>
<td>I &amp; II</td>
<td>485</td>
<td>(\delta (\text{CO})) glycosidic</td>
</tr>
<tr>
<td>517</td>
<td>I &amp; II</td>
<td>518</td>
<td>(\delta (\text{COC})) glycosidic</td>
</tr>
<tr>
<td>II</td>
<td>575</td>
<td></td>
<td>(\delta (\text{COC})) ring</td>
</tr>
<tr>
<td>702</td>
<td>I &amp; II</td>
<td>703</td>
<td>(\rho (\text{CH}_2))</td>
</tr>
<tr>
<td>891</td>
<td>I &amp; II</td>
<td>895</td>
<td>(\nu (\text{CC}), \nu (\text{CO}))</td>
</tr>
<tr>
<td>966</td>
<td>I</td>
<td></td>
<td>(\nu (\text{COC})), glycosidic; ring breathing, symmetric</td>
</tr>
<tr>
<td>1096</td>
<td>I &amp; II</td>
<td>1096</td>
<td>(\nu (\text{COC})), glycosidic; ring breathing, symmetric</td>
</tr>
<tr>
<td>1118</td>
<td>I &amp; II</td>
<td>1106</td>
<td>(\nu (\text{CC}), \nu (\text{CO})) ring breathing, asymmetric</td>
</tr>
<tr>
<td>1150</td>
<td>I &amp; II</td>
<td>1146</td>
<td>(\nu (\text{CC}), \nu (\text{CO})) ring breathing, asymmetric</td>
</tr>
<tr>
<td>II</td>
<td>1260</td>
<td></td>
<td>(\tau (\text{CH}_2), \delta (\text{HCC}), \delta (\text{HCO}), \delta (\text{COH}))</td>
</tr>
<tr>
<td>II</td>
<td>1291</td>
<td></td>
<td>(\tau (\text{CH}_2), \delta (\text{HCC}), \delta (\text{HCO}), \delta (\text{COH}))</td>
</tr>
<tr>
<td>1324</td>
<td>I &amp; II</td>
<td>1332</td>
<td>(\omega (\text{CH}_2), \delta (\text{HCC}), \delta (\text{HCO}), \delta (\text{COH}))</td>
</tr>
<tr>
<td>1379</td>
<td>I &amp; II</td>
<td>1384</td>
<td>(\delta (\text{CH}_2), \delta (\text{HCC}), \delta (\text{HCO}), \delta (\text{COH}))</td>
</tr>
<tr>
<td>1464</td>
<td>I &amp; II</td>
<td>1454</td>
<td>(\delta (\text{CH}_2)) scissors, (\delta (\text{COH})),</td>
</tr>
</tbody>
</table>
Figure 3-1  Raman spectra of ramie (cellulose Iβ), mercerized ramie (cellulose II) and ionic liquid (IL): The ellipses were drawn to show the clear differences between cellulose Iβ and cellulose II. The dotted lines were drawn to show the features that match with cellulose Iβ, cellulose II and IL.
Figure 3-2  Raman spectra of Avicel (cellulose Iβ), mercerized Avicel (cellulose II) and IL: The ellipses were drawn to show the clear differences between cellulose Iβ and cellulose II. The dotted lines were drawn to show the features that match with cellulose Iβ, cellulose II and IL.
3.3.3 Recrystallization of ramie and mercerized ramie upon IL treatment at 50°C

Recrystallization of cellulose Iβ (ramie) and cellulose II (mercerized ramie) after IL dissolution was also followed by Raman spectroscopy. The samples for Raman experiments were treated in the same manner as X-ray fiber diffraction samples. Ramie and mercerized ramie samples were tautly wound on a sample pin holder similar to the X-ray fiber diffraction experiments. Raman data were acquired on the native ramie and mercerized ramie samples. Native and mercerized ramie were then treated with IL at 50°C for about 40 minutes and 3 hours, respectively, and washed with water to remove the IL. IL has a strong Raman signal and interferes with the weak raman cellulose signal. Raman data was collected on the wet samples. There was no interference from water since water has very weak Raman signal. The wet samples were allowed to recrystallize by drying at 50°C in a vacuum oven and Raman data were again collected on the recrystallized ramie and mercerized ramie samples.

The dominant peaks in the Raman spectra observed for untreated cellulose Iβ (native ramie) were also observed in the wet and re-crystallized samples, indicating that there was no transformation to cellulose II upon IL treatment at 50°C and recrystallization with water (Figure 3-3). There were no peaks in the recrystallized cellulose Iβ spectrum with wave numbers that correspond uniquely to cellulose II (Figure 3-3). This is consistent with the X-ray fiber diffraction experiments where ramie treated with IL at 50°C retained its initial crystal structure upon recrystallization with water (Figure 3-9).
Similarly, cellulose II (mercerized ramie) upon IL treatment and re-crystallization with water retained the initial cellulose II structure as evidenced by the Raman spectra (Figure 3-4). There were no wave numbers that correspond to cellulose Iβ exclusively. These results are consistent with the X-ray fiber diffraction studies where IL treated mercerized cellulose regenerated to the same crystal polymorph as the starting material (Figure 3-11).

Although X-ray fiber studies indicated an almost complete loss of diffraction from the cellulose fibrils in IL treated samples at 50°C (Figures 3-9 and 3-11), Raman spectroscopy suggests persistence of residual crystallinity (Figures 3-3 and 3-4) due to partial drying of sample. The Raman spectra of amorphous cellulose has been found to possess a peak at 1462 cm\(^{-1}\) and loss of a peak at 1481 cm\(^{-1}\) with gradual loss in intensity of glycosidic bond vibrations at 1090 and 1120 cm\(^{-1}\) wave numbers in cellulose I [81]. However, in our studies, the region from 1400 to 1500 cm\(^{-1}\) for both cellulose Iβ and cellulose II appears to have very broad peaks, making these features largely indistinguishable. The amorphous sample (IL treated and water washed samples) exhibited broad peaks and lower intensities compared to the native sample. Retention of residual structure in the Raman spectra indicates that samples may be more appropriately referred to as partially amorphous.

The residual crystallites of cellulose Iβ and cellulose II may serve as nuclei for the recrystallization to cellulose Iβ and cellulose II respectively. The recrystallization to cellulose I due to persistence of crystallinity in lignocellulosic biomass with IL treatment at 50°C was observed also by X-ray fiber diffraction experiments in Chapter 2.
Figure 3-3  Raman spectra of ramie, cellulose Iβ treated with IL at 50°C, washed with water and dried at 50°C: Ramie treated with IL at 50°C and washed with water (partially amorphous wet sample), and recrystallized sample (dried at 50°C).
Figure 3-4  Raman spectra of mercerized ramie, cellulose II treated with IL at 50°C, washed with water and dried at 50°C: Mercerized ramie treated with IL at 50°C and washed with water (partially amorphous wet sample), and recrystallized sample (dried at 50°C).
3.3.4 Recrystallization of ramie upon IL treatment at room temperature

Ramie samples were soaked in IL in a scintillation vial for about 24 hours at room temperature. The sample appeared completely dissolved in IL and formed a thick gel after addition of water. The gel was then washed with water to remove the IL. Raman data were collected on the wet precipitated sample. The wet precipitated sample has very broad peaks at 1090 and 1120 cm\(^{-1}\) wave numbers with peaks in common to IL and cellulose II, mercerized ramie (Figure 3-5). No peaks were observed at wave numbers unique to cellulose I. The raman data for the precipitated sample suggest that the sample was largely amorphous. The washed sample was then allowed to recrystallize by drying at 50°C in a vacuum oven. Raman data were again collected on the recrystallized sample. Peaks at wave numbers specific to cellulose II (mercerized ramie) were observed in recrystallized ramie spectrum. After IL treatment and complete re-crystallization, the raman peak intensity appears to be less than that of native or mercerized samples. From this we infer that the re-crystallization of the fiber samples is not complete and is largely amorphous. This is consistent with the fiber data in Chapter 2.
Figure 3-5  Raman spectra of ramie cellulose Iβ treated with IL at 25°C, washed with water and dried at 50°C: The solid black ellipses were drawn to show the features resulting from cellulose II. The dotted ellipse on the amorphous sample indicates the loss of intensity compared to the native cellulose Iβ sample. The dotted lines on the amorphous sample represent the features that arise from the IL. On the figure the legend Ramie 25C wet represents amorphous sample and Ramie 25C dry represents recrystallized sample. The sharp intense peaks at around 450cm⁻¹ on the amorphous samples do not match with either cellulose or IL.
3.3.5 Avicel recrystallization

For comparison to ramie, with a DP reported between 1686 and 3400 [82, 83], Avicel, a highly crystalline substrate (CrI of 78) of low DP of ~225 [78], was selected for IL treatment and re-crystallization. Raman data indicate that Avicel recrystallized to cellulose II similar to ramie recrystallization at 25°C after complete dissolution in IL (Figure 3-5). X-ray data indicate that Avicel transforms to cellulose II upon IL treatment at both mild and harsh pretreatment conditions (Chapter 2).

Avicel was dissolved in IL at 50°C for 12 to 14 hours or at 120°C for 1 hour, followed by IL displacement with water. Samples appeared completed dissolved with the long incubation time at 50°C. The dissolution was not complete at the short incubation time at 120°C (one hour) with a small amount of sample observed visually in the glass vial. The powder XRD of wet samples showed evidence of a hydrate of cellulose II for both IL treatment temperatures (Figure 3-6). This hydrate was also observed by Wada et al. [40] in mercerization of cellulose I using sodium hydroxide. The hydrate, upon drying, recrystallized to cellulose II, but of lower crystallinity compared to mercerized cellulose. This indicates that IL treatment produces an extremely disordered sample and that the regeneration process is not complete. This is consistent with the Raman data for Avicel recrystallization (Figure 3-7).

Raman data suggests that Avicel becomes amorphous with IL incubation at 50°C for 12 to 14 hours or at 120°C for 1 hour with loss of intensity (i.e., broad peaks) at 1090 and 1120 cm⁻¹ wave numbers compared to the crystalline cellulose peaks (Figure 3-7).
The amorphous Avicel upon washing with water and drying at 50°C recrystallized to cellulose II (mercerized Avicel) (Figure 3-7). The peaks that correspond to mercerized Avicel (cellulose II) were observed on recrystallized IL treated Avicel samples (Figure 3-7) suggesting that Avicel had transformed to cellulose II crystal phase.

In similar studies by Shakeri et al [84], the ionic liquid 1-butyl-3-methylimidazolium formate was used to dissolve and regenerate microcrystalline cellulose (MCC) with an initial DP of 225. The structure and morphology of regenerated cellulose was investigated by wide angle X-ray diffraction. The authors found that the regenerated cellulose exhibited transformation from cellulose Iβ to cellulose II after IL dissolution, displacement with water and drying. This transformation could be due to the presence of more deformed or disordered regions in cellulose micro fibrils of MCC. This is consistent with our studies of IL treated Avicel (Figures 3-6 and 3-7).
Figure 3-6  X-ray powder diffraction spectra of Avicel wet and dry treated with IL at 50 and 120°C: X-ray powder diffraction patterns of Avicel (cellulose I), mercerized Avicel, cellulose II (wet and dry) and IL treated Avicel (washed with water and dried). The IL treated Avicel has an XRD pattern consistent with cellulose.
Figure 3-7  Raman spectra of Avicel treated with IL at 50 or 120°C, washed with water and dried at 50°C: The solid black ellipses were drawn to show the features resulting from cellulose II. The dotted ellipse on the amorphous sample indicates the loss of intensity compared to the native cellulose Iβ sample. The dotted lines on the amorphous sample represent the features that arise from the IL. On the figure the legend, Avicel 50 and 120 C wet represents amorphous sample and Avicel 50 and 120 C dry represents recrystallized sample. The sharp intense peaks at around 450cm⁻¹ on the amorphous samples do not match with either cellulose or IL.
### 3.3.6 Comparative enzymatic hydrolysis to cellulose structural changes

The impact on cellulose structural changes with IL pretreatment was observed (Figures 3-5, 3-6 and 3-7). Since IL treated cellulose I can re-crystallize to cellulose II, comparison of hydrolysis kinetics for the two polymorphs was made.

Avicel (cellulose Iβ), mercerized Avicel (cellulose II), cellulose II from Avicel treated with IL at 50°C and 120°C, and cellulose II hydrate from Avicel treated with IL at 50°C and 120°C were hydrolyzed enzymatically using Spezyme CP (15 FPU/g glucan) and Novozyme 188 (30 CBU/g glucan) (Table 3.2).

Table 3.2 Enzymatic hydrolysis of wet and dry Avicel treated with IL at 50 and 120°C: Avicel (cellulose I), mercerized Avicel (cellulose II), Avicel treated with IL at 50 and 120°C and recrystallized upon washing and drying (cellulose II) and Avicel treated with IL at 50 and 120°C and washed (wet Avicel) were enzymatically hydrolyzed at 1% (w/v) with Spezyme CP at 15 FPU/g glucan and with Novozyme 188 at 30 CBU/g glucan enzyme loadings. The crystallinity indices (CrI) were calculated [85] for the native and recrystallized samples and not for amorphous samples from wet Avicel as water obscures the signal from cellulose.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>IL treatment</th>
<th>% Glucose Yield</th>
<th>% CrI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>Avicel</td>
<td>-</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>Mercerized Avicel</td>
<td>-</td>
<td>39±4</td>
<td>87±9</td>
</tr>
<tr>
<td>Recrystallized Avicel</td>
<td>120°C</td>
<td>20±6</td>
<td>55±10</td>
</tr>
<tr>
<td>Wet Avicel</td>
<td>120°C</td>
<td>65±12</td>
<td>87±9</td>
</tr>
<tr>
<td>Recrystallized Avicel</td>
<td>50°C</td>
<td>100±1</td>
<td>100±1</td>
</tr>
<tr>
<td>Wet Avicel</td>
<td>50°C</td>
<td>54±7</td>
<td>88±7</td>
</tr>
</tbody>
</table>

Mercerized cellulose (cellulose II) hydrolyzed more rapidly than Avicel (cellulose Iβ) with 24 hour glucose yields of 87% and 35%, respectively (Table 3.2). This is
consistent with recently reported comparative hydrolysis kinetics by Wada et al. [40]. Wada et al. attributed the increase in hydrolysis kinetics of cellulose II to an increase in spacing between pyranose rings in adjacent fibrils of cellulose II compared to cellulose Iβ allowing water to penetrate more readily. However, the CrI of mercerized cellulose in our case is lower than that of the untreated Avicel which may increase digestibility of cellulose to an even greater extent than the changes in cellulose structure.

Avicel samples showed enhanced hydrolysis at 50°C compared to 120°C with significant differences between wet and dry samples (Table 3.2). The 24 hour hydrolysis of wet Avicel samples IL treated at 50°C and 120°C appear to be similar to that of mercerized Avicel (Table 3.2). The enhanced hydrolysis in wet samples could be because of their expanded structure of the cellulose II hydrate (Figure 3-6).

The hydrolysis yields for recrystallized Avicel IL treated at 50°C appears to be essentially complete within 5 hours. The enhanced hydrolysis could be due to presence of cellulose II as a result of prolonged incubation in IL. The hydrolysis yields for recrystallized Avicel IL treated at 120°C appears to fall between that of untreated and mercerized Avicel, a mixture of cellulose Iβ and cellulose II. However, this is not clearly evident from powder XRD data (Figure 3-6) or Raman data (Figure 3-7).

Avicel dissolution in IL was observed to be complete at 50°C and incomplete at 120°C. IL treatment at 50°C with prolonged incubation led to complete dissolution of sample with formation of a gel upon addition of water. The lack of residual cellulose I and complete dissoultion could have led to recrystallization exclusively to cellulose II. For samples incubated at 120°C in IL for one hour, residual cellulose I is expected with
primarily cellulose II formation upon recrystallization. Some evidence of this can be seen in the XRD data (Chapter 2) where the doublet at 2θ of 19 and 20° should exhibit greater intensity at the lower diffraction angle in cellulose II. The peak intensities are nearly equal, suggesting an underlying peak at higher intensity corresponding to cellulose I.

The hydrolysis yields of recrystallized Avicel IL treated at 50°C appear to be greater than that of recrystallized Avciel IL treated at 120°C despite the higher crystallinity index with IL treatment at 50°C (Table 3.2). The crystallinity index may not be a major indicator of the hydrolysis kinetics for recrystallized samples. The major indicator could be the relative amounts of cellulose I and cellulose II. Cellulose II was found to be a more easily hydrolyzed substrate.

The enhanced enzymatic hydrolysis with respect to cellulose II formation was also observed in biomass samples in Chapter 2. For the biomass samples, enhanced hydrolysis was observed with IL treatment at 120°C compared to 50°C with no clear differences in CrI’s. Hence the major contributor for enhanced biomass hydrolysis was observed to be increased cellulose II formation observed with IL treatment at 120°C.

3.3.7 Dissolution of cellulose I and cellulose II in IL

The dissolution of cellulose II in IL appears to occur less readily in comparison to cellulose Iβ (Figure 3-8). Cellulose II loses crystallinity with incubation in IL over a longer time period (~ 2 to 3 hours) than cellulose Iβ (~ 40 minutes). It is hypothesized that the slower dissolution is due to the lower solubility of cellulose II than cellulose I in the IL. We measured Avicel and mercerized Avicel solubility at 50°C
(Figure 3-8). Cellulose Iβ has solubility of 20 % (wt/wt) and cellulose II has solubility of 12 % (wt/wt) in the IL (EMIM-Ac). This appears to be consistent with the time course of dissolution in IL seen in X-ray fiber diffraction data for ramie cellulose I (Figure 3-9) and mercerized ramie cellulose II (Figure 3-11). A great reduction in the intensity of crystalline cellulose reflections were observed after 40 minutes incubation in IL for cellulose I and after 3 hours incubation in IL for cellulose II.

![Image](image.png)

Figure 3-8 Solubility of cellulose I (Avicel) and cellulose II (mercerized Avicel) in IL at 50°C: 1- 20% (w/w) Avicel in IL and 2- 15% (w/w) mercerized Avicel in IL that appears partially dissolved.

3.3.8 Time course experiments for cellulose I and cellulose II solubility in IL

The time course experiments on ramie and mercerized ramie samples were performed on the Rigaku FRE X-ray diffractometer. The experimental procedure was described in Chapter 2.
The time course required for the transformation of ramie (cellulose I) from crystalline state to amorphous state with IL treatment at 50°C was carefully followed (Figure 3-9). As ramie is treated with IL at 50°C (Figure 3-9) in situ or the equatorial and meridional reflections start to disappear indicating that the treated ramie becomes amorphous. Ramie becomes amorphous after soaking it in IL for about 40 minutes at 50°C (Figure 3-9 D).

Diffuse scattering from IL dominates as scattering from cellulose diminishes. The amorphous cellulose can more properly be called disordered cellulose as reflections become fainter. As the sample dissolves it is impossible to distinguish between the contribution of ramie and IL in the diffraction images. The diffraction image of IL alone was shown in Figure 3-10. When the IL is displaced from the sample with water the amorphous cellulose re-crystallizes to an unresolved crystalline form of cellulose in the wet state. This intermediate structure (Figures 3-9E) has equatorial reflections falling at lower angles of diffraction (2θ= 7 or 7.5°or 11.5 A°) on either sides of the beam stop that does not match with either cellulose Iβ or cellulose II unit cell parameters. This intermediate structure was considered to arise due to the IL intercalation between the cellulose micro fibrils expanding the structure of cellulose. The intermediate structure formation was explained in Chapter 2 and also observed with biomass dissolution.

Upon further washing and drying the intermediate structure recrystallized back to cellulose Iβ at 50°C IL treatment (Figure 3-9F). The recrystallization to cellulose Iβ at 50°C IL treatment may be due to incomplete dissolution of the sample in tension on the stainless steel sample pin.
After IL treatment and complete re-crystallization, the ramie fiber diffraction appears to be less intense. From this we infer that the re-crystallization of the fiber samples is not complete and is still partially amorphous. This is consistent with the above explanation on biomass recrystallization in the comparative enzymatic hydrolysis section. The recrystallization to cellulose Iβ process is consistent with ball milled microcrystalline cellulose [86] but the existence of the intermediate structure during the recrystallization process was not reported elsewhere.

Figure 3-9 F shows diffraction consistent with the lattice parameters for cellulose Iβ. The positions of the reflections do not change $d$-spacing: there is no evidence of lattice changes during or after the recrystallization process.
Figure 3-9  Time course for ramie, cellulose I dissolution in IL at 50°C: X-ray fiber diffraction of ramie treated with IL at 50°C. Panel A is Ramie (cellulose Iβ), Panel B is ramie soaked in IL for 10 minutes, Panel C is ramie soaked in IL for 20 minutes, Panel D is ramie soaked in IL for 40 minutes (amorphous ramie), Panel E is wet intermediate structure of ramie washed with water and re-crystallized and Panel F is recrystallized ramie as cellulose I, washed with water and dried at 50°C.
Similarly, the time course required for the transformation of mercerized ramie (cellulose II) from a crystalline state to an amorphous state with IL treatment at 50°C was carefully followed (Figure 3-11). Mercerized ramie (cellulose II) was treated with IL at 50°C in situ. The equatorial and meridional reflections start to disappear indicating that cellulose II becomes disordered upon IL treatment. Mercerized ramie becomes amorphous after soaking it in IL for about 3 hours (Figure 3-11E). Upon re-crystallization it crystallizes back to cellulose II (Figure 3-11F) with the same trends as observed with cellulose Iβ re-crystallization after 50°C IL treatment. But the formation of intermediate structure upon recrystallization was not observed in cellulose II. The polymorphic transformation from cellulose I to cellulose II is irreversible. Cellulose II is a stable state whereas cellulose I is a metastable crystalline state.
Figure 3-11  Time course for mercerized ramie, cellulose II dissolution in IL at 50°C: X-ray fiber diffraction of mercerized ramie (cellulose II) treated with IL at 50°C. Panel A is mercerized ramie (cellulose II), Panel B is mercerized ramie soaked in IL for 10 minutes, Panel C is mercerized ramie soaked in IL for 20 minutes, Panel D is mercerized ramie soaked in IL for 40 minutes, Panel E is mercerized ramie soaked in IL for 3 hours (amorphous ramie), Panel E is recrystallized mercerized ramie as cellulose II, washed with water and dried at 50°C.

3.4. Conclusions

The X-ray fiber diffraction and Raman spectroscopy results were in agreement. The changes in cellulose structure with IL treatment and the interplay of these changes with enzymatic digestibility were successfully tracked. Cellulose Iβ transforms to cellulose II when cellulose is completely soluble in IL. Complete dissolution is achieved with IL treatment at high temperatures or prolonged incubation in IL. The hydrolysis results in relation to the changes in cellulose structure indicate that cellulose II is a
preferred substrate for enhanced hydrolysis by enzymes due to its expanded structure in comparison to cellulose I. The lower solubility of cellulose II compared to cellulose Iβ is consistent with other findings which indicate that cellulose II is the more stable polymorph.

The observation of the formation of an intermediate structure in the process of recrystallization to cellulose I as observed for lignocellulosic biomass (Chapter 2) was also observed for cellulose samples. This provides scope for further investigation to ascertain cellulose intermediate in cellulose II.

The recrystallization was observed to be incomplete with ionic liquid pretreated biomass. The substrate remains amorphous upon drying after IL treatment. This is a fascinating finding which infers that complete pore closure on drying does not appear to occur for ionic liquid pretreated biomass.

In summary, these results underscore the importance of maintaining hydrated samples after IL pretreatment for applications where hydrolysis of the feedstock to monomeric sugars is a processing goal. For materials applications, where cellulose fractionation and re-crystallization produces the desired end product, these results indicate the ILs may be useful for extraction of cellulose from biomass and formation of crystalline fibers.
Chapter 4

Saccharification of Ionic Liquid Pretreated Biomass at Low Solid Loadings with Commercial Enzyme Mixtures

4.1. Introduction

Cellulose and hemicellulose in biomass can be hydrolyzed by enzymes into simple sugars and fermented to biofuels or converted to other chemicals through a sugar platform. Ethanol is one such biofuel that is largely produced from first generation biomass substrates such as corn and sugarcane. Efforts for over a decade have focused on replacing the first generation biomass (food) with lignocellulosic biomass substrates [87] to avoid impacts on food sources.

Ethanol can be produced from biomass through a biochemical platform consisting of sequential or simultaneous steps. These include (i) pretreatment to produce a more
easily hydrolyzed substrate; \((ii)\) enzyme hydrolysis to convert cellulose and hemicellulose components to their sugar monomers; and \((iii)\) fermentation or conversion of sugars to ethanol [88] or other products. The highly crystalline nature of cellulose, a major fraction of lignocellulosic biomass, provides a major impediment to substrate accessibility of cellulase enzymes and the subsequent conversion of glucan (cellulose) to glucose [57].

In order to improve the enzyme accessibility and overcome the recalcitrance of lignocellulose to hydrolysis, biomass must be pretreated. Pretreatment with ionic liquids (IL) reduces the crystallinity of cellulose, partially removes hemicellulose and lignin and does not produce degradation products inhibitory to hydrolysis enzymes or fermentation micro-organisms [57, 89]. Most pretreatments such as dilute acid hydrolysis [32], pH controlled liquid hot water treatment [33], and lime [31] result in biomass substrates that lack components (often hemicellulose) and are highly crystalline. Ammonia fiber explosion (AFEX) technique [90] retains hemicellulose, but cellulose remains highly crystalline with the formation of a cellulose III\textsubscript{1} polymorph and increased enzyme access to cellulose [53]. In our studies, most hemicellulose remains intact in the biomass substrates with ionic liquid pretreatment. There is a lack of research reported for optimization of enzyme mixtures for biomass hydrolysis with amorphous substrates and intact hemicellulose, since these types of substrates were largely unattainable prior to the
advent of IL pretreatment. Variation in pretreatment strategy affects the requisite enzyme mixture employed for polysaccharide hydrolysis.

Enzymes often used for polysaccharide hydrolysis are produced by a variety of fungi and bacteria [91]. Commercial cellulase enzymes are cocktails that include individual endoglucanases, exoglucanases, β-glucosidase, and xylanolytic enzymes expressed most often from *Trichoderma reesei*. The cellulolytic enzymes include exoglucanases (cellbiohydrolases) which hydrolyze the crystalline cellulose chains to cellobiose from reducing or non-reducing ends, producing a glucose dimer (cellobiose) and some glucose trimers; and endoglucanases which act on the amorphous regions of cellulose at random points along the glucan chain, producing glucan oligomers [91]. β-glucosidase acts on soluble glucan oligomers and cellobiose to produce glucose monomers. The xylanolytic enzymes hydrolyze the hemicellulosic portion of biomass and include a number of subcategories of enzymes that are selective for depolymerization of the backbone xylan chain and its substituents.

Pretreatment technologies have been proposed to produce amorphous cellulose. Phosphoric acid pretreatment of lignocellulose produces amorphous cellulose largely lacking in the hemicellulose constituents in contrast to IL pretreatment. A combination of endoglucanase and β-glucosidase activities produced glucose on hydrolysis of phosphoric acid swollen cellulose (PASC) [92-95]. Endoglucanase can hydrolyze amorphous cellulose but has little activity against crystalline cellulose substrates [96].

In this study, Spezyme CP and Accelerase 1000, widely used commercial cellulase enzymes, Primafast Luna CL and Indiage Super L, biofinishing cellulase
enzymes and Multifect Xylanase were used to hydrolyze ionic liquid pretreated substrates. All the commercial enzyme mixtures were supplemented with β-glucosidase (Novozyme 188) to limit end product inhibition. The commercial enzymes were characterized for their specific enzyme activities against defined substrates. The molecular weight (MW) and iso-electric point (pI) of major protein components of the biofinishing enzymes were also determined. The performance of these enzymes was evaluated based on a comparative study of the hydrolyzability of different feedstocks by varying enzyme mixtures. From the results, it was observed that Primafast is a mixture of endoglucanase activities and further characterization was performed on this fabric biofinishing enzyme.

Fabric biofinishing enzymes are often formulated to hydrolyze amorphous cellulose for fabric softening, to prevent fabric pilling and fibrillation, for surface fiber removal, and for preserving the strength of fabric. They are expected to have high endoglucanase activity [97-99]. The Primafast enzyme was later supplemented with Multifect Xylanase to enhance the yields of both cellulose and hemicellulose sugars. It was compared to the widely used cellulase enzyme, Spezyme CP. The present work focuses on the evaluation of the performance of these cellulase enzyme mixtures for hydrolysis of glucan and xylan components of ionic liquid pretreated poplar, switchgrass and Avicel, a highly crystalline cellulose substrate.
4.2. Materials and Methods

4.2.1 Pretreatment

Poplar (provided by the National Renewable Energy Laboratory), switchgrass (provided by USDA-ARS, Peoria, IL) and Avicel PH-101 (Fluka Chemicals) were pretreated with the ionic liquid, 1-ethyl 3-methyl imidazolium acetate, EmimOAc, (Sigma Aldrich, MO, USA). The biomass was mixed with EmimOAc, 5% (w/w), and incubated at 120°C for 30 minutes. The sample was then mixed with water at room temperature to precipitate the biomass/cellulose from the IL solution. The precipitant and wash solutions were separated by centrifugation. The precipitated solids were washed repeatedly with deionized (DI) water to displace the IL from the sample until the wash solution appeared colorless.

The carbohydrate and lignin content in the untreated and pretreated biomass were determined in duplicate using concentrated acid hydrolysis followed by dilute acid hydrolysis according to the standard laboratory analytical procedure developed by the National Renewable Energy Laboratory (NREL) [100]. Following washing with water, the pretreated samples were dried in a vacuum oven at 45°C until a constant weight was achieved. The monomeric sugars were quantified by high performance liquid chromatography with refractive index detection using a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate column and a mobile phase of de-ionized water at a flow rate of 0.6 ml/min. The temperature of the column was maintained at 80°C.
4.2.2 Enzyme activity assays

The Avicelase, CMCase and xylanase activities for the commercial enzymes were determined using defined substrates Avicel, carboxy methyl cellulose (CMC) and Birchwood xylan (Sigma Aldrich, MO, USA), respectively, using the Nelson Somogyi colorimetric assay for soluble reducing sugars [101]. The assay was carried out in a 50mM Na acetate buffer, pH 5.0. Known concentration of enzyme (diluted prior in the buffer) was added to the substrates in buffer and was incubated at 50°C. Samples were drawn at timed intervals for approximately 30 minutes. The aliquots were immediately mixed with copper reagent and boiled at 100°C in a water bath for 10 minutes to deactivate the enzymes. The samples were then cooled to room temperature (in ice or cold water) and arsenomolybdate reagent was added and incubated for 15-40 min at room temperature. The absorbance of color developed was measured on a UV-spectrophotometer at 500nm. The initial rates of the released sugars were quantified with a standard curve developed with glucose or xylose for cellulosic or xylan substrates, respectively. The intensity of the color change is proportional to the amount of sugar released. One unit of enzyme is defined as the activity producing 1μmol of reducing sugars (expressed as glucose equivalent) per minute [102]. Specific enzyme activity is expressed in international units per mg of protein and with one international unit of activity equivalent to one micromole/min/mg, i.e. IU/mg.

The β-glucosidase and β-xylosidase activities were determined by the release of p-nitrophenol using p-nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-β-D-xylopyranoside respectively as substrates [103]. The assay was carried out in a 50mM Na
acetate buffer, pH 5.0. The substrate was added to the buffer in a culture tube. Known concentration of enzyme (diluted prior in the buffer) was added to the substrates in buffer and was incubated at 50°C. Samples were drawn every 5 to 10 minutes and the reaction was stopped by adding 0.5M Na₂CO₃, a high pH solution. The release of p-nitrophenol was measured on a UV-spectrophotometer at 400 nm through a standard curve developed using known concentrations of p-nitrophenol. The specific activity of the enzyme was calculated based on the amount of enzyme used to release p-nitrophenol per minute. Activity is expressed in international units (IU/mg (micro moles of p-nitrophenol released per min / mg of enzyme).

4.2.3 Enzymatic Hydrolysis

Commercial cellulase enzymes: Spezyme CP, Accelerase 1000, Primafast Luna CL and xylanase enzymes: Indiage Super L and Multifect Xylanase were provided by Genencor International Rochester, NY, USA. β-glucosidase, Novozyme 188, was purchased from Sigma Aldrich, MO, USA. The activities of Spezyme CP, Accelerase 1000 and Primafast Luna CL were also determined by measurement of soluble reducing sugars via a 2,5-dinitrosalicylic acid assay [104] and expressed as filter paper units, FPU. The protein content in Spezyme CP, Accelerase 1000, Primafast Luna CL, Indiage Super L and Multifect Xylanase was estimated using a Bradford assay with bovine serum albumin as the standard [105].

Hydrolysis was carried out with 1% (w/v) substrate at 45°C, in 0.05 M sodium citrate buffer, pH 4.8 in a New Brunswick (NJ, USA) water bath orbital shaker (C-76) at 200 rpm. The enzyme hydrolysis procedure was based on a protocol from NREL [106].
The pretreated substrate was hydrolyzed with all the commercial cellulose and xylanase enzymes and supplemented with β-glucosidase at 30 CBU/g glucan. Cellobiase activity is determined by a cellobiose hydrolysis assay [107] and expressed as cellobiose units (CBU) per gram of glucan. Aliquots of the reaction mixture were sampled periodically and the enzyme reaction was stopped by boiling the sample at 100°C for 5 minutes.

4.2.4 HPLC Analysis

The sugar analysis of hydrolysis samples was performed using high performance liquid chromatography with refractive index detection and a Bio-Rad (Richmond, CA) Aminex HPX-87H carbohydrate analysis column. The mobile phase was HPLC grade 5mM sulfuric acid at a flow rate of 0.6 ml/min with a column temperature of 65°C.

4.2.5 XRD Measurement

X-ray powder diffraction (XRD) is an analytical technique used to characterize the crystallinity of a material. XRD data for pretreated and untreated poplar, switchgrass and Avicel were measured at 25°C with an X’PERT PRO powder diffractometer PAN analytical with X’celerator detector using Nickel filtered Cu-Kα radiation. Samples were scanned over the range of 5 to 35° (2θ), with a step size of 0.05° and step time of 10 seconds. Crystallinity of biomass was quantified from X-ray powder diffraction data using a crystallinity index, CrI, for cellulose [85].
4.3. Results and Discussion

4.3.1 Compositional analysis pre and post IL treatment

The Avicel, poplar and switchgrass substrates appeared to dissolve completely during incubation in the IL. All three samples produced solid precipitate upon washing with water. The compositions of the untreated samples and IL-pretreated precipitate were analyzed (Table 4.1) and reported as dry weight of major components; glucan, xylan, arabinan, and lignin (acid soluble and insoluble). The loss of cellulose (glucan) to the IL/wash solutions was negligible. However, both xylan and lignin appear to partition into the IL and wash phases. For IL pretreated poplar, a reduction in mass of approximately 15% of initial xylan and 7% lignin was observed. For IL pretreated switchgrass, a larger reduction of approximately 32% of initial xylan and 27% initial lignin was observed. Avicel compositions are essentially unchanged with IL pretreatment and no significant glucan losses were observed.
Table 4.1  The weight percent of major components of untreated and IL-treated poplar, switchgrass and Avicel: Lignin is the sum of acid soluble and acid insoluble lignin. The percentage of recovered mass of 300 mg samples after IL pretreatment, washing and drying is 93% for poplar, 82% for switchgrass and 96% for Avicel. All measurements are an average of duplicate samples that deviated by ±6%.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Poplar</th>
<th>Switchgrass</th>
<th>Avicel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
<td>Untreated</td>
</tr>
<tr>
<td>Glucan</td>
<td>37</td>
<td>41</td>
<td>30</td>
</tr>
<tr>
<td>Xylan</td>
<td>12</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Arabinan</td>
<td>0.5</td>
<td>0.4</td>
<td>5</td>
</tr>
<tr>
<td>Lignin</td>
<td>23</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>CrI</td>
<td>38</td>
<td>8</td>
<td>21</td>
</tr>
</tbody>
</table>

In a similar comparative study, significant losses of both cellulose and hemicellulose fractions with substantial delignification for maple wood flour pretreated with EMIMOAc were reported [57]. Samples were pretreated in IL at varying temperatures for a 90 minute incubation time followed by biomass recovery and washing with ethanol. A removal of 15% cellulose was observed at 110°C and 130°C. Reported losses of xylan were 16 and 26%, at 110°C and 130°C, respectively, similar to those found in our experiments (~15 to 32%). Reported losses of lignin were 46 and 63%, at 110°C and 130°C, respectively, compared to 7 to 27% in our experiments. The higher lignin and cellulose losses reported by Lee et al. may be due to the longer pretreatment incubation time (90 versus 30 minutes), differences in wash solution (ethanol versus water in our case), or structural and chemical differences in biomass sources.
4.3.2 Structural changes upon IL treatment

The structural changes after pretreatment with IL were assessed using X-ray powder diffraction (XRD) experiments (Figure 4-3). The XRD patterns for the substrates pretreated with IL show a decrease in crystallinity compared with untreated substrates with reduced intensity of the diffraction peak at $2\theta = 22^\circ$, corresponding to cellulose I [85]. The crystallinity index (CrI), was calculated using XRD data and found to decrease substantially for all samples incubated in IL (Table 4.1).

![XRD patterns for IL-treated and untreated substrates](image)

Figure 4-1  XRD patterns for IL-treated and untreated substrates: Untreated Avicel (A), poplar (B), and switchgrass (C) exhibited a significantly greater degree of crystallinity than that of the regenerated samples. Samples (D), (E), (F) correspond to IL-treated Avicel, poplar and switchgrass respectively. Samples were incubated in EMIMOAc at 120°C for 30 minutes and washed with water. CrI of samples are listed in Table.4.1.

4.3.3 Characterization of commercial enzyme mixtures

The commercial enzyme mixtures used in the saccharification of different feedstocks exhibit differences in their constituent enzymes as assessed by SDS polyacrylamide gel electrophoresis (PAGE). The commercial enzyme mixtures were also characterized to assess the substrate specificity of the enzyme mixtures.
4.3.4 Molecular weight distribution and iso-electric focusing of commercial enzymes

The commercial enzyme mixtures in this study were characterized by SDS polyacrylamide gel electrophoresis (PAGE). The composition of the Primafast Luna CL enzyme system was not published; it was further characterized using one and two dimensional SDS-polyacrylamide gel electrophoresis and isoelectric focusing to determine approximate molecular weights and isoelectric points, pI, of constituent proteins (Figure 4-3). Major bands for Primafast were observed at 48 and 35 kilo Daltons (kDa) with pI’s between ~4.7 to 5.3 (Figure 4-3). The dominant endoglucanases in T. reesei are EG I and EG II (each 5 to 10% of total proteins) [25, 94, 108]. Molecular weights and pI’s are: 48 kDa, pI 4.7 for EG I; 48 kDa, pI 5.0 for EG II and 35 kDa, pI 5.0 for EG IV [109]. The molecular weights and pI’s for Primafast appear to correspond to the T. reesei endoglucanases. Cellobiohydrolase (CBH I) is the main constituent of the T. reesei system, comprising about 60% of the cellulases [25] with a molecular weight of 54 kDa and a pI of 5.6 [109] or with a molecular weight of 63 kDa and a pI of 4.5[110]. The molecular weights and pI of the Primafast constituents do not appear to correspond to CBH I.
Spezyme and Accelerase appear to have major bands at around ~63 kDa and at ~50 and 36 kDa (Figure 4-3) corresponding to CBH I, EG I or II, and IV, respectively. Both enzyme mixtures also appear to possess a minor band at around 81kDa (Figure 4-3). The molecular weight of β-glucosidase from the *Aspergillus niger* and *T. reesei* strains is approximately 81kDa [110, 111]. The band at 81kDa for Spezyme CP appears to be consistent with β-glucosidase from *T. reesei*. Novozyme 188 (from *A. niger*) has a very prominent band at ~81kDa (Figure 4-3). This appears to be consistent with the β-glucosidase from the *A. niger*. Novozyme 188 also has major bands at ~66 kDa and at ~50kDa (Figure 4-3). The molecular weight of *A. niger* CBH II is 67 kDa [112] and the
molecular weight of *A. niger* endoglucanase is 50 kDa [113]. This appears to be consistent with CBH II and EG of *A. niger*.

![SDS PAGE gel of commercial enzyme mixtures](image)

**Figure 4-3** SDS PAGE gel of commercial enzyme mixtures

Multifect Xylanase has a major band at around 25 and 81kDa (Figure 4-3). The molecular weight of ~25 kDa is consistent with that of *T. reesei* EG III [102, 109] and the molecular weight of 81kDa is consistent with that of *T. reesei* ß-glucosidase. Spezyme and Accelerase also have a band at around 25 kDa. Indiage is purported to be composed of primarily of EG III (personal communication). A single band is evident for Indiage at ~25kDa that is consistent with *T. reesei* EG III.

### 4.3.5 Specific activities of commercial enzymes

To investigate the specific activities of the enzyme mixtures, the key activities were determined against defined substrates. The endoglucanase activity was estimated by measurement of release of soluble reducing sugars from amorphous cellulose substrate
carboxymethyl cellulose (CMC). The exoglucanase activity was similarly assessed with the crystalline cellulose substrate Avicel. Endoxylanase activity was measured against the substrate Birchwood xylan. β-glucosidase activity was measured against the substrate para-nitrophenyl β-glucopyranosidase. β-xylosidase activity was measured against the substrate para-nitrophenyl β-xylopyranosidase.

Table 4.2 Specific enzyme activities of commercial enzyme mixtures: Specific activities (U/mg of enzyme) of Spezyme CP, Accelerase 1000, Primafast Luna CL, Multifect Xylanase, Indiagese Super L and Novozyme 188 were measured at 50°C against defined substrates. ND corresponds to not determine. (The PNP-GP and PNP-XP activities on all the enzymes and all the specific activities of Novozyme 188 were determined by Christopher J Barr).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CMCase</th>
<th>Avicelase</th>
<th>Xylanase</th>
<th>PNP-GP</th>
<th>PNP-XP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spezyme</td>
<td>4.27±0.38</td>
<td>0.11 ± 0.04</td>
<td>3.96 ± 0.12</td>
<td>0.88</td>
<td>0.2</td>
</tr>
<tr>
<td>Accelerase</td>
<td>0.12</td>
<td>0.15</td>
<td>0.66</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Primafast</td>
<td>0.42 ± 0.05</td>
<td>0.16 ± 0.03</td>
<td>0.51 ± 0.040</td>
<td>0.9</td>
<td>0.012</td>
</tr>
<tr>
<td>Xylanase</td>
<td>44</td>
<td>0.19</td>
<td>105</td>
<td>1.29</td>
<td>1.43</td>
</tr>
<tr>
<td>Novozyme</td>
<td>0.15</td>
<td>0.022</td>
<td>0.056</td>
<td>5.74 ± 0.46</td>
<td>0.0497</td>
</tr>
</tbody>
</table>

The specific activity data were found to be consistent with the SDS PAGE results. Spezyme CP has higher endoglucanase and xylanase activity and equivalent cellobiohydrolase activity compared to Accelerase (Table 4.2). Accelerase and Spezyme have considerable amounts of β-glucosidase and β-xylosidase activities. Spezyme can hydrolyze both crystalline and amorphous cellulose substrates with intact hemicellulose as it contains both cellulase and hemicellulase enzymes. Accelerase can hydrolyze crystalline and amorphous cellulose substrates lacking in hemicellulose content as it has
very low xylanase activity. The $\beta$-glucosidase and $\beta$-xylosidase activities for Accelerase were not determined. Primafast has cellbiohydrolase and xylanase activity similar to that of Accelerase. Primafast has higher endoglucanase activity compared to Accelerase and hence can hydrolyze amorphous cellulose more rapidly. Primafast also has considerable $\beta$-glucosidase and $\beta$-xylosidase activities. Primafast has lower endoglucanase and xylanase activity than Spezyme CP and comparable cellbiohydrolase activity compared to Spezyme. Primafast cannot hydrolyze xylan because of its lack of xylanase activity. The performance of Primafast enzyme on different feedstocks was evaluated in detail in the following sections.

Multifect Xylanase, used for xylan hydrolysis, was observed to have significant xylanase activity (Table 4.2). It also has significantly higher endoglucanase, $\beta$-glucosidase and $\beta$-xylosidase activities compared to other the enzymes mixtures in Table 4.2. Novozyme 188, known to be specific for cellobiose hydrolysis, has significant $\beta$-glucosidase activity. It also has significant endoglucanase activity with minimal cellbiohydrolase, xylanase and $\beta$-xylosidase activities compared to the cellulase and xylanase enzymes.

4.3.6 Saccharification with commercial enzyme mixtures

Enzymatic conversions of IL treated and untreated substrates (Avicel, poplar and switchgrass) were compared to ascertain the effectiveness of ionic liquid pretreatment. The IL treated and untreated samples were hydrolyzed with commercial cellulase enzyme systems: 15 FPU (38 mg protein) / g glucan Spezyme CP; 15 FPU (23 mg protein) / g
glucan Accelerase; 38 mg protein /g glucan Primafast Luna CL; 38 mg protein /g glucan Indi age Super L. The cellulase systems were supplemented with Novozyme 188, a β-glucosidase, (30 CBU/ g glucan) to avoid end product inhibition by cellobiose. Cellobiase activity is determined by a cellobiose hydrolysis assay [107] and expressed as cellobiose units (CBU) per gram of glucan. The yield of glucose and xylose from glucan and xylan, respectively, was reported as a percentage of theoretical yields of monomeric sugars based on the glucan and xylan analysis of untreated substrates in Table 4.1.

Higher 24 hour hydrolysis yields were obtained for most substrates with Spezyme compared to hydrolysis with Accelerase, Primafast or Indi age (Tables 4.3 and 4.4).

Table 4.3  Enzymatic hydrolysis of pretreated poplar, switchgrass and Avicel with Spezyme CP and Accelerase 1000: Hydrolysis results after 5 and 24 hours for 5% (w/w) IL-treated substrates, poplar, switchgrass and Avicel. The percentage theoretical yield of glucose (% Glu) from glucan and xylose (% Xyl) from xylan is based on glucan and xylan analysis of untreated substrates in Table 4.1. Substrates were hydrolyzed with Spezyme CP (38 mg protein/g glucan), Accelerase (23 mg protein/g glucan) at 15 FPU/g glucan supplemented with Novozyme 188 at 30 CBU/g glucan. Measurements are an average of duplicate samples which deviated by ± 6 to10%.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Spezyme (15FPU, 38 mg)</th>
<th>Accelerase (15 FPU, 23mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Glu</td>
<td>% Xyl</td>
</tr>
<tr>
<td>Substrate</td>
<td>5 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Poplar</td>
<td>39</td>
<td>85</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>46</td>
<td>91</td>
</tr>
<tr>
<td>Avicel</td>
<td>77</td>
<td>100</td>
</tr>
</tbody>
</table>
Complete conversion of Avicel was observed with Spezyme and Accelerase within 24 hours of hydrolysis (Table 4.3). Spezyme CP and Accelerase have almost comparable Avicelase activity (Table 4.2). Spezyme has higher CMCase activity than Accelerase. The complete conversion with both Spezyme and Accelerase indicates that cellulase activity is adequate in both mixtures. The higher CMCase of Spezyme is likely not required for complete 24 hour hydrolysis.

Spezyme and Accelerase performance with biomass do show differences in saccharification yields (Table 4.3). The 24 hour yield of glucose from glucan for poplar was 85% with Spezyme CP and 71% with Accelerase. Similarly the 24 hour yield of glucose from glucan for switchgrass was 91% with Spezyme CP and 65% with Accelerase. In a similar way, the 24 hour yield of xylose from xylan for poplar was 76% with Spezyme CP and 43% with Accelerase. Similarly the 24 hour yield of xylose from xylan for switchgrass was 71% with Spezyme CP and 36% with Accelerase. With Spezyme, the xylose yields were essentially complete based on the composition of xylose in pretreated poplar and switchgrass (Table 4.1). The xylose yields with Accelerase were lower by 40% compared to the xylose yields with Spezyme (Table 4.3) due to the very low xylanase activity in Accelerase (Table 4.2). The higher glucose yields with Spezyme could be due to the higher CMCase (endoglucanase) activity in Spezyme compared to Accelerase (Table 4.2). However, the lower yields with biomass are more likely due to the incomplete hemicellulose hydrolysis with Accelerase. Hemicellulose components can impede access of cellulose to cellulases.
Table 4.4  Enzymatic hydrolysis of pretreated poplar, switchgrass and Avicel with Primafast Luna CL and Indiage Super L: Hydrolysis results after 5 and 24 hours for 5% (w/w) IL-treated substrates, poplar, switchgrass and Avicel. (a) The percentage theoretical yield of glucose (% Glu) from glucan and xylose (% Xyl) from xylan is based on glucan and xylan analysis of untreated substrates in Table 4.1. Substrates were hydrolyzed with Indiage at 38 mg/g glucan and Primafast at 38 mg protein/g glucan. All mixtures were supplemented with β-glucosidase at 30 CBU/g glucan. Measurements are an average of duplicate samples which deviated by ± 6 to10%. The measurements with Indiage were single runs.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Primafast (38mg)</th>
<th>Indiage (38 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Glu</td>
<td>% Xyl</td>
</tr>
<tr>
<td>Substrate</td>
<td>5 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Poplar</td>
<td>21</td>
<td>48</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>Avicel</td>
<td>42</td>
<td>59</td>
</tr>
</tbody>
</table>

With Primafast the conversion of Avicel (59%) (Table 4.4) was observed to be lower compared to Spezyme and Accelerase (Table 4.3). This could be due in part to the lower CMCase activity of Primafast compared to Spezyme (Table 4.2). However, Accelerase and Primafast have similar CMCase and Avicelase activities (Table 4.2). From the SDS PAGE distinct bands for EG I, EG II and EG IV were observed for both Primafast and Accelerase (Figure 4-1). The EG’s have different substrate specificities. EG I has broad specificity and has been found to be active against amorphous cellulose, β-1,3–1,4-glucan (a major hemicellulose component of the primary cell wall of terrestrial plants [114]), xyloglucan and xylan [115]. In addition to the above substrates, EG I was also found to adsorb very strongly on Avicel and primarily produces cellobiose and glucose[94]. EG II also adsorbs strongly on Avicel but primarily produced cellotriose in addition to cellobiose and glucose [94]. The cellotriose hydrolysis is very slow in EG II.
compared to EG I [94]. EG II has narrower specificity with activity against amorphous cellulose and soluble cellulose derivatives[115]. EG II is cellulose specific because it can cleave only the unsubstituted β-1,4 linkages in β-1,3-1,4-glucan with no activity against xylan or xyloglucan [115]. EG IV has low activity against 1,3–1,4-β-D-glucan, Avicel and xylan [116]. It has higher activity against cellotetraose and cellopentaose producing cellobiose and glucose [117]. The accumulation of cellobiose or cello oligomers was not observed due to the added cellobiase activity of Novozyme 188.

Primafast and Accelerase have similar CMCase, Avicelase and xylanase activities. Accelerase was initially designed for dilute acid pretreated biomass and may lack broad hemicellulase activity. The ratio of xylanase to CMCase activity is higher in Accelerase compared to Primafast (Table 4.2). Since EG I has activity against xylan with broader specificity against cellulose substrates, Accelerase appears to have higher EGI activity than Primafast. The lower xylanase to the CMCase activity in Primafast compared to Accelerase (Table 4.2) infers that Primafast could have higher EGII or EG IV and less EG I than Accelerase. EG IV is not active against Avicel and EG II results in slower hydrolysis of Avicel to glucose. The lower hydrolysis yields may be due to Primafast’s lack of CBH activity compared to Accelerase. Similar to the Avicel hydrolysis, the sugar yields from biomass were also significantly lower with Primafast compared to Spezyme and Accelerase. The 24 hour yield of glucose from glucan for poplar was 48% and 29% for switchgrass with Primafast. The 24 hour yield of xylose from xylan for poplar was 39% and 14% for switchgrass with Primafast. The lower glucose yields could be due to the lower CMCase activity and the lower xylose yields
could be due to the lower xylanase activity (Table 4.2) compared to Spezyme. The lower glucose and xylose yields with Primafast compared to Accelerase could be due to the higher activity of EG II or EG IV than EG I. Since EG II and EG IV show no activity against xylan, xylose remained unconverted and hindered the glucose conversion. For efficient glucan and xylan conversions in biomass with Primafast, supplementation with xylanases is essential.

With Indiage the conversion of Avicel is almost negligible compared to other enzyme mixtures. From the gel data (Figure 4-3), it appears that Indiage could be primarily EG III. EG III has very weak adsorption on Avicel [94]. EG III is active against xyloglucan (a polysaccharide that interacts with cellulose micro fibrils via hydrogen bonding) [118] and substituted and unsubstituted β-D-glucan with very low CMCase activity compared to EG I and EG II [115]. The lower CMCase activity and the weak adsorption on Avicel appear to be correlated with the lower Avicel conversions. The lower glucose yields from biomass appear to be consistent with the Avicel hydrolysis data. The xylose yields from xylan for poplar and switchgrass were 78% and 58%, respectively. The xylose yields with Indiage appear to be essentially complete based on the composition of xylose in pretreated poplar and switchgrass (Table 4.1). It appears that Indiage could possess the activity that cleaves the bonding connecting the β-1, 4-D-glucan backbone to xylose and xylan substituents. It appears to have very low activity against the β-1, 4-D-glucan backbone of cellulose.

Based on the above saccharification screening results, it appears that the enzymes should also be characterized against additional hemicellulose substrates such as tamarind
(xyloglucan), arabinoxylan, and polygalacturonic acid and cellulose substrates such as barley (β-D-glucan), laminarin, lichenin, hydroxyl ethyl cellulose (HEC) to give a deeper understanding of the specific activities of the enzyme mixtures.

4.3.7 Multifect xylanase supplementation to Primafast

To improve the hydrolysis of biomass rich in hemicellulose, supplementation with either cellulases or hemicellulases (xylanases) appears essential. In order to enhance the cellulose hydrolysis by Primafast, the protein loading was increased almost two-fold to 67 mg protein / g glucan. The hydrolysis of untreated and pretreated Avicel was almost unchanged with an increase in Primafast loading since the IL pretreated Avicel exhibited a higher CrI than biomass samples (Table 4.1). Even at high loadings of Primafast, the higher crystallinity of pretreated Avicel constitutes a core barrier for complete hydrolysis. But, the hydrolysis of biomass was enhanced at high loadings of Primafast (Table 4.5a). Five hour yields of glucose from glucan increased approximately by three-fold (63%) for pretreated poplar and over two-fold for switchgrass (42%) when compared to the data from lower enzyme loading. Primafast hydrolyzed increasing amounts of the amorphous cellulose portions of lignocellulosic material roughly proportional to increased loadings (Table 4.5a) with less than 10% yield for all the untreated substrate (data not tabulated).

The yield of xylose from xylan for untreated and IL treated poplar and switchgrass appears essentially unchanged with increased enzyme loadings (Table 4.5b). We attribute the increased glucan and unchanged xylan hydrolysis seen for the lignocellulosic substrates at higher enzyme loadings to be the result of the greater activity
of added cellulose selective enzymes, and the lack of strong xylan specific activity in the Primafast enzyme mixture. The relative lack of hemicellulase activity in the commercial mixture of Primafast Luna CL at low enzyme loading results in the lower conversion of glucan for biomass rich in hemicellulose. The unconverted xylan fraction has been found to hinder glucan hydrolysis and impede access of glucanases to their substrates [119, 120]. Hydrolysis of substrates with higher xylan content can be improved by supplementing the cellulases with xylanases [121]. Primafast Luna CL was hence supplemented with Multifect Xylanase, a commercial mixture, at 10% of the lower Primafast loading 3.8 mg protein/ g glucan [122]. β-glucosidase was added to the mixture to prevent inhibition from cellobiose accumulation. Multifect Xylanase alone was also added to IL treated (Table 4.5) and untreated substrates.
Table 4.5  Enzymatic hydrolysis of pretreated poplar, switchgrass and Avicel with Primafast supplemented with Multifect Xylanase: (a) The percentage theoretical yield of glucose (% Glu) from glucan and (b) xylose (% Xyl) from xylan is based on glucan and xylan analysis of untreated substrates in Table 1. Substrates were hydrolyzed with Spezyme CP at 38 mg protein/g glucan noted as Spezyme (38mg) or Primafast Luna CL at 38 or 67 mg protein/g glucan noted as Primafast (38 mg) and Primafast (67 mg), respectively. Primafast/Mult Xyl is a combination of Primafast Luna CL at a concentration of 38 mg protein/g glucan supplemented with Multifect Xylanase at 3.8mg protein/g glucan. Multi Xyl is Multifect Xylanase at 3.8mg protein/g glucan. Spezyme and Primafast were supplemented with β-glucosidase at 30 CBU/g glucan. Measurements are an average of duplicate samples which deviated by ± 6 to10%. The average and one standard deviation are shown for samples run in triplicate.

(a) Theoretical glucose yield.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Spezyme (38 mg)</th>
<th>Primafast (38 mg)</th>
<th>Primafast (67mg)</th>
<th>Primafast/Mult Xyl</th>
<th>Multi Xyl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Glu</td>
<td>% Glu</td>
<td>% Glu</td>
<td>% Glu</td>
<td>% Glu</td>
</tr>
<tr>
<td>Substrate</td>
<td>5 h</td>
<td>24 h</td>
<td>5 h</td>
<td>24 h</td>
<td>5 h</td>
</tr>
<tr>
<td>Poplar</td>
<td>39</td>
<td>85</td>
<td>21</td>
<td>48</td>
<td>63</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>46</td>
<td>91</td>
<td>18</td>
<td>29</td>
<td>42</td>
</tr>
<tr>
<td>Avicel</td>
<td>77</td>
<td>100</td>
<td>42</td>
<td>59</td>
<td>45</td>
</tr>
</tbody>
</table>

(b) Theoretical xylose yield.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Spezyme (38 mg)</th>
<th>Primafast (38 mg)</th>
<th>Primafast (67mg)</th>
<th>Primafast/Mult Xyl</th>
<th>Multi Xyl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Xyl</td>
<td>% Xyl</td>
<td>% Xyl</td>
<td>% Xyl</td>
<td>% Xyl</td>
</tr>
<tr>
<td>Substrate</td>
<td>5 h</td>
<td>24 h</td>
<td>5 h</td>
<td>24 h</td>
<td>5 h</td>
</tr>
<tr>
<td>Poplar</td>
<td>39</td>
<td>76</td>
<td>19</td>
<td>39</td>
<td>8</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>26</td>
<td>71</td>
<td>5</td>
<td>14</td>
<td>5</td>
</tr>
</tbody>
</table>

93
The addition of xylanases to the Primafast mixture led to additional improvements in the yields of both glucose and xylose sugars for pretreated substrate after 24 hour of hydrolysis when compared to the hydrolysis data of Primafast alone. But the addition of Xylanase did not show any improvement in the yields of sugars for untreated substrate. The increase in xylan hydrolysis at 24 hours is approximately the sum of that measured with Primafast and Multifect Xylanase alone for both poplar and switchgrass (Tables 4.5). For switchgrass and Avicel the glucan hydrolysis for the mixture of Primafast and xylanase is also approximately equal to the sum of the individual components (Table 4.5). The yield of glucose was increased from 29% to 46% for pretreated switchgrass with relatively low xylan hydrolysis of about thirty percent for the Primafast / Xylanase mixture. In contrast, poplar xylan was completely hydrolyzed in the Primafast / Xylanase mixture. The resulting 24 hour glucan hydrolysis of poplar with the Primafast / Xylanase mixture exceeded the sum of the individual components by 27% (Figure 4-4), indicating that the hemicellulosic components of biomass can impede hydrolysis of amorphous cellulose. In switchgrass, with low xylan hydrolysis with added xylanases, the glucan hydrolysis appears to remain impeded and no significant synergistic effects on glucan hydrolysis with addition of xylanase were observed in contrast to that of poplar.
Synergism between Primafast and Multifect Xylanase: Twenty four hour glucan conversion of IL pretreated biomass hydrolyzed with Primafast and Multifect Xylanase. The glucan conversions using a combined mixture of Primafast and Multifect Xylanase enzyme systems are greater than the sum of their individual contributions, indicating synergism between Primafast and Multifect Xylanase in the hydrolysis of biomass.

The impedance of glucan hydrolysis and low xylan hydrolysis in switchgrass compared to poplar may be attributed to differences in chemical composition of hemicellulose in herbaceous crops and hardwoods. The principal hemicelluloses in herbaceous crops such as switchgrass are arabinoxylans and that in hardwoods are 4-O-methyl glucuronoxylans [123]. Arabinoxylan consists of alpha-L-arabinofuranose residues attached to the xylose backbone. In addition, ferulic acids and p-coumaric acids
may be covalently linked to arabinoxylans. These substitutions on the xylose backbone can retard xylanase activity and inhibit the hydrolysis of both cellulose and hemicellulose. To achieve complete xylan hydrolysis the enzyme mixture should possess both side group cleaving and depolymerizing activities for highly substituted xylan substrates [124]. In addition to xylan other non-cellulosic polysaccharides that coat the cellulose fibers is pectin. Pectin is a heteropolysaccharide, consisting of a complex set of polysaccharides that are present in most primary cell walls of the non-woody parts of terrestrial plants. Hence, supplementation with pectinase along with xylanase helps stimulate cellulose hydrolysis in switchgrass [125].

4.3.8 Specific enzyme activities of enzyme mixtures

The hydrolysis results appear to be correlated with the specific activities of the enzyme mixtures (Table 4.6). The specific activity of the Spezyme/Novozyme enzyme mixture is comparable to that of the Primafast/Xylanase/Novozyme mixture. Since, the xylanase activity was high in both of these mixtures complete hydrolysis of xylan in poplar was reached. This is in contrast to switchgrass possibly due to structural differences between these substrates. The Primafast 38 /Novozyme enzyme mixture lacked xylanase, endoglucanase and β-glucosidase activity. As seen in the hydrolysis data shown in Table 4.5, the low xylan hydrolysis may have impeded access of cellulases and the low glucan conversion to glucose. In the Primafast 67/ Novozyme enzyme mixture with limited xylanase activity, xylan conversion was not enhanced compared to low Primafast loadings. The Xylanase/ Novozyme mixture lacked
cellulbiohydrolase activity which resulted in very low conversions of glucan but significant conversion of xylan due to high xylanase activity.

Table 4.6 Specific enzyme activities (U/g glucan or xylan) of Primafast/ Multifect Xylanase enzyme mixtures: SP is Spezyme CP; NV is Novozyme 188; PF is Primafast CL; XY is Multifect Xylanase. The activities were calculated from their individual specific activities (U/ mg protein) from Table 4.2.

<table>
<thead>
<tr>
<th>Activities</th>
<th>SP 38 NV 30</th>
<th>PF 38 NV 30</th>
<th>PF 67 NV 30</th>
<th>PF 38 XY 11.6 NV 30</th>
<th>XY 11.6 NV 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCase</td>
<td>163</td>
<td>17</td>
<td>29.2</td>
<td>184</td>
<td>168.3</td>
</tr>
<tr>
<td>Avicelase</td>
<td>4.3</td>
<td>6.2</td>
<td>10.8</td>
<td>6.9</td>
<td>0.87</td>
</tr>
<tr>
<td>Xylanase</td>
<td>465</td>
<td>61</td>
<td>127</td>
<td>1279</td>
<td>1219</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>72</td>
<td>74.4</td>
<td>100.5</td>
<td>79</td>
<td>45</td>
</tr>
<tr>
<td>β-xylosidase</td>
<td>24</td>
<td>2.5</td>
<td>3.55</td>
<td>19</td>
<td>17.66</td>
</tr>
</tbody>
</table>

4.4. Conclusions

The commercial T. reesei enzymes, Spezyme CP, Accelerase 1000, Primafast Luna CL and Indiage Super L, used in this work differed in the specificity of saccharification of glucan and xylan components of lignocellulose. Spezyme hydrolyzed both cellulose and hemicellulose components of IL pretreated substrates completely in 24 hours. Accelerase hydrolyzed Avicel completely and appears to have high EG I activity. EG I is active against both Avicel and amorphous cellulose. Significant glucan hydrolysis in biomass was observed with Accelerase. Lower xylan hydrolysis in biomass with Accelerase was due to the lower xylanase activity compared to Spezyme. Indiage,
primarily EG III, hydrolyzed the xylan fraction of biomass and little glucan. Primafast appears to primarily hydrolyze the amorphous cellulose fraction of biomass. Primafast, a mixture of endoglucanases (EG I, EG II and EG IV) appears to have higher EG II and EG IV activities than EG I. Primafast showed very low activity against xylan as EG II and EG IV have very low xylan activity. Incomplete xylan hydrolysis may lead to lower glucan hydrolysis. Primafast in combination with Multifect Xylanase hydrolyzed both the cellulose and hemicellulose fractions of poplar, but was less effective with switchgrass. The increased digestibility in cellulose and hemicellulose fractions of poplar appears to arise from synergism between Primafast cellulase and Multifect Xylanase rather than from the additional cellulase activity of Multifect Xylanase.
Chapter 5

Enzymatic Hydrolysis of Ionic Liquid Pretreated Poplar at High Solids Loadings

5.1. Introduction

Conversion of biomass to concentrated product streams through saccharification and fermentation at high solid loadings is both a rate and cost limiting process for ethanol and other fermentation products [116]. The goal of the current study is to maximize the sugar yields at higher biomass slurry concentrations to create a platform for designing a sequential or simultaneous saccharification of biomass with xylanase and cellulases and fermentation by micro-organisms to ethanol or other products. High solids enzymatic hydrolysis increases the product concentration, reduces the capital costs and improves the process economics [44, 126, 127]. The rates of enzymatic hydrolysis tend to decrease as the biomass concentration increases. This decrease in the rates compared to low biomass
concentrations could be due to mass transfer limitations, steric hindrances due to lignin deposition, soluble inhibitors or end product inhibition [128].

Efficient conversion of sugars from biomass involves uniform and adequate distribution of heat and enzymes into the biomass slurries and efficient transport of sugars back into the liquid medium [129]. At low slurry concentrations the biomass particles are freely suspended in the liquid medium with negligible hydrodynamic interactions between the particles and fewer transport issues [130]. At high slurry concentrations, the volume of liquid present can be equal to the interparticle void volume as a minimum limit. Dense suspensions can be formed with entanglement of particles resulting in interparticle interactions and complex slurry rheology [131]. As slurry concentration increases, achieving efficient mixing without mass transfer limitations is quite a challenge.

Despite potential challenges, efficient release of sugars from cellulose and hemicellulose components in high biomass slurries with a two stage treatment was reported [44, 132-137]. In the first stage, the biomass was pretreated with dilute acid to remove major portions of the hemicellulose and lignin rich components in a liquid phase and residual cellulose in a solid phase. In the second stage, the separated cellulose and hemicellulose fractions can be hydrolyzed separately with cellulases and hemicellulases, respectively, and subsequently fermented with glucose and xylose specific microorganisms. In contrast to dilute acid pretreatment, ionic liquid pretreatment results in amorphous cellulose with largely intact hemicellulose and lignin content [26]. Hydrolysis of cellulosic biomass with large amounts of hemicellulose and lignin fractions at high
slurry concentrations is a challenge. Potential problems such as end product inhibition, nonproductive binding of enzymes and impedance of enzyme access by various components may prevail in mixed sugar saccharification.

In the present study the lignocellulosic biomass, poplar was pretreated with the IL, 1-ethyl, 3-methyl imidazolium acetate, at 5% (w/w) solid loadings at 120°C. The pretreated poplar was enzymatically hydrolyzed at 10, 12 and 15% (w/v) with commercial cellulase and xylanase enzyme mixtures. The pretreated poplar was enzymatically hydrolyzed in two stages for sequential saccharification and fermentation of xylose followed by saccharification and fermentation of glucose sugars. The sequential saccharification and fermentation strategy was developed to overcome hurdles of diauxy exhibited by many micro-organisms. In the first step of hydrolysis, commercial xylanases were added to the lignocellulosic biomass slurries to hydrolyze the hemicelluloses to xylose at 50°C. In the second step, the hydrolysis temperature was dropped to 25°C for fermentation of xylose to ethanol with the yeast P. stipitis. Commercial cellulases were then added to the reaction mixture to hydrolyze the cellulose portion of biomass. The enzymatic hydrolysis experiments were carried out on bell rollers to mix solid loadings of 15% (w/v) or less. Hodge et al., 2005 [138] found that mass transfer limitations were not apparent when solid loadings were less than ~25% (w/v).
5.2. Materials and Methods

5.2.1 Pretreatment

Poplar (provided by the National Renewable Energy Laboratory) was pretreated with the ionic liquid, 1-ethyl 3-methyl imidazolium acetate, EmimOAc, (Sigma Aldrich, MO, USA). The biomass was mixed with EmimOAc, 5% (w/w), and incubated at 120°C for 30 or 60 minutes. The sample was then mixed with water at room temperature to precipitate the biomass from the IL biomass solution. The precipitant and wash solutions were separated by centrifugation. The precipitated solids were washed repeatedly with deionized (DI) water to displace the IL from the sample until the wash solution appeared colorless.

5.2.2 Compositional analysis

The carbohydrate and lignin content in the untreated and pretreated biomass were determined in duplicate using concentrated acid hydrolysis followed by dilute acid hydrolysis according to the standard laboratory analytical procedure developed by the National Renewable Energy Laboratory (NREL) [100]. Following washing with water, the pretreated samples were dried in a vacuum oven at 45°C until a constant weight was achieved. The monomeric sugars were quantified by high performance liquid chromatography with refractive index detection using a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate column and a mobile phase of de-ionized water at a flow rate of 0.6 ml/min. The temperature of the column was maintained at 80°C.
5.2.3 Powder XRD measurement

XRD data for untreated and pretreated poplar were measured at 25°C with an X’PERT PRO powder diffractometer PAN 188 analytical with X’celerator detector using Nickel filtered Cu Kα radiation. Samples were scanned over the range of 5–35° (2θ), with a step size of 0.05 and step time of 10 s. The crystallinity indices, CrI were calculated for the samples [58].

5.2.4 Enzymatic hydrolysis

Commercial cellulase/hemicellulase enzyme mixture, Spezyme CP, and xylanase enzyme mixture, Multifect Xylanase, were provided by Genencor International Rochester, NY, USA. β-glucosidase, Novozyme 188, was purchased from Sigma Aldrich, MO, USA. β-xylosidase, from Selenomonas ruminantium (SXA) of glycoside hydrolase family 43 was provided by Douglas Jordan, USDA ARS, NCAUR, Peoria, IL. The activity of Spezyme CP was determined by measurement of soluble reducing sugars via a 2,5-dinitrosalicylic acid assay [104] and expressed as filter paper units, FPU. The protein content in Spezyme CP and Multifect Xylanase was estimated using a Bradford assay with bovine serum albumin as the standard [105]. The protein content in SXA, β-xylosidase was determined by measuring the absorbance of the protein at 280nm with an extinction coefficient of 129,600 M⁻¹ cm⁻¹ [139].

Hydrolysis was carried out with 10, 12 and 15% (w/v) poplar in 0.05 M sodium citrate buffer, pH 4.8 (with added 30mg/l cyclo heximide and 40 mg/l tetracycline), in 30ml glass scintillation vials on a Bellco roller mixer placed in a incubator for
temperature control. The pretreated substrate was hydrolyzed with the commercial cellulase and xylanase and supplemented with β-glucosidase (Novozyme 188 from Sigma Aldrich, MO, USA) at 30 CBU/g glucan. Cellobiase activity is determined by a cellobiose hydrolysis assay [107] and expressed as cellobiose units (CBU) per gram of glucan. Aliquots of the reaction mixture were sampled periodically and the enzyme reaction was stopped by boiling the sample at 100°C for 10 minutes.

5.2.5 HPLC analysis

The sugar analysis of hydrolysis samples was performed using high performance liquid chromatography with refractive index detection on Bio-Rad (Richmond, CA) Aminex HPX-87P and HPX-87H analytical columns. The xylobiose detection can be done on 87P analytical column and cellobiose detection can be done on either of these two. The mobile phase for the 87P analytical column was HPLC grade DI water at a flow rate of 0.6 ml/min with a column temperature of 80°C. The mobile phase for the 87H column was 5mM sulfuric acid at a flow rate of 0.6 ml/min with a column temperature of 65°C. The sugar concentrations were calculated based on a standard calibration curve developed for mixed sugar standards.

5.3. Results and Discussion

5.3.1 Comparison of enzymatic hydrolysis at low and high biomass loadings

Saccharification was carried out at high solid loadings with the some of the Spezyme and Primafast enzyme mixtures used in Chapter 4. Enzymatic hydrolysis with pretreated poplar at high biomass loadings (10% w/v) is compared to low loadings (1%
w/v) for poplar pretreated in IL at 120°C, 30 minutes. The data for low biomass loadings were presented in Chapter 4.

With SP 38 NV 30 (38 mg Spezyme and 30 CBU Novozyme 188 per gram of glucan) significant 24 hour hydrolysis yields at both low and high biomass loadings were observed (Figure 5-1). With Primafast and Novozyme mixtures, the hydrolysis at 10% (w/v) compared to 1% (w/v) was severely inhibited with low glucan and xylan hydrolysis. Upon supplementation with β-xylosidase, SXA, both xylose and glucose yields improve but the overall hydrolysis is significantly lower than that of low biomass loading. With MX 23.2 NV 30 mixture, the lower yields of sugars at high biomass loadings compared to the low biomass loadings may be due to end product inhibition of xylanase by higher concentration of xylobiose in solution at 10% solid loadings. Glucose yields are unaffected due to low cellulase activity of Mulitfect Xylanase.
5.3.2 Comparison of enzymatic hydrolysis with extended IL incubation

Screening of pretreatment conditions were also performed with IL incubation at 120°C for 30 and 60 minutes. Poplar was incubated in IL at 5% (w/w) biomass loadings. The pretreatment condition of 120°C, 30 minutes was chosen as pretreated poplar produced near complete conversions of sugars at low biomass loadings (Chapter
The pretreatment condition 120°C, 60 minutes was chosen as cellulose II was observed in samples with extended incubation times (Chapters 2 and 3). Cellulose II was found to hydrolyze more rapidly than cellulose I.

The compositions of the untreated samples and IL-pretreated precipitate were analyzed (Table 5.1) and reported as dry weight of major components; glucan, xylan, arabinan, and lignin (acid soluble and insoluble). For poplar pretreated in IL at 5% (w/w) at 120°C for 30 minutes, negligible losses of cellulose and lignin to the IL washes were observed. Xylan losses of 15% were observed (as reported in Chapter 4). For poplar pretreated in IL at 5% (w/v) 120°C for 60 minutes, 24% loss of glucan and 21% loss of xylan were observed with negligible loss of lignin.

Table 5.1 Compositional analysis of untreated and pretreated poplar at 120°C, 30 and 60 minutes: The weight percent of major components of untreated and IL-treated at 120°C for 5% (w/w) poplar are given below. Lignin is the sum of acid soluble and acid insoluble lignin. The percentage of recovered mass of 300 mg samples after IL pretreatment, washing and drying at 120°C, 30 and 60 minutes after IL pretreatment, washing and drying is 93% and 95.66 % respectively. All measurements for poplar at 120°C, 30 minutes are an average of duplicate samples that deviated by ±6% and the measurements for poplar at 120°C, 60 minutes were done in triplicates that deviated by less than ±8%.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Poplar Untreated</th>
<th>Poplar treated 120°C, 30min</th>
<th>Poplar treated 120°C, 60min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>37</td>
<td>41</td>
<td>29±8</td>
</tr>
<tr>
<td>Xylan</td>
<td>12</td>
<td>11</td>
<td>10±2</td>
</tr>
<tr>
<td>Arabinan</td>
<td>0.5</td>
<td>0.4</td>
<td>2±0.7</td>
</tr>
<tr>
<td>Lignin</td>
<td>23</td>
<td>23</td>
<td>25±3</td>
</tr>
<tr>
<td>CrI</td>
<td>38</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

The two pretreatment conditions resulted in reduction in crystallinity of cellulose (Table 5.1) with CrI of 8 and 16 for 30 and 60 minute IL incubation, respectively. The
higher CrI at longer incubation time could be due to greater swelling of cellulose with extended incubation. This increased swelling could result in increased recrystallization and formation of cellulose II when IL was expelled from IL biomass solutions. This is consistent with studies by Cheng et al. 2011, [140] where Avicel pretreated with the IL, 1-ethyl, 3-methyl imidazolium acetate at harsher pretreatment conditions resulted in higher CrI than at milder pretreatment conditions.

The enzymatic hydrolysis for poplar pretreated in IL at 120°C, 30 minutes is compared to the poplar pretreated in IL at 120°C, 60 minutes at high biomass loadings (10% w/v). The enzymatic hydrolysis on poplar was carried out with various combinations of commercial enzymes at a solid loading of 10% (w/v). Yield of glucose and xylose, respectively, were calculated based on glucan and xylan in the feedstock (Table 5.1). The overall 24 hour hydrolysis yields of sugars from poplar pretreated for 30 minutes appear to be lower than that for the extended incubation time of 60 minutes (Figure 5-2). For most enzyme mixtures the increase in yields at 120°C, 60 minutes could be due in part to loss of glucan and xylan to the IL washes increasing the enzyme loading per gram of polysaccharide (Table 5.1). The longer incubation in IL could also lead to the greater the swelling and mobility of cellulose chains and the formation of cellulose II hydrate at 120°C. This was seen in Chapters 2 and 3 where IL treated Avicel transformed to cellulose II hydrate. The formation of cellulose II (vs I) hydrate is hard distinguish in XRD spectra of wet biomass samples due to the strong signal from water, obscuring the cellulose signal.

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Figure 5-2  Comparison of enzymatic hydrolysis of pretreated poplar with extended IL incubation: 24 hour enzymatic hydrolysis data of 5% (wt/wt) poplar pretreated with IL at 120°C incubated for 30 and 60 minutes. Pretreated poplar of 10% (w/v) is hydrolyzed by different commercial enzymes and the enzyme loadings were as follows: SP 38 – Spezyme CP @ 38 mg/g glucan, PF 38, 50 - Primafast Luna CL @ 38mg, 50mg /g glucan, XY 11.6, XY 23.2 - Multifect Xylanase @11.6mg, 23.2mg/g xylan, SXA 11.6 -β-xylosidase @ 11.6 mg/g xylan, NV 30- Novozyme 188 @ 30CBU/g glucan. Sugar yields are based on the pretreated poplar composition from Table 5.1.

Significant sugar yields with Spezyme / Novozyme mixture were observed at the two pretreatment conditions due to significant Avicelase, CMCase, xylanase and β-xylosidase activities in the enzyme mixture (Table 5.2). Hydrolysis results with SP38 NV30 enzyme mixture resulted in the 24 hour yields of glucose and xylose of 72% and 68%, respectively, for 30 minute IL pretreatment (Figure 5-2). For pretreatment
incubation time of 60 minutes the yields of glucose and xylose were 87% and 75%, respectively. The increase in glucose and xylose yields at extended incubation time could be due to the greater losses in glucan and xylan during pretreatment with extended incubation. Spezyme has greater Avicelase, CMCase, xylanase and \( \beta \)-xylosidase activities (i.e., more protein content/g of sugar) at extended incubation pretreatment conditions.

Table 5.2  The activities of the enzyme mixtures (U/g glucan or xylan) of Primafast/Multifect Xylanase/SXA: SP 38- Spezyme @ 38mg/g glucan, NV 30-Novozyme 188 @ 30CBU/g glucan, XY 11.6- Multifect Xylanase @11.6mg/g xylan, PF 38- Primafast Luna CL @ 38mg/g glucan, SXA 23.3-\( \beta \)-xylosidase @ 23.2 mg/g xylan.

<table>
<thead>
<tr>
<th>Activities (( \mu ) mol per min x sugar oligomer)</th>
<th>SP 38 NV 30</th>
<th>PF38 XY 11.6 NV 30</th>
<th>PF 38 XY 11.6 SXA 23.2 NV 30</th>
<th>MX 23.2 NV 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCCase</td>
<td>163</td>
<td>184</td>
<td>184</td>
<td>335</td>
</tr>
<tr>
<td>Avicelase</td>
<td>4.3</td>
<td>6.9</td>
<td>6.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Xylanase</td>
<td>465</td>
<td>1290</td>
<td>1290</td>
<td>2340</td>
</tr>
<tr>
<td>( \beta )-glucosidase</td>
<td>72</td>
<td>44</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>( \beta )-xylosidase</td>
<td>24</td>
<td>18</td>
<td>237</td>
<td>33</td>
</tr>
</tbody>
</table>

*The enzyme mixture activities were determined against defined substrates (Table 5.2) according to the procedure described in Chapter 4.

The Primafast / Novozyme enzyme mixture was supplemented with Multifect Xylanase due to lack of xylanase activity in Primafast. Cellulose hydrolysis can be hindered by the unconverted hemicellulose fraction. This effect was observed in Chapter 4 with low biomass loadings. The Hydrolysis results with the enzyme mixture PF 38 XY 11.6 NV30 resulted in the 24 hour yields of glucose and xylose of 32% and 26%, respectively, for 30 minute IL pretreatment. For pretreatment incubation time of 60
minutes, the yields of glucose and xylose dramatically increased to 83% and 73%, respectively. Near complete conversion of sugars were observed with Primafast/Multifect Xylanase/Novozyme enzyme mixture from poplar pretreated at 120°C, 60 minutes. The hydrolysis yields from poplar incubated for 30 minutes was observed to be very low (Figure 5-2). The xylanase, CMCase and Avicelase activity in the Primfast/Multifect Xylanase/Novozyme enzyme mixture did not seem to be sufficient in hydrolyzing significant amounts of glucose and xylose (Table 5.2). It appears to be inhibited by xylose oligomer accumulation.

With the addition of SXA to the Primafast/Multifect Xylanase mixtures (PF 50 XY 11.6 SXA 11.6 NV30), the 24 hour yields of glucose and xylose were 62% and 67%, respectively, for 30 minute IL pretreatment, a sharp increase from mixtures without SXA supplementation. For pretreatment incubation time of 60 minutes the yields of glucose and xylose increased modestly to 89% and 77%, respectively. Supplementation with SXA (β-xylosidase) to Primafast/Multifect Xylanase mixture appears to increase the xylose yield. An increase in xylose yield appears to be correlated to the increase in glucose yields (Figure 5-2). Xylose and xylose oligomer appears to inhibit the glucose yields. Similarly, for dilute acid and AFEX pretreated cornstover Wyman et al., 2011 observed glucose hydrolysis inhibition from xylose and xylose oligomer accumulation [141].

The xylose oligomer accumulation was also observed with Multifect Xylanase/Novozyme hydrolysis from poplar pretreated at 120°C, 30 minutes. The 24 hour yields of glucose and xylose were 13% and 33% respectively for 30 minute IL
pretreatment with the enzyme mixture XY 23.2 NV30. For pretreatment incubation time of 60 minutes the yields of glucose and xylose were 7% and 75% respectively. The increase in xylose yield at 60 minutes was due to the greater loss of xylan to the IL washes. The xylanase activity present in the Multifect Xylanase/Novozyme enzyme mixture is sufficient in hydrolysis the lower fraction of xylan at 60 minutes than at 30 minutes. The lower glucose yields with Multifect Xylanase/Novozyme enzyme at 30 and 60 minutes incubation was due to the very low Avicelase activity in the enzyme mixture (Table 5.2).

The overall hydrolysis rates suggest that xylanase and β-xylosidase activities are crucial in hydrolyzing high biomass slurries with significant hemicellulose fractions.

Despite higher conversions of sugars with IL incubation extended from 30 to 60 minutes, 60 minute pretreatment condition was not considered optimal due to large losses of sugars. The shorter incubation time of 30 minutes was used in further studies of saccharification at high biomass loadings.

5.3.3 High solids enzymatic hydrolysis for sequential saccharification and fermentation

Saccharification was carried out in two steps. In the first step, the saccharification was carried out with Multifect Xylanase and SXA (β-xylosidase) to hydrolyze a major portion of the hemicellulose and produce xylose. In the second step, the cellulosic portion was hydrolyzed to glucose by Spezyme CP and Novozyme 188. Inoculation with yeast
will be made during the second step in combined saccharification and fermentation experiments (Chapter 6).

The above mentioned strategy could be a positive approach in fermenting both glucose and xylose efficiently with *P. stipitis*. *P. stipitis*, a xylose fermenting yeast, experiences diauxic lag during mixed sugar fermentations. When grown on glucose, the switch from glucose to xylose is severely stalled due to slow expression of xylose reductase and xylitol dehydrogenase enzymes even though there seems to be xylose transport into the yeast cells [142]. These enzymes are essential for the isomerization of xylose to xylulose which is the preferred substrate for the yeast. The suppressed expression of these enzymes results in xylose or xylitol accumulation that is inhibitory to the glucose consumption resulting in diauxic lag. To avoid diauxic lag, the cells can be primed with xylose rich streams. The yeast cells are initially grown on xylose as opposed to glucose. Initial saccharification with Multifect Xylanase and SXA (β-xylosidase) releases xylose that helps in priming the cells with xylose to avoid diauxic lag when both glucose and xylose are available in mixed sugar hydrolyzates.

The hydrolysis with xylanase and cellulases was carried out with poplar pretreated with IL at 120°C for 30 minutes. Biomass solid loadings during hydrolysis were 10%, 12% and 15% (w/v) solid with various enzyme mixtures. Spezyme with Novozyme (SP 38 NV 30) exhibited high yield of sugars at both low and high biomass slurry concentrations (Figures 5-1 and 5-2). The xylanase and β-xylosidase activities in SP38 NV30 enzyme mixture were used as the basis for initial Multifect Xylanase and SXA enzyme loading. The enzyme loadings were 4.5 mg / g xylan of Multifect Xylanase
equivalent to about 465 U / g xylan of xylanase activity in SP38 NV30 mixture (Table 5.2). 1.8 mg / g xylan of SXA (β-xylosidase) was equivalent to about 24 U / g xylan of β-xylosidase activity in SP38 NV30 enzyme (Table 5.2).

Initial screening experiments at variable solids loading were performed (Table 5.3). In the first step, saccharification of pretreated poplar is carried out with Multifect Xylanase and SXA (β-xylosidase) at 50°C for about 24 to 32 hours. In the second step temperature was reduced to 37°C since many yeast strains cannot withstand higher temperatures. The cellulase enzyme Spezyme CP was added at 13 mg /g glucan (5 FPU/g glucan at 50°C) with 7mg Novozyme 188/g glucan (30CBU/g glucan).

Table 5.3  Sequential saccharification at 50 and 37°C of pretreated poplar at high solid loadings: Enzymatic hydrolysis of poplar pretreated with IL at 120°C, 30 minutes. The enzyme loadings were 4.5 mg / g xylan of Multifect Xylanase and 1.8 mg / g xylan of SXA (β-xylosidase) at 50°C and 5 FPU / g glucan (13 mg/g glucan) Spezyme CP and 30 CBU / g glucan (7mg/g glucan) Novozyme 188 at 37°C. Glucose and xylose yields are given in parentheses.

<table>
<thead>
<tr>
<th>Solid loadings</th>
<th>Initial Enzyme mixture</th>
<th>Supplementation</th>
<th>Time (hr)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>MX/SXA+</td>
<td>SP/NV (37°C)</td>
<td>32</td>
<td>3 (8%)</td>
<td>10 (83%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>64</td>
<td>16 (43%)</td>
<td>13 (100%)</td>
</tr>
<tr>
<td>12%</td>
<td>MX/SXA+</td>
<td>SP/NV (37°C)</td>
<td>48</td>
<td>2 (5%)</td>
<td>9.3 (65%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>14 (32%)</td>
<td>12 (83%)</td>
</tr>
<tr>
<td>15%</td>
<td>MX/SXA+</td>
<td>SP/NV (37°C)</td>
<td>48</td>
<td>1.57±0.13 (4%)</td>
<td>5±0.47 (28%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
<td>11.5±0.83 (21%)</td>
<td>5.12±0.37 (28%)</td>
</tr>
</tbody>
</table>

MX- Multifect Xylanase, SXA-β-xylosidase, SP-Spezyme CP and NV- Novozyme 188 (β-glucosidase). *The complete yield of glucose corresponds to 37 mg/ml at 10 % (w/v), 44.4 mg/ml at 12 % (w/v) and 55.5 mg/ml at 15 % (w/v). Similarly the complete yield of xylose corresponds to 12mg/ml at 10% (w/v), 14.4 mg/ml at 12 % (w/v) and 18mg/ml at 15 % (w/v).
The yields of glucose were very low for 10, 12 and 15 % (w/v) most likely due to low activity of Spezyme at 37°C. The yields of xylose were near completion due to the high activity of Multifect Xylanase at 50°C. The xylose yield of 83% at 10% (w/v) solid loading at 50°C after 32 hours compared to the xylose yield of 30% at 10% (w/v) solid loading in Figure 5-1 (Multifect Xylanase without addition of SXA). Results suggest that xylose conversion was inhibited by xylobiose or xylose oligomers without added SXA. A combination of Multifect Xylanase and β-xylosidase appears essential for complete xylan hydrolysis at high biomass slurries.

Table 5.4  Sequential saccharification at 50 and 37°C of pretreated poplar with 10 FPU/g glucan Spezyme CP: Enzymatic hydrolysis of poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 4.5 mg / g xylan of Multifect Xylanase and 1.8 mg / g xylan of SXA (β-xylosidase) at 50°C and 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 30 CBU / g glucan (7mg/g glucan) Novozyme 188 at 37°C. Glucose and xylose yields are given in parentheses.

<table>
<thead>
<tr>
<th>Solid loadings</th>
<th>Initial Enzyme mixture</th>
<th>Supplementation</th>
<th>Time (hr)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>MX/SXA+</td>
<td>SP/NV (37°C)</td>
<td>24</td>
<td>2 (5%)</td>
<td>9 (75%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>48</td>
<td>27 (73%)</td>
<td>12 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>72</td>
<td>36 (97%)</td>
<td>14 (100%)</td>
</tr>
<tr>
<td>12%</td>
<td>MX/SXA+</td>
<td>SP/NV (37°C)</td>
<td>24</td>
<td>1 (23%)</td>
<td>4 (28%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>48</td>
<td>31 (70%)</td>
<td>13 (90%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>72</td>
<td>43 (97%)</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>15%</td>
<td>MX/SXA+</td>
<td>SP/NV (37°C)</td>
<td>24</td>
<td>1 (2%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>48</td>
<td>22 (40%)</td>
<td>11 (61%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>72</td>
<td>30 (54%)</td>
<td>13 (72%)</td>
</tr>
</tbody>
</table>

MX- Multifect Xylanase, SXA-β-xylosidase, SP-Spezyme CP and NV- Novozyme 188 (β-glucosidase). *The complete yield of glucose corresponds to 37 mg/ml at 10 % (w/v), 44.4 mg/ml at 12 % (w/v) and 55.5 mg/ml at 15 % (w/v). Similarly the complete yield of xylose corresponds to 12mg/ml at 10% (w/v), 14.4 mg/ml at 12 % (w/v) and 18mg/ml at 15 % (w/v).
In the next set of screening experiments, the cellulase loading was increased by two fold to 10FPU/g glucan to achieve higher yields of glucose (Table 5.4). The yields of both glucose and xylose were observed to be almost complete at 10 and 12% (w/v). At 15% (w/v) an increase in the xylose yield was observed from 28% (Table 5.3) to 72% and the glucose yield was observed to have increased from 21% (Table 5.3) to 54% at the end of 72 hours hydrolysis (Table 5.4). In the next set of experiments the cellobiose and xylobiose accumulation were carefully followed at 15% (w/v) solid loading (Table 5.5).

Table 5.5  Sequential saccharification at 50 and 30°C of 15% (w/v) pretreated poplar: Enzymatic hydrolysis of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 4.5 mg / g xylan of Multifect Xylanase and 1.8 mg / g xylan of SXA (β-xylosidase) at 50°C and 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 30 CBU / g glucan (7mg/g glucan) Novozyme 188 at 30°C. Glucose and xylose yields are given in parentheses.

<table>
<thead>
<tr>
<th>Initial Enzyme Mixture</th>
<th>Supplementation</th>
<th>Time (hr)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
<th>Cellobiose (g/l)</th>
<th>Xylobiose (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX/SXA</td>
<td>-</td>
<td>6</td>
<td>1 (2%)</td>
<td>3 (17%)</td>
<td>0.44</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>SP/NV (30°C)</strong></td>
<td>24</td>
<td>2 (4%)</td>
<td>7 (39%)</td>
<td>0.14</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>15 (27%)</td>
<td>10 (56%)</td>
<td>~0</td>
<td>~0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>28 (50%)</td>
<td>14 (78%)</td>
<td>~0</td>
<td>~0</td>
<td></td>
</tr>
</tbody>
</table>

MX- Multifect Xylanase, SXA-β-xylosidase, SP-Spezyme CP and NV- Novozyme 188 (β-glucosidase). *The complete yield of glucose and xylose corresponds to 55.5 mg/ml and 18mg/ml respectively at 15 % (w/v).

The saccharification temperature was further dropped to 30°C for *P.stipitis* fermentation. Significant xylobiose and cellobiose accumulation was observed initially (Table 5.5) but appeared completely converted to monomeric sugars by 48 hours likely
due to added ß-xylosidase and cellobiase activity in Spezyme and Novozyme 188. When the temperature was dropped to 30°C from the previous attempt of 37°C (Table 5.4) there were significant decreases in the yields of sugars after 72 hours of hydrolysis.

Since xylobiose and cellobiose accumulation was observed in the first step of hydrolysis the Multifect Xylanase and SXA enzyme mixture was supplemented with Novozyme 188 (Table 5.6). Cellobiose binds in the active site of ß-xylosidase and can inhibit its activity [143]. Cellobiose was formed in the first step of hydrolysis due to some cellulase activity in the Multifect Xylanase (see Table 4.2, Chapter 4). The cellulase activity in Multifect xylanase was apparent in Figure 5-1 where Multifect Xylanase and Novozyme 188 at 10% (w/v) mixture formed glucose. No cellobiose accumulation was observed with added Novozyme 188 (Figure 5-1).

In another set of experiments, Multifect Xylanase and SXA are added to IL pretreated poplar in the first step of hydrolysis at 50°C. The temperature in the second step, where cellulase was added, was further dropped to 25°C (Table 5.6). Slininger et al. observed that the optimal temperature for fermentation of sugars to ethanol with P. Stipitis was 25°C. At higher temperatures glucose and xylose consumption was observed to be severely stalled with xylitol accumulation [144].

The addition of Novozyme 188 in the first step did help in complete removal of cellobiose and xylobiose. However, no remarkable change in the overall hydrolysis of glucose and xylose was observed with the addition of Novozyme 188 in the first step (Table 5.6). There was no significant reduction in glucose and xylose yields when the temperature was dropped from 30 to 25°C but yields were consistently lower than
hydrolysis at 37°C. The overall hydrolysis yields were higher when Spezyme and Novozyme were added along with Multifect Xylanase and SXA compared to sequential hydrolysis and fermentation experiments. Hydrolysis with SP/NV in the first step at 50°C resulted in higher conversions of xylose compared to the hydrolysis with MX/SXA or MX/SXA/NV at 50°C. The comparison of the glucose yields at a temperature combination of 50 and 25°C to the glucose yields at a temperature of 50°C shows that Spezyme CP and Novozyme 188 activities were indeed significantly reduced at lower temperatures. Significant cellobiose accumulation was also observed as a result of decreased cellobiase activity at lower temperatures. Cellobiose is a strong inhibitor of cellulase, reducing glucan conversion to glucose.
Table 5.6  Sequential and simultaneous saccharification at 50 and 25°C of 15% (w/v) pretreated poplar: Enzymatic hydrolysis of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 4.5 mg / g xylan of Multifect Xylanase and 1.8 mg / g xylan of SXA (β-xylosidase) at 50°C for the first 24 hours of hydrolysis. Temperature was reduced to 25°C and 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 30 CBU / g glucan (7mg/g glucan) Novozyme 188 were added. Glucose and xylose yields are given in parentheses.

<table>
<thead>
<tr>
<th>Initial Enzyme mixture</th>
<th>Supplementation</th>
<th>Time (hr)</th>
<th>Cellobiose (g/l)</th>
<th>Xylobiose (g/l)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
</tr>
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<tbody>
<tr>
<td>MX/SXA+ SP/NV (25°C)</td>
<td></td>
<td>24</td>
<td>0.3</td>
<td>0.2</td>
<td>1 (1%)</td>
<td>7 (40%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>48</td>
<td>3</td>
<td>18 (32%)</td>
<td>11 (61%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>72</td>
<td>3</td>
<td>24 (43%)</td>
<td>12 (61%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>96</td>
<td>5</td>
<td>27 (49%)</td>
<td>13 (61%)</td>
</tr>
<tr>
<td>MX/SXA/NV+ SP (25°C)</td>
<td></td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>2 (4%)</td>
<td>9 (48%)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>48</td>
<td>2</td>
<td>10 (18%)</td>
<td>6 (33%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>72</td>
<td>3</td>
<td>22 (40%)</td>
<td>12 (67%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>96</td>
<td>4</td>
<td>25 (45%)</td>
<td>12 (67%)</td>
</tr>
<tr>
<td>MX/SXA/SP/NV 50°C</td>
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<td>4</td>
<td>-</td>
<td>25 (45%)</td>
<td>11 (61%)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>72</td>
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<td>23 (41%)</td>
<td>7 (39%)</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td>96</td>
<td>6</td>
<td>40 (72%)</td>
<td>13 (72%)</td>
</tr>
</tbody>
</table>

MX- Multifect Xylanase, SXA-β-xylosidase, SP-Spezyme CP and NV- Novozyme 188 (β-glucosidase). *The complete yield of glucose and xylose corresponds to 55.5 mg/ml and 18mg/ml respectively at 15 % (w/v).

Novozyme 188 was increased to 40 CBU/g glucan from 30CBU/g glucan (Table 5.7). Multifect Xylanase loading was also increased two fold (from 9 mg from 4.5 mg/ g xylan).
Table 5.7  Sequential saccharification at 50 and 25°C of 15% (w/v) pretreated poplar at increased Multifect Xylanase and Novozyme loadings: Enzymatic hydrolysis of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 9 mg / g xylan of Multifect Xylanase and 1.8 mg / g xylan of SXA (β-xylosidase) at 50°C and 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 30 and 40 CBU / g glucan Novozyme 188 at 25°C. Glucose and xylose yields are given in parentheses.

<table>
<thead>
<tr>
<th>Initial Enzyme mixture</th>
<th>Supplementation</th>
<th>Time (hr)</th>
<th>Cellobiose (g/l)</th>
<th>Glucose (g/l) (%)</th>
<th>Xylose (g/l) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2MX/SXA+</td>
<td>SP/NV40 (25°C)</td>
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<td>2 (4%)</td>
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<td>9 (50%)</td>
</tr>
<tr>
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<td></td>
<td>48</td>
<td>3.1</td>
<td>3 (5%)</td>
<td>13 (72%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>4.0</td>
<td>27 (49%)</td>
<td>14 (78%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96</td>
<td>4.7</td>
<td>31 (56%)</td>
<td>15 (83%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>5.0</td>
<td>34 (61%)</td>
<td>15 (83%)</td>
</tr>
<tr>
<td>2MX/SXA+</td>
<td>SP/NV30 (25°C)</td>
<td>12</td>
<td>0.2</td>
<td>1 (2%)</td>
<td>7 (45%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.3</td>
<td>1 (2%)</td>
<td>7 (45%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>2.0</td>
<td>13 (23%)</td>
<td>10 (45%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>3.0</td>
<td>20 (36%)</td>
<td>12 (45%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96</td>
<td>3.7</td>
<td>24 (43%)</td>
<td>13 (45%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>4.2</td>
<td>28 (50%)</td>
<td>14 (45%)</td>
</tr>
</tbody>
</table>

MX- Multifect Xylanase, SXA-β-xylosidase, SP-Spezyme CP and NV- Novozyme 188 (β-glucosidase). *The complete yield of glucose and xylose corresponds to 55.5 mg/ml and 18mg/ml respectively at 15 % (w/v).

Increasing the Novozyme loading did not seem to completely avoid celllobiose accumulation. The overall conversion of glucose and xylose at the end of 120 hours of hydrolysis was improved by increasing the Novozyme 188 enzyme loadings. The xylose yield was observed to increase in the first step with increase in the Multifect Xylanase.
loading. Complete yield of xylose was not achieved. Another set of experiments were performed where cellulase was supplemented after 24 hours of xylanase hydrolysis (Table 5.8). The Novozyme 188 enzyme loading was reduced to 30CBU/g glucan keeping rest of the enzyme loadings similar. Significant improvement in xylose conversion was observed but there was incomplete at 24 hours of hydrolysis (Table 5.8). With increase in xylan conversion, glucan conversion also increased. The 144 hour hydrolysis yields with MX/SXA/SP/NV30 in Table 5.8 appear to be comparable to the 120 hour hydrolysis yields with MX/SXA/SP/NV40 in Table 5.7. Lower cellobiase activity resulted in slower hydrolysis. The overall xylose yield is significant but the glucose yield is very low due to the lower activity of Spezyme and Novozyme 188 at low temperatures.

Based on the observed hydrolysis trends (Table 5.8), to run the sequential saccharification and fermentation experiments it would be logical to carry out the saccharification at 9 mg / g xylan of Multifect Xylanase and at 1.8 mg / g xylan of SXA (β-xylosidase) at 50°C to release most of the xylose. In the second step, the saccharification temperature is reduced to 25°C, optimal for \textit{P.stipitis} fermentation. Increasing Spezyme CP and Novozyme 188 loadings higher than 10 FPU / g glucan (25 mg/g glucan) and 30 CBU / g glucan, respectively, may hydrolyze cellulose portion to completion.
Table 5.8  Sequential saccharification at 50 and 25°C of 15% (w/v) pretreated poplar at extended hydrolysis with increased Multifect Xylanase loadings: Enzymatic hydrolysis of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 9 mg / g xylan of Multifect Xylanase and 1.8 mg / g xylan of SXA (β-xylosidase) at 50°C and 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 30 CBU / g glucan Novozyme 188 at 25°C.

<table>
<thead>
<tr>
<th>Initial Enzyme mixture</th>
<th>Supplementation</th>
<th>Time (hr)</th>
<th>Cellobiose (g/l)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2MX/SXA+ SP/NV30 (25°C)</td>
<td>-</td>
<td>24</td>
<td>-</td>
<td>2 (4%)</td>
<td>10 (55%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>48</td>
<td>2.7</td>
<td>19 (34%)</td>
<td>12 (67%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>72</td>
<td>3.0</td>
<td>20 (36%)</td>
<td>13 (72%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>96</td>
<td>3.5</td>
<td>25 (45%)</td>
<td>14 (78%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>120</td>
<td>3.9</td>
<td>28 (50%)</td>
<td>15 (83%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>144</td>
<td>5.0</td>
<td>34 (61%)</td>
<td>15 (83%)</td>
</tr>
</tbody>
</table>

MX- Multifect Xylanase, SXA-β-xylosidase, SP-Spezyme CP and NV-Novozyme 188 (β-glucosidase). *The complete yield of glucose and xylose corresponds to 55.5 mg/ml and 18mg/ml respectively at 15 % (w/v).

5.4. Conclusions

The hydrolysis at high biomass loadings (10%) and low biomass loading (1%) with Spezyme CP at 50°C was observed to be significant. It was found that at high solid loadings xylan conversion was inhibited by xylose oligomer accumulation. For complete xylan conversion xylosidase activity in addition to xylanase activity is crucial.

At 10% and 12% (w/v) solid loadings, the sequential hydrolysis of xylan with low enzyme loadings of Multifect Xylanase and SXA (β-xylosidase) at 50°C followed by glucan hydrolysis with Spezyme CP ( 5 to 10 FPU/g glucan) and Novozyme 188 (30 CBU/ g glucan at 37°C was observed to be complete. Significant hydrolysis at 15% (w/v) solid loading was also observed at 30 and 37°C. An interesting scenario is sequential
saccharification and fermentation experiments with a microorganism that ferments sugars
in the temperature range of 30-35°C. Celllobiose accumulation was evident in the second
step of sequential saccharification screening experiments as the temperature was dropped
from 37 to 30°C attributed to a decrease in Novozyme 188 activity. Further drop in
temperature from 30 to 25°C resulted in even greater accumulation of celllobiose.

The lower yields of glucose at low hydrolysis temperatures were attributed to
reduced activity of cellulase. This was observed to be the primary roadblock for complete
saccharification of cellulose at low temperatures. Increasing the cellulase loadings which
in turn increases the activity of cellulases was considered to facilitate complete
conversion of glucose at 25°C at 15% (w/v) solid loading. Increasing the Novozyme 188
from 30CBU to 40CBU/ g glucan enzyme loading did result in modest increases in
glucan hydrolysis but did not completely avoid not celllobiose accumulation. A further
increase in Novozyme 188 loading is expected to hydrolyze the celllobiose and cellulose
completely while keeping the Multifect Xylanase and SXA loadings similar to those in
Table 5.8.
Chapter 6

Sequential Saccharification and Fermentation of Ionic Liquid Pretreated Lignocellulosic Biomass

6.1. Introduction

In the United States, corn fiber was targeted as a source of renewable fuel for over a decade because of its high carbohydrate and low lignin content [145]. In recent years, research has focused on identifying abundant renewable feedstock that can preserve the economic and energy security of the country [146]. Crop derived residues such as lignocellulosic biomass were found to replace food crops such as corn, barley, and wheat due to their high energy content and availability [12]. Polysaccharides from sustainable sources such as lignocellulosic biomass derived from agricultural and forest based residues can be hydrolyzed by enzymes to a sugar platform that produces both hexose and pentose sugars, comprised primarily of glucose and xylose. These sugars can
be fermented by naturally occurring yeast, bacterial, filamentous fungal strains or by genetically modified yeast or bacterial organisms (GMO’s) [147] [148-153] to liquid fuels, most commonly to bio-ethanol.

The overall biofuel production process via a sugar platform is not viable unless xylose is fermented to ethanol as it constitutes the second most abundant polysaccharide fraction in lignocellulosic biomass after glucose [154, 155]. Most naturally occurring strains do not have the potential to efficiently metabolize mixed sugar hydrolyzates [156]. Naturally occurring yeast or bacterial strains have not yet been found to possess characteristics to withstand high ethanol concentration and tough industrial operating conditions [157, 158]. Some research efforts have been directed towards engineered GMO’s that can ferment both glucose and xylose with efficient ethanol yields in an industrial environment [148-152, 159]. The GMO’s are engineered to tolerate inhibitory products produced from biomass and withstand aerobic conditions [148-153]. The long term viability of such genetically modified strains is still a challenge [160].

Amongst the naturally occurring strains for ethanol production are *Saccharomyces cerevisiae*, also called Baker’s yeast, *Pachysolen tannophilus* or *Pichia stipitis* (yeast) and *Zymomonas mobilis*, a bacterial strain. In general, the yeast strains are favored over the bacterial strains, with thick cell wall, less rigorous nutrient requirement, better growth at low pH and a tolerance for contamination [161]. With glucose and xylose available in the same hydrolyzate, potential processing issues arise. *Saccharomyces cerevisiae* readily metabolizes xylulose and glucose and cannot metabolize xylose even though there is cellular transport for xylose. This is due to the
lack of xylose isomerase (XI) which can isomerize xylose to xylulose [156, 162]. Researchers have developed strategies where mixed sugar fermentations in lignocellulosic biomass are achievable with Saccharomyces cerevisiae with efficient ethanol yields. These process strategies include exogenous induction of the isomerase enzyme [156, 162], engineering xylose utilizing recombinant strains of S. cerevisiae that can metabolize glucose and xylose [163], or isomerizing xylose in the fermentation media [164].

However, for cost effective ethanol production from lignocellulosic biomass there is still a necessity to investigate readily available naturally occurring native yeast strains that can effectively metabolize both hexose and pentose sugars. P. stipitis, a xylose fermenting yeast can metabolize both glucose and xylose sugars in lignocellulosic biomass hydrolyzates [165]. It was reported by duPreez et al., 1986 [166] that glucose is metabolized preferentially by P. stipitis followed by xylose and cellobiose only after exhaustion of glucose.

During mixed sugar fermentation, the native or engineered pentose metabolizing yeast experiences severe diauxic lag. Agbogbo pointed out that efficient sugar uptake rate requires understanding of sugar repression systems with improving ethanol tolerance, as well as strengthening promoters to reduce the effects of diauxy [165]. Slininger and coworkers have studied the above mentioned effects in depth [167]. It was reported by Slininger et al., 2010 [167] that a severe lag could be as long as 10 hours when glucose and xylose are available at the same time. The D-glucose represses enzymes such as xylose reductase (XR) and xylitol dehydrogenase (XDH) that are required for D-xylose
metabolism in the oxo-reductive pathway [167]. In this pathway, the XR reduces xylose to xylitol using the co-factors NADH and NADPH and the XDH oxidizes xylitol to D-xylulose using the co-factor NAD. The D-xylulose is phosphorylated by an ATP utilizing xylulo kinase (XK) to D-xylulose-5-phosphate which is an intermediate in the pentose pathway. D-xylulose-5-phosphate is converted to glyceraldehyde-3-phosphate and fructose-6-phosphate. The glyceraldehyde-3-phosphate is then converted to carbon dioxide and ethanol and fructose-6-phosphate converts back to D-xylulose-5-phosphate. Glucose through glycolysis is converted to pyruvate and then to acetaldehyde and ethanol releasing carbon dioxide [168].

Slininger *et al.*, 2010 [167] have proposed a technique where resuspension of yeast cells in large concentrations of xylose sugar can eliminate the lag in sugar transition from glucose to xylose. They successfully demonstrated that growing the *P. stipitis* cells on xylose induced the XR and XDH xylose specific enzymes before entering into the mixed hydrolyzates for fermentation. The technique of priming the cells with xylose resulted in faster fermentation rates of both glucose and xylose well below the inhibitory levels of ethanol accumulation.

In the present work, based on the technique of priming cells with xylose, we have investigated strategies of sequential or simultaneous saccharification of ionic liquid pretreated lignocellulosic biomass with xylanases and cellulases and fermentation (SSF) with *P. stipitis* to ethanol. Hydrolysis of hemicellulose was carried out with Multifect Xylanase and SXA (β-xylosidase) at 50°C to xylose and in the second step the hydrolyzates were inoculated at 25°C with a high cell density of *P. stipitis* that was
initially grown on xylose sugar. The released xylose was then converted to ethanol. After about 12 hours of fermentation in controlled aerobic conditions, the hydrolyzates were supplemented with Spezyme CP and Novozyme 188 to hydrolyze the cellulose portion to glucose. The released glucose was then successively fermented to ethanol. Pure sugar standards were also run in addition to the biomass samples. Complete assimilation of sugars and production of ethanol took about 96 hours with residual cellobiose accumulation. 0.51 grams of ethanol can theoretically be produced from one gram of glucose or xylose sugar [169, 170]. *Ethanol yields are expressed as a percentage of this theoretical conversion. The overall yield of ethanol from ionic liquid pretreated hydrolyzates was around 56% and with pure mixed sugars the overall yield of ethanol was around 70%. Lower theoretical ethanol yields with biomass hydrolyzates appear due to primarily insufficient hydrolysis of glucan at the low temperatures of *P.stipitis* fermentation.

\[ \text{Percentage of Theoretical Maximum Ethanol Yield (\%) } = \frac{\text{Ethanol produced (g)}}{\left( \text{Initial sugar, glucose + xylose (g}} \right) x 0.511 } x 100 \]

6.2. **Materials and methods**

**Note:** Care should be taken to perform all the below suggested steps in a biological laminar flow hood. Use of clean alcohol sterilized gloves is highly recommended during contact with the media or organism to avoid possible contamination.
6.2.1 Organism and purity streaking

The organism handling and inocula preparation were performed as directed by Dr. Patricia Slininger, USDA-ARS, NCAUR, Peoria, IL, USA. The YM plates were streaked with Scheffersomyces (Pichia) stipitis NRRL Y-7124 (CBS 5773) and were obtained from the ARS Culture Collection (NCAUR, Peoria, IL). The plates were stored in a refrigerator at 2-4°C and incubated for about 48-72 hours. The YM plates were restreaked by taking a consistently colonized section of the yeast lawn (no colonies showing odd morphologies). The plates were incubated for 48 hours at 25°C prior to loop transfer to liquid pre-cultures until a uniform lawn of cells (slightly off white) were developed. These were stored up to a week in the refrigerator until transfer. The YM-Yeast Malt plates that were re-streaked to a second YM plate and allowed to grow out a second time, were flooded with 15 mL of sterile 10% glycerol and scraped to resuspend cells. These cells can then be pipetted to sterile cryovials (Fisher Scientific, Pittsburgh, PA, USA) and stored at -80°C. The YM agar plates were poured warm after autoclaving (121°C) the following ingredients per L of water: 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g dextrose, 20 g agar and incubated for 24 hours. The agar sterile plastic plates and sterile transfer loops were purchased from Fisher Scientific, Pittsburgh, PA, USA.

6.2.2 Composition of Optimum Defined Media (ODM)

ODM consists of the following constituents mixed in water. Carbon: 150g/L Xylose. Purines/Pyrimidines: 10mg/L Adenine, Cytocine, Guanine, uracil, and Thymine. Trace Minerals: 5.5 mg/L ZnSO$_4$$2$$H_2$$O$, 1.6 mg/L CoCl$_2$$6$$H_2$$O$, 12.5
mg/L MnCl₂·4H₂O, 5.0 mg/L (NH₄)₆(Mo₇O₂₄).4H₂O, 8.0 mg/L CuSO₄·5H₂O, 28.0 mg/L CaCl₂·2H₂O, 250.0mg/L EDTA, 10.0mg/L NaCl. **Mineral Salts**: 1g/L K₂HPO₄, 1g/L KH₂PO₄, 0.75g/L MgSO₄·7H₂O. **Vitamins**: 0.5mg/L Thiamin, 0.5mg/L Riboflavin, 0.5mg/L Calcium Pantothenate, 0.5mg/L Niacin, 0.5mg/L Pyridoxamine, 0.5mg/L Thioctic Acid, 0.5mg/L Biotin, 0.05mg/L Folic Acid, 0.05mg/L B12, Iron- 50.0 mg/L FeSO₄·7H₂O. **Urea**: (N = 80% from Urea) 3.56g/L. **Amino Acids**: (N = 20% from Amino Acids) - 10 g/L Casamino Acids, 0.1 g/L Tryptophan, 0.4 g/L Cysteine, 0.4 ml/L HCL.

### 6.2.3 Optimum Defined Medium preparation for pre-cultures and test cultures

The protocol for the ODM preparation was shared by Dr. Patricia Slininger, USDA-ARS, NCAUR, Peoria, IL, USA. All the minerals, purines, vitamins, amino acids, chemicals and sterile filter units were purchased from Sigma Aldrich, MO, USA. The protocol was designed for 2L batch and can be scaled down to the desired quantities. Stock solutions were prepared as directed below and can be stored in a refrigerator.

**Group A:**

**PO₄ Mineral Salts**: 8g K₂HPO₄ and 8g KH₂PO₄ dilute to 200ml dH₂O and autoclave.

**Mg Mineral Salts**: 4.50g MgSO₄·7H₂O dilute to 150ml dH₂O and autoclave.

**Trace Minerals**: 0.044g ZnSO₄·7H₂O, 0.0128g CoCl₂·6H₂O, 0.1g MnCl₂, 0.04g (NH₄)₆(Mo₇O₂₄), 0.064g CuSO₄·5H₂O, 0.08g NaCl, 0.224g CaCl₂·2H₂O (*Add last to check for precipitation problem), 2.0g EDTA, Add each to water while stirring. Dilute to 200ml with deionized (DI) H₂O and autoclave. (Recommended to make fresh on the day of use or else add EDTA separately at 1g/100ml of the stock).
Purines/Pyrimidines: 0.12g of each (adenine, cytocine, guanine, uracil, & thymine). Boil with 300ml DI H$_2$O and 60 ml concentrated HCl until dissolved. Dilute to 600mls. Stock need not be sterilized.

Iron: 0.50g FeSO$_4$·7H$_2$O in 100ml dH$_2$O. Filter sterilize the solution. Prepare fresh on the day of use.

Vitamins (1X): 0.025g each (thiamin, riboflavin, calcium pantothenate, niacin, pyridoxamine, thioctic acid, Biotin) and 0.0025g of each (folic acid, B12) dilute to 1000ml with DI H$_2$O and filter sterilize.

Group B:

Xylose – 15g Xylose per 100ml of complete media.

Group C:

Amino Acids: Add 25g casamino acids, 0.25g tryptophan, 1.0g cysteine, 1.0ml conc. HCL and dilute up to 100ml DI H$_2$O and autoclave.

Urea: 27.36g Urea dilute to 200mls with DI H$_2$O and autoclave.

All the stock solutions were combined as per the table below in ml quantities. Total produces 2L media and xylose is added in the concentration of 150g/l after all the stock solutions were combined. Use 6N NaOH to pH media to 6.5 +/- 0.1(optimum range is 4-7 for \textit{P. stipitis} NRRL Y-7124). The filter sterilized media is stored in a sterile container in the refrigerator at 2-4°C until inoculated.

<table>
<thead>
<tr>
<th>PO$_4$</th>
<th>Tr.Min.</th>
<th>MgSO$_4$</th>
<th>P/P</th>
<th>Fe</th>
<th>Vit</th>
<th>Urea</th>
<th>AA</th>
<th>Water</th>
<th>Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>52</td>
<td>80</td>
<td>1558</td>
<td>300g</td>
</tr>
</tbody>
</table>
6.2.4 Culture Conditions

Pre-cultures

Pre-cultures of Y-7124 were inoculated by loop from YM agar plates and grown for 24 hours. Cultivations were in 75 mL ODM with 150 g/L xylose in 125 mL flasks capped with Bellco silicone foam closures at 25°C and 150 rpm on an orbital shaker. Initial pH of the media was 6.5. The OD of the pre cultures after 24 hours measured at 600 nm on UV-spectrophotometer was around 2.5 with an absorbance of 0.123.

Growth Cultures

Growth cultures of Y-7124 were inoculated from pre-cultures to an optical density (OD) of 0.123 at 620 nm and incubated for 96 hours on ODM with 150 g/L xylose. Cultivars were grown in 200 mL ODM in 300 mL flasks with Bellco silicone foam closures at 25°C and 150 rpm (1” diameter eccentricity). Initial pH was 6.5 for Y-7124. At 96 hours the OD at 600 nm is ~10 units under these conditions and reached the stationary phase with no further change in the OD. Pichia requires oxygen while propagating. Once cells are grown, they can ferment anaerobically, but a minimal supply of oxygen would be needed to allow enough cell growth to replace dead cells. The objective here is to grow the yeast on xylose so that the XR and XDH were induced in the Pichia cells before inoculation.
6.2.5 Inocula

Once the *Pichia* cells were grown to the stationary phase the cell suspension was transferred into the 50ml sterile centrifuge tubes (Fisher Scientific, PA, and USA) and centrifuged at 3000 rpm at 4°C. The supernatant media was removed with autoclaved serological glass pipette dispensers (Fisher Scientific, PA, USA). The cell paste was resupended in sterile isotonic saline (9 g/L NaCl in water) and serially diluted to an OD of ~60 units. The hydrolyzates about 3.24 ml were inoculated with 350μl of OD 60 cell suspension.

6.2.6 Pretreatment

Poplar (provided by the National Renewable Energy Laboratory) was pretreated with the ionic liquid, 1-ethyl 3-methyl imidazolium acetate, EmimOAc, (Sigma Aldrich, MO, USA). The biomass was mixed with EmimOAc, 5% (w/w), and incubated at 120°C for 30 or 60 minutes. The sample was then mixed with water at room temperature to precipitate the biomass/cellulose from the IL solution. The precipitant and wash solutions were separated by centrifugation. The precipitated solids were washed repeatedly with deionized (DI) water to displace the IL from the sample until the wash solution appeared colorless.

6.2.7 Sequential saccharification and fermentation

The experiments for sequential saccharification and fermentation were carried out at a combination of 50°C and 25°C. Hydrolysis was carried out with 15% (w/v) substrate in 0.05 M sodium citrate buffer, pH 4.8 (with added 30mg/l cyclo heximide and 40 mg/l
tetracycline), in 30ml glass scintillation vials equipped with septa and cap (Fisher Scientific, Pittsburgh, PA, USA) on Bellco roller mixer placed in an incubator for temperature control. After inoculation, the septa of the scintillation glass vials were pierced with a needle and plugged with ethanol soaked cotton to allow release of CO$_2$ during fermentation. The pretreated substrate was hydrolyzed with commercial cellulase and xylanase enzymes supplemented with Novozyme 188 (β-glucosidase). Aliquots of the reaction mixture were sampled periodically and the enzyme and the yeast reaction were stopped by boiling the sample at 100°C for 10 minutes.

Commercial cellulase mixture, Spezyme CP, and commercial xylanases, Multifect Xylanase, were provided by Genencor International Rochester, NY, USA. Novozyme 188, a β-glucosidase, was purchased from Sigma Aldrich, MO, USA. β-xylosidase, from *Selenomonas ruminantium* (SXA) of glycoside hydrolase family 43 was provided by Douglas Jordan, USDA ARS, NCAUR, Peoria, IL.

### 6.2.8 HPLC Analysis

The sugar and alcohol analysis of the sequential hydrolysis and fermentation samples was performed using high performance liquid chromatography with refractive index detection and a Bio-Rad (Richmond, CA) Aminex HPX-87H carbohydrate analysis column. The mobile phase was HPLC grade 5mM sulfuric acid at a flow rate of 0.6 ml/min with a column temperature of 65°C. The concentration of residual sugars and ethanol released were measured using a calibration curve that was prepared with mixed sugar standards and ethanol standards. The ethanol prior to the standard curve preparation was stored on molecular sieves to avoid water absorption.
6.3. Results and Discussion

Fermentations with *P. stipitis* were first performed on pure sugars (glucose and xylose) individually or using sugar mixtures. Glucose and xylose sugar concentrations were adjusted to closely match to that of fully hydrolyzed poplar substrate at a concentration of 15% (w/v) poplar for the hydrolysis step. Only glucose and xylose concentrations were monitored through the course of experiments as they compose the major fraction of polysaccharides in biomass. In Chapter 5, it was shown that for poplar, glucose composition was around 37% (w/w) and xylose composition was around 12% (w/w). At 15% (w/v) solids in the hydrolysis step, the maximum concentration of glucose and xylose is 55.5 and 18 g/l respectively. Assuming a theoretical yield of 0.51 grams of ethanol per gram of glucose and xylose [169, 170], the ethanol concentration would be 28 and 9 g/l from glucose and xylose, respectively. Fermentation with glucose/xylose solutions with *P. stipitis* with and without ODM (excluding xylose) was initially assessed. To prime cells with xylose [167], the first set of fermentation experiments with monomeric sugars S.No.1, was carried out with xylose added initially, followed by 24 hours of fermentation, then glucose addition (Table 6.1).

As seen in Table 6.1 little ethanol was produced in the first 24 hours even though xylose was consumed. Ethanol production appears inhibited. Similar trends were observed after glucose addition. The fermentation appears stalled between 48 and 72 hours. In experiment S. No.2, both glucose and xylose are introduced with the initial inoculum of *P. stipitis*. At the end of 72 hours the ethanol concentration was similar to that of experiment, S. No.1 with little xylose consumption. In the next two sets of
experiments with pure xylose, S. No.3, and pure glucose, S. No. 4, it appears that fermentation to ethanol is stalled. In S.No.3 the ethanol production was observed to be stalled at 2 g/l even though there appears to be no residual xylose. The little ethanol produced from mixed sugar hydrolyzates appeared to be primarily from glucose and comparable to the S. No. 4 experiment (Table 6.1).

Table 6.1  Sequential and simultaneous fermentation with pure sugars: Fermentations were carried out with 55.5 g/l pure glucose sugar and 18 g/l pure xylose sugar suspended in 0.05M sodium acetate buffer, pH 4.8 at 15% (w/v) loading and inoculated with \textit{P. stipitis} at OD ~ 60 at 25°C. All the samples were inoculated at time zero.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Time</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
<th>Ethanol (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>-</td>
<td>18.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>\textbf{55.5}</td>
<td>\textbf{8.7}</td>
<td>\textbf{1.2}</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>39.6</td>
<td>8.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>36.0</td>
<td>8.7</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>55.5</td>
<td>18.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>37.0</td>
<td>16.0</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>33.2</td>
<td>15.7</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>28.7</td>
<td>15.6</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>-</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
<td>8.3</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>3.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
<td>0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>55.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>37.5</td>
<td>-</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>32.7</td>
<td>-</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>25.2</td>
<td>-</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Similar set of experiments were carried out with 5% (w/w) poplar pretreated at 120°C, 30 minutes (Table 6.2). The enzyme mixture loadings used in the hydrolysis experiments were similar to those described in Chapter 5. Xylitol accumulation was not observed in any samples consistent with xylose fermentation at 25°C [144]. In the
experiment S. No.1, (Table 6.2) it was observed that xylose is not consumed and most glucose appears to convert to ethanol. It appears that glucose is readily fermented. The consumption of xylose appeared to be very slow. In the second and third experiments S.No.2 and 3, similar trends of xylose concentration and glucose fermentation were observed.

The fermentation of lignocellulosic biomass hydrolyzates appears to follow the same trends as the pure sugars. The ethanol yields from pure sugars without nitrogen, mineral or vitamin supplementation (Table 6.1) were significantly lower compared to that of the lignocellulosic biomass hydrolyzates (Table 6.2). This could be due to the lack of nutrients in sugar solutions. Lignocellulosic biomass generally possesses variety of nutrients in the form of salts, minerals, proteins and acids. The yeast cells when deprived of nutrients may not express the XR and XDH enzymes that are essential for xylose isomerization to xylulose or enzymes required for glucose fermentation to ethanol. Lack of XR and XDH enzymes results in xylose accumulation with no xylitol production [144, 167]. There was no evidence of xylitol accumulation with both pure sugars and lignocellulosic biomass.
Table 6.2  Sequential and simultaneous saccharification and fermentation of pretreated poplar: Enzymatic hydrolysis and fermentation of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 4.5 mg / g xylan of Multifect Xylanase, 1.8 mg / g xylan of SXA (β-xylosidase),10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 30 CBU / g glucan (7mg/g glucan) Novozyme 188. Hydrolyzates were inoculated with \textit{P.stipitis} at OD ~ 60 at 25°C. All the measurements were duplicate measurements that deviated by less than 5%.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Initial Enzyme mixture</th>
<th>Enzyme Supplementation &amp; Inoculation</th>
<th>Time (hr)</th>
<th>Cellobiose (g/l)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
<th>Ethanol (g/l)</th>
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<td>0.55</td>
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</table>
The nutrients were supplemented in the biomass hydrolyzates and pure sugars by adding the vitamins, purines, minerals, iron and amino acids in the same concentration as in the ODM. A 10X filter sterilized ODM (as described in materials and methods section) at pH 6.5 with no added sugar was added to the hydrolyzates in a volume ratio of 10:90 (ODM:hydrolyzates) before inoculation (Table 6.3). The 15% (w/v) biomass solids were diluted to 13% (w/v) after nutrient supplementation and inoculation.

The fermentation rates were rapid after nutrient supplementation. Solutions with single sugars, glucose or xylose appear to be rapidly fermented to ethanol within 24 hours (Table 6.3). The ethanol yields with pure glucose and pure xylose were equivalent to the ethanol yields of mixed sugars. With pure glucose (S.No.4) and pure xylose (S.No.3) ethanol production was 74% and 65% of theoretical yield, respectively, with complete sugar depletion. Similarly, mixed sugars (S.No.2) and sequential addition of sugars (S.No.1) produced an overall 70% theoretical ethanol yield. Ethanol may be lost due to consumption of ethanol (carbon source) at the end of fermentation due to sugar depletion (personal communication with Dr. Patricia Slininger, USDA-ARS, NCAUR, Peoria, IL).
Table 6.3  Sequential and simultaneous fermentation with pure sugars supplemented with nutrients:

Fermentations were carried out with 55.5 g/l pure glucose sugar and 18 g/l pure xylose sugar suspended in 0.05M sodium acetate buffer at 15% (w/v) loading and inoculated with *P. stipitis* at OD ~ 60 at 25°C. All the samples were inoculated at time zero and prior to inoculation ODM was supplemented at 10% of the total reaction volume.

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<th>Ethanol (g/l)</th>
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Supplementation of biomass hydrolyzates with nutrients also resulted in significant enhancement in the fermentation to ethanol (Table 6.4). After nutrient supplementation the theoretical ethanol yield increased from 27% (Table 6.2) to 43% (Table 6.4) for both sequential and simultaneous saccharification and fermentation steps. These yields from biomass are lower compared to 70% ethanol yield from pure sugars (Table 6.3). Lower ethanol yields from biomass compared to pure sugars could be due to
the lower glucan hydrolysis and sugar production at 25°C. On comparison with the controls with no inoculum (Table 5.6 in Chapter 5) approximately 50% theoretical ethanol yield should be observed for sequential saccharification and fermentation and 72% theoretical ethanol should be observed for simultaneous saccharification (all enzymes added in the first step). The lower ethanol yields compared to the controls run at similar conditions could be due to incomplete xylan hydrolysis in the first step of hydrolysis resulting in inefficient priming. The lower ethanol yields could also be due to xylose and cellobiose accumulation.

The xylose release in S.No.1, 2 and 3 at 50°C are consistent with the xylose release at 50°C in the first step of sequential saccharification experiments with no inoculum (Table 5.6 in Chapter 5). The ethanol yields in the experiments S.No.1 and S.No.2 are similar (Table 6.4). Saccharification with MX/SXA at 50°C resulted in cellobiose and xylobiose accumulation. Since cellobiose accumulation can inhibit the β-xylosidase activity stalling the xylan conversion (Chapter 5), Novozyme 188 was added in the first step of saccharification (S. No.2) at 50°C. Novozyme 188 supplementation at 50°C increased the xylan conversion by less than 10% but resulted in a greater cellobiose accumulation at the end of 96 hours of saccharification and fermentation in S.No.2 experiment compared to S.No.1 experiment (Table 6.4). The greater accumulation could be due to the low activity of Novozyme in the later stages of saccharification.
Table 6.4  Sequential and simultaneous saccharification and fermentation of pretreated poplar supplemented with nutrients: Enzymatic hydrolysis and fermentation of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 4.5 mg / g xylan of Multifect Xylanase, 1.8 mg / g xylan of SXA (β-xylosidase), 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 30 CBU / g glucan (7mg/g glucan) Novozyme 188. Hydrolyzates were inoculated with *P.stipitis* at OD ~ 60 at 25°C. Prior to inoculation ODM was supplemented at 10% of the total reaction volume. All the measurements were duplicate measurements that deviated by less than 5%.

<table>
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<th>S.No.</th>
<th>Initial Enzyme mixture</th>
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<th>Cellobiose (g/l)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
<th>Ethanol (g/l)</th>
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<td>13.0</td>
<td>15.7</td>
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</table>
The ethanol yields from sequential (all enzymes added at once) hydrolysis and fermentation experiment (S.No.3) were comparable to the sequential saccharification and fermentation experiments (S.No.1 and 2) (Table 6.4). The ethanol yield in this case was not higher despite the higher sugar conversions in the initial saccharification step. The lower ethanol yields could be due to diauxy resulting from high glucose concentration. As a result there was no consumption of xylose. Severe cellobiose accumulation was also observed. The ethanol production appeared primarily from glucose (Table 6.4).

In order to increase the theoretical ethanol yields by achieving complete xylan conversion in the first step of saccharification at 50°C, Multifect xylanase loading was increased by two fold from 4.5 mg to 9 mg/g xylan (Table 6.5). The Novozyme 188 loading was also increased from 30 to 40 CBU/g glucan to avoid cellobiose accumulation observed in (Table 6.4). Cellulase enzymes are strongly inhibited by end product cellobiose. The enzyme loadings for the SXA and Spezyme were unchanged.

The Xylanase and SXA were added first at 50°C and with inoculation of the hydrolyzates at 25°C after 12 hours of saccharification. Spezyme CP and Novozyme 188 are added after the yeast cells were primed with xylose 12 hours after inoculation (Table 6.5). The addition of cellulase along with yeast did not appear to result in efficient priming of yeast cells with xylose, with xylose uptake appearing stalled upon cellulase addition (Table 6.4). The released glucose may have repressed xylose metabolism.

The sequential saccharification and fermentation at increased enzyme loadings was performed with S.No.1 enzyme mixture supplementation case and not with S.No.2
(Table 6.5). There were no significant differences between the two cases. The xylose consumption appeared to be very slow and appears to be consumed only after the glucose consumption (after 48 hours), consistence with duPreez et al., 1986 [166]. The increase in Novozyme 188 did not seem to avoid the cellobiose accumulation but increased the overall ethanol yield by 27% (~51%) (Table 6.5). The ethanol yield when compared to the S.No.1 in Table 6.4 to S.No.1b in Table 6.5 appears to be similar. Increasing the xylanase loading did not significantly increase in the overall ethanol yields.

Increasing the Multifect Xylanase and Novozyme 188 loadings resulted in the overall theoretical ethanol yield of 51% (Table 6.5). Comparing this to the controls with no inoculum (Table 5.7 in Chapter 5) approximately 66% theoretical ethanol yield would be observed with fermentation of all sugars in hydrolysate to ethanol. The lower ethanol yields appear largely due to incomplete hydrolysis.

The increased Multifect Xylanase enzyme loading had little impact on xylan hydrolysis (Tables 6.4 to 6.6) The xylose yield in S.No.1a increased by 2g/l but the xylose yield in S.No.1b decreased by 1g/l (Table 6.5) compared to the xylose yields in Table 6.4. Complete conversion of xylan was not achieved by increasing the Multifect Xylanase loadings (Table 6.5).
Table 6.5  Sequential saccharification and fermentation of pretreated poplar with increased Multifect Xylanase and Novozyme loadings: Enzymatic hydrolysis and fermentation of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 9 mg / g xylan of Multifect Xylanase, 1.8 mg / g xylan of SXA (β-xylosidase), 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 40 (40 NV) and 30 CBU (30 NV) / g glucan (9.2 and 7mg respectively /g glucan) Novozyme 188. Hydrolyzates were inoculated with P.stipitis at OD ~ 60 at 25°C. Prior to inoculation ODM was supplemented at 10% of the total reaction volume. All the measurements were duplicate measurements that deviated by less than 5%.

<table>
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<th>S.No.</th>
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<th>Enzyme Supplementation &amp; Inoculation</th>
<th>Time (hr)</th>
<th>Cellobiose (g/l)</th>
<th>Glucose (g/l)</th>
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The saccharification with Multifect Xylanase and SXA was extended to 24 hours with inoculation at 24 hours. Spezyme CP and Novozyme 188 were supplemented 12 hours after inoculation in an effort to prime cells with xylose (Table 6.6). A significant improvement in xylose consumption was observed compared to results in Table 6.5 prior to cellulase supplementation. More xylose was released in the extended 24 hour saccharification with MX at 50°C. A similar increase in xylose conversion with the controls was also observed in Table 5.8 in Chapter 5. A minor improvement in glucose conversion was also observed. The overall increase in ethanol yield was observed to be around 10% (Table 6.6) compared to Table 6.5. Cellobiose appears to accumulate even at increased Novozyme 188 enzyme loading. A further increase in Novozyme 188 enzyme loading would be worth attempting in the future experiments.

The overall theoretical ethanol yield observed with increased Novozyme loadings (40 CBU/g glucan) was 56% and with reduced Novozyme loadings (30 CBU/g glucan) the overall theoretical ethanol yield observed was 49% (Table 6.6) compared to a possible 66% from hydrolyzates at reduced Novozyme loadings (30 CBU/g glucan) (Table 5.8).
Table 6.6  Sequential saccharification and fermentation of pretreated poplar at extended hydrolysis with increased Multifect Xylanase and Novozyme loadings: Enzymatic hydrolysis and fermentation of 15% (w/v) poplar pretreated at 120°C, 30 minutes. The enzyme loadings were 9 mg / g xylan of Multifect Xylanase, 1.8 mg / g xylan of SXA (β-xylosidase), 10 FPU / g glucan (25 mg/g glucan) Spezyme CP and 40 (40 NV) and 30 CBU (30 NV) / g glucan (9.2 and 7 mg respectively / g glucan) Novozyme 188. Hydrolyzates were inoculated with P.<i>stipitis</i> at OD ~ 60 at 25°C. Prior to inoculation ODM was supplemented at 10% of the total reaction volume. All the measurements were duplicate measurements that deviated by less than 5%.

<table>
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<th>S.No.</th>
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<th>Time (hr)</th>
<th>Cellobiose (g/l)</th>
<th>Glucose (g/l)</th>
<th>Xylose (g/l)</th>
<th>Ethanol (g/l)</th>
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Our results are consistent with those of Slininger et al., 2011[167] in xylose priming of *Scheffersomyces (Pichia) stipitis* NRRL Y-7124 (CBS 5773). They observed as high as 60g/l ethanol with highly concentrated xylose streams, accounting for 78% of theoretical ethanol yield from 95g/l pure glucose and 55g/l pure xylose. We observed 26 g/l ethanol i.e., 70% theoretical ethanol yield from 55.5g/l pure glucose and 18g/l pure xylose. The theoretical ethanol yield from lignocellulosic biomass was 56%. The further lower ethanol yields from lignocellulosic biomass as compared to pure sugars may be due to the complex nature of the substrate and the reduced activity of cellulase enzyme at low temperature.

6.4. Conclusions

Addition of cellulase after efficient priming of cells with xylose appeared to enhance the ethanol yields. Xylose sugar appears to be assimilated only after the glucose is consumed. Cellobiose accumulation was evident at enzyme loadings investigated here and appears to impede both xylan and glucan hydrolysis. An increase in Novozyme 188 appears merited.

The strategy of sequential saccharification with xylanase and cellulases and fermentation with yeast *P.stipitis* appears to be practically viable with mixed sugar hydrolyzates. Nutrient supplementation observed to be necessary in fermentation of pure sugars and biomass hydrolyzates. 70% of theoretical ethanol yield with complete consumption of sugars was observed with pure sugars. About 56% of theoretical ethanol yield was achieved with lignocellulosic biomass hydrolyzates. The lower ethanol yield in lignocellulosic biomass was in part due to the lower glucose production from Spezyme
CP/Novozyme 188 at 25°C due to the low enzyme activity and incomplete xylan hydrolysis.

Slininger et al., 1990 [144] showed that optimal temperature of *P. stipitis* fermentation to ethanol varies with substrate. 34°C is the optimal temperature for glucose fermentation. However, xylose is converted primarily to xylitol at 34°C resulting in decreasing the ethanol production. Xylose is converted primarily to ethanol at 25°C. The key for achieving enhanced ethanol yields with *P. stipitis* at a combination of 50 and 25°C may be to increase the cellulase and cellobiase loadings.

From the sequential or simultaneous saccharification controls (without inoculum), a maximum of 66% of theoretical ethanol yield is possible (Chapter 5). The observed lower theoretical ethanol yield of 56% was due to the incomplete hydrolysis of xylan at 50°C even with increased Multifect Xylanase loadings with added SXA. It appears that the xylose conversion was inhibited by xylan and glucan (cellobiose) oligomers. The xylose conversion may be enhanced by the addition of cellobiase and other accessory enzymes such as acetylxylan esterase (AXE). AXE hydrolyzes the ester linkages of acetylated xylan from hardwood [171].

In summary, the results underscore the importance of enhancing the activity of cellulase enzymes at lower temperatures. The sequential or simultaneous saccharification and fermentation with *P. stipitis* shows that it has potential applications in efficient glucose and xylose fermentations.
Chapter 7

Future Directions

7.1. Biomass characterization

In chapter 2, the cellulose I in poplar, switchgrass and cornstover transformed to cellulose II at harsher pretreatment conditions. This transformation was hypothesized to be due to lignin redistribution. In particular, switchgrass retained some fibrous structure even at the harsher IL pretreatment (120°C) condition. Poplar and cornstover exhibited powder pattern with no residual fiber structure, post IL treatment at 120°C and recrystallization. This may be due to a higher glass transition temperature ($T_g$) for switchgrass. At $T_g$ the lignin has increased mobility and can coalesce and redistribute. Delignification from lignin carbohydrate complexes (LCC) is also a possible scenario. It is important to establish the glass transition temperatures of the various biomasses in order to assess the effect of lignin $T_g$ on pretreatment. Since lignin is more mobile at $T_g$, the dissolved lignin could partition into the IL wash phases. The extracted lignin can be
converted into other value added products with recycling and reusing the IL to maintain the process economics.

The recrystallization of cellulose Iβ to cellulose II was apparent upon complete dissolution in IL. In this process of transformation an intermediate structure of cellulose was observed when the IL was partially expelled from the biomass/cellulose mixtures. This provides scope for further investigation to ascertain if the cellulose intermediate is observed in the pathway of cellulose II recrystallization or cellulose I to cellulose II transformation. Elucidation of the intermediate structure may provide insight into the physiochemistry of cellulose structural transformations with IL pretreatment.

Polymorphic transformations in biomass were observed at low biomass to IL loadings. It is important to study the transformations at high biomass to IL loadings which would be of commercial relevance.

7.2. Enzyme characterization and saccharification

The commercial enzymes used in this dissertation were characterized against defined substrates such as Avicel, CMC, Birchwood xylan, p-nitro phenyl substituted pyranosidase to estimate various activities (i.e. endoglucanase, exoglucanase, xylanase). These activities provide limited understanding of the saccharification kinetics. It is
crucial to assess the activities of commercial enzyme mixtures to establish the cross reactivity or inhibition from the substrates. Characterized against additional hemicellulose substrates such as tamarind (xyloglucan), arabinohylan, and polygalacturonic acid and cellulose substrates such as barley (β-D-glucan), lamanarin, lichenin, hydroxyl ethyl cellulose (HEC) may give a deeper understanding of the specific activities of the enzyme mixtures. Research focused towards mono-component enzyme mixtures may provide greater insight into activities required for saccharification of IL treated substrates.

Switchgrass showed significantly lower hydrolysis at low and high biomass loadings. To increase the hydrolysis rates in switchgrass supplementation with pectinase along with xylanase may increase cellulose and hemicellulose hydrolysis in switchgrass. Pectin is a heteropolysaccharide, consisting of a complex set of polysaccharides that are present in most primary cell walls of the non-woody parts of many terrestrial plants.

High solid loadings in both pretreatment and hydrolysis steps are to be performed which are crucial on commercial scale. The hydrolysis kinetics shown for high solid loading in chapter 5 presents preliminary results for these studies.

7.3. Fermentation of IL pretreated hydrolyzates

The maximum theoretical ethanol yields observed here from sequential saccharification and fermentation with *P. stipitis* of pure sugars are 70% and 56% from IL treated lignocellulosic biomass hydrolyzates. The lower ethanol yields for biomass were due to the incomplete xylose hydrolysis in the first step of hydrolysis at 50°C, low activity of Spezyme/Novozyme at 25°C and accumulation of cellobiose. To achieve
complete yields of xylose from hemicellulose hydrolysis, Multifect Xylanase/SXA enzyme mixture may require additional accessory enzymes, a recommended subject of future research.

The *P.stipitis* strains used in fermentation can further be evaluated to test the tolerance from degradation products and ethanol. Nutrient optimization (*i.e.* additions of urea, amino acids or vitamins) for IL treated biomass hydrolyzates requires further study. The fermentation with *P.stipitis* should be extended to other lignocellulosic biomass substrates.

Sequential saccharification at high solid loading at 37°C was observed to be almost complete. Testing of xylose and glucose fermenting micro-organisms that produces ethanol in the temperature range 30-37°C would of interest for sequential saccharification and fermentation.
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